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Genetic, ecological, and behavioural divergence between two sibling snapping shrimp species (Crustacea: Decapoda: *Alpheus*)

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Abstract

Examination of genetic and ecological relationships within sibling species complexes can provide insights into species diversity and speciation processes. *Alpheus angulatus* and *A. armillatus*, two snapping shrimp species with overlapping ranges in the north-western Atlantic, are similar in morphology, exploit similar ecological niches and appear to represent recently diverged sibling species. We examined phylogenetic and ecological relationships between these two species with: (i) sequence data from two mitochondrial genes (16*S* rRNA and *COI*); (ii) data on potential differences in microhabitat distribution for *A. armillatus* and *A. angulatus*; and (iii) data from laboratory experiments on the level of reproductive isolation between the two species. DNA sequence data suggest *A. armillatus* and *A. angulatus* are sister species that diverged subsequent to the close of the Isthmus of Panama, and that haplotype diversity is lower in *A. armillatus* than in *A. angulatus*. Both species are distantly related to *A. heterochaelis* and *A. estuariensis*, two species with which *A. angulatus* shares some similarities in coloration. Ecological data on the distribution of *A. angulatus* and *A. armillatus* from two locations revealed differences in distribution of the two species between habitat patches, with each patch dominated by one or the other species. However, there was no apparent difference in distribution of the two species within habitat patches with respect to microhabitat location. Ecological data also revealed that heterospecific individuals often occur in close proximity (i.e. within metres or centimetres) where sympatric. Behavioural data indicated that these species are reproductively isolated, which is consistent with speciation in transient allopatry followed by post-divergence secondary contact. Our data further resolve taxonomic confusion between the sibling species, *A. armillatus* and *A. angulatus*, and suggest that sympatry in areas of range overlap and exploitation of similar ecological niches by these two recently diverged species have selected for high levels of behavioural incompatibility.

Keywords: *Alpheus*, ecological divergence, mtDNA, reproductive isolation, sibling species, speciation

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Introduction

Speciation has long been thought to involve a process of genetic divergence between populations coupled with a secondary acquisition of morphological differences (Mayr 1963). However, in some cases, daughter species accumulate genetic differences without accompanying morphological divergence. In these cases, distinction by human observers may be difficult or impossible by morphological characters alone. Such cases are known as 'sibling species' groups or complexes, and this phenomenon is by no means rare (Mayr 1963, 1969). Taxonomic studies of marine groups have been especially plagued by the prevalence of sibling species. Sibling species have been described as 'ubiquitous' among marine groups, especially invertebrates (Knowlton 1993).

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Marine sibling species occur commonly in sympatry at least over part of their ranges (Knowlton 1993). This is inconsistent with the traditional view that marine species, especially those with planktonic dispersal stages in development, should be characterized by low levels of genetic differentiation among populations. This traditional view implies that most marine speciation processes should occur after rare colonization events, such as transoceanic migration, or major geographical processes, such as the closing of the Isthmus of Panama. This paradigm has long been supported by substantial empirical evidence revealing little genetic structure over broad spatial scales in apparently highly dispersive marine species (Palumbi 1992). However, a number of investigators have found evidence for genetic differentiation among populations of species with potentially dispersive larval stages (Benzie & Williams 1997; Palumbi *et al*. 1997), which has been attributed either to selection (Koehn *et al*. 1976, 1984; Hedgecock 1986) or larval behaviour (Burton & Feldman 1982). In addition, a number of authors have suggested the importance of vicariant events or local ecological barriers to gene flow in leading to population differentiation in marine species (Reeb & Avise 1990; Benzie & Williams 1997; Hellberg 1998). This suite of recent empirical work introduces the possibility of a paradigm shift in marine population genetics, and some authors have suggested that population differentiation over relatively small scales may not be unusual in marine species (Palumbi 1994; Hilbish 1996).

Although sibling species complexes have been documented in a broad range of marine invertebrates, those among the decapod crustaceans are of particular interest for a number of reasons. First, they are among the most diverse of the marine invertebrate groups and comprise extensive commercial fisheries, making species identification and conservation of particular importance. Second, because this group is characterized by a sclerotized exoskeleton and an associated diversification in ecological specialization, life history pattern, and body form, members of this group generally possess a comparably large number of phylogenetically informative morphological characters (relative to soft bodied invertebrates). As a result, the presence of decapod sibling species complexes that are morphologically indistinguishable suggests relatively recent divergence from a common ancestor, rather than either an older divergence followed by little morphological change or convergence in morphology because of similar ecological specialization. This factor makes decapod sibling species complexes particularly useful for studies of speciation processes and marine biogeography.

The genus *Alpheus* is a highly speciose caridean taxon of at least 250 recognized species (Kim & Abele 1988), many of which represent unresolved complexes of species that are difficult to distinguish morphologically (Knowlton & Weigt 1998). Together with members of the genus *Synalpheus*, these small decapods, commonly called snapping shrimp, have a worldwide distribution in tropical and subtropical habitats (Banner & Banner 1966; Chace 1988; Kim & Abele 1988), and display a range of ecological specializations, from apparently obligate associations with animal hosts such as sponges or anemones, in some cases combined with eusociality (Knowlton 1980; Duffy 1996), apparently facultative associations with burrow-dwelling thalassinidean shrimps (DLF personal observation) and gobies (Karplus 1987), to socially monogamous, burrow-constructing assemblages (Nolan & Salmon 1970). However, except in cases of extreme ecological specialization, most snapping shrimp species are very similar in morphology, even in the presence of high genetic or protein divergence (Knowlton *et al*. 1993; McClure & Greenbaum 1994; Knowlton & Weigt 1998). This has led to taxonomic confusion, and decapod taxonomists working in the western Atlantic and the Caribbean have long informally recognized the existence of multiple 'species complexes' (Knowlton & Mills 1992). *Alpheus angulatus* is extremely similar in morphology to *A. armillatus*, its putative sibling species (*A. angulatus* keys to *A. armillatus* according to Chace 1972 and Abele & Kim 1986). *A. angulatus* is distinguishable from *A. armillatus* only by the shape of the margin of the ventral carapace and by colour patterns (McClure 1995), which, though consistent in live specimens, fade rapidly in specimens stored in preservatives. Although colour differences can be of value in taxonomic identification (Knowlton & Mills 1992; Chan & Chu 1996), intraspecific variation in individual colour patterns both between and among populations of some crustacean species (Thacker *et al*. 1993; Ra'anan & Sagi 1985; McGaw *et al*. 1992) requires that colour differences be used cautiously in diagnoses. Superficial similarities in coloration have led some workers to confuse *A. angulatus* with other alpheids, including *A. heterochaelis* and *A. estuariensis* (McClure & Greenbaum 1994; McClure 1995), despite clear morphological differences.

Although *A. angulatus* and *A. armillatus* may have different overall ranges, with *A. angulatus* having more temperate distribution, they overlap along much of the Atlantic coast of Florida and possibly in the Caribbean (Chace 1972; L. M. Mathews, personal observation). Previous collections at Ft. Pierce, FL (this study) suggested that these sibling species also exploit nearly identical microhabitats.

The purpose of this study was to elucidate the phylogenetic and ecological relationships of the putative sibling species *A. armillatus* and *A. angulatus* in the western Atlantic and Gulf of Mexico. Three different lines of investigation were used. We sequenced DNA of two mitochondrial genes from the two putative sibling species as well as three (two Atlantic, one Pacific) other species of the genus to examine genetic differentiation and phylogenetic relationships. We also sampled natural populations in south Florida to obtain data on possible divergence in ecological specialization and microhabitat preferences of the two

Table 1 List of species, collecting sites, collection numbers and known distributions of species used in genetic analysis

Species	Site(s)*	Collection numbers†	Distribution‡
Alpheus angulatus	1	4585, 4586	North Carolina & Gulf of Mexico, USA, to Haiti[1]
	3	4590	
	4	4588	
	5	4589	
Alpheus armillatus	1	4584	North Carolina, USA to Sao Paulo, Brazil[2]
	2	4587	
Alpheus estuariensis	1	4582	Florida & Gulf of Mexico, USA to Caribbean Sea[3]
Alpheus heterochaelis	1	4583	North Carolina, USA to Surinam[2]
Alpheus tenuis	6	4581	Panama (Pacific Coast)[4]
Automate gardineri	1	4580	Atlantic: North Carolina, USA to Yucatan Peninsula, Mexico[2]

*Site numbers refer to locations listed in Fig. 1.
†University of Louisiana at Lafayette Zoological Collection.
‡As described previous to this study.
[1]McClure (1995); [2]Chace (1972); [3]Christoffersen (1984); [4]Kim & Abele (1988).

putative sibling species. Finally, we conducted laboratory experiments to obtain data on reproductive isolation between *A. armillatus* and *A. angulatus*.

Materials and methods

Shrimp were collected from a total of six sites (Table 1, Fig. 1) in 1998 and 1999 and identified to species with the keys of Chace (1972), Abele & Kim (1986), and Kim & Abele (1988). Individuals of the putative sibling species, *Alpheus angulatus* and *A. armillatus*, were identified by coloration: individuals of *A. armillatus* have distinct dark bands transversely across the abdomen, and white speckles concentrated on the chelae, whereas individuals of *A. angulatus* are pale brown to olive green, with no banding pattern and no speckling (McClure 1995). Our collections did not include individuals of intermediate coloration.

Shrimp for use in the genetic analysis were preserved in 70% ethanol. All other shrimp were returned live to the Smithsonian Marine Station at Fort Pierce (SMS) or the University of Louisiana at Lafayette (UL) for measurements and use in behavioural experiments.

Genetic data for phylogenetic reconstruction

Mitochondrial DNA (mtDNA) sequence data were obtained from eight individuals of each of the two sibling species: two *A. angulatus* from each of four collecting locations, and four *A. armillatus* from each of two collecting locations (Fig. 1, Table 1). In addition, we included in the genetic analysis one individual from each of three other congeners (Fig. 1, Table 1) and one individual of *Automate gardineri* Coutière 1902. We extracted DNA from abdominal muscle of fresh or recently (within one year) preserved individuals using a phenol–chloroform method (Kocher *et al*. 1989). The extracted DNA was precipitated with 100% ethanol and sodium acetate, and then rinsed with 70% ethanol. Dried DNA was resuspended in TE buffer.

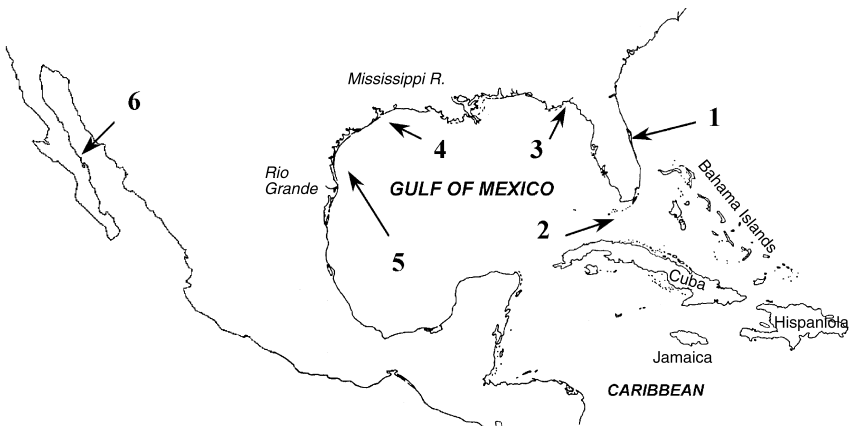

Fig. 1 Map showing collecting locations of all species used in genetic analyses. 1, Ft. Pierce, Florida, USA (FPFL); 2, Key West, Florida, USA (KWFL); 3, Florida State University Marine Laboratory, Florida, USA (FSUML); 4, Galveston, Texas, USA (GLTX) (supplied by M. McClure); 5, Port Aransas, Texas, USA (PATX); 6, Playa Santispac, Mexico (PSMX).

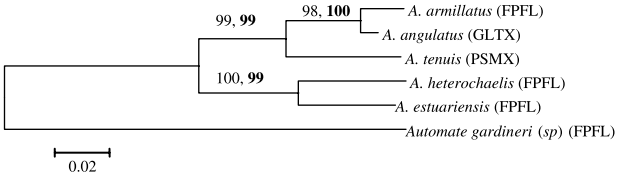

Fig. 2 Phylogenetic tree for species of *Alpheus* (A) and *Automate gardineri* based on 16*S* rRNA mitochondrial gene (most common haplotypes). Numbers above the branches are confidence values from a maximum parsimony (MP) analysis (2000 bootstrap replications); length = 238, consistency index (CI) = 0.882, retention index (RI) = 0.708, or from a neighbour-joining (NJ) analysis (Kimura 2-parameter, 2000 bootstrap replications; in bold). (See Fig. 1 for collecting location codes).

For polymerase chain reactions (PCR), we used the primers 16S-L2 (5′-TGCCTGTTTATCAAAAACAT-3′; designed by CDS) and 16S-1472 (5′-AGATAGAAACCAACCTGG-3′; Schubart *et al*. 2000) to amplify a 545 bp region of the large subunit ribosomal RNA (16*S*) gene, and the primers COIa (5′-AGTATAAGCGTCTGGGTAGTC-3′) and COIf (5′-CCTGCAGGAGGAGGAGAYCC-3′; Palumbi *et al*. 1991) or COI-H4 (5′-GGYATACCRTTDARTCCTARRAA-3′; designed by CDS) to amplify a 639 bp region of the cytochrome *c* oxidase subunit I (*COI*) gene. PCR reactions were carried out in 25 μL volumes containing each primer at 20 μM, 1.25 mM dNTPs, 10× buffer, Taq Gold polymerase®, template DNA, and millipore water. To amplify the 16*S* gene, samples were held at 94 °C for 10 min and then underwent 38–40 cycles of 98 °C for 1 min, 48 °C for 2 min, and ramped to 72 °C in 2 min, where they remained for 2 min. To amplify the *COI* gene, samples underwent 38–40 cycles of 98 °C for 1 min, 48–55 °C for 2 min, and ramped to 72 °C in 2 min, where they remained for 2 min. PCR products were purified with Microcon 100 filters, and then underwent a sequencing PCR reaction. Products were spin filtered in Sephadex columns, dried, resuspended in 20–22 μL of resuspension buffer, and run on an ABI Prism 310 automated sequencer.

For all samples, both forward and reverse strands were sequenced for confirmation of sequences. Sequences were aligned manually using the program ESEE Version 3.0 (Cabot & Beckenbach 1989). We constructed phylogenetic trees using distance methods with the program MEGA Version 2.0, and maximum parsimony with PAUP, using *Automate gardineri* (Fig. 2) or *Alpheus tenuis* (Figs 3 and 4) as outgroups. We compared differences in haplotype diversity (H, as defined by Nei 1987) between *A. angulatus* and *A. armillatus* with a randomization procedure. The absolute value of the observed difference $|H_{angulatus} - H_{armillatus}|$ was compared with the distribution of values for 20 000 replicates in which the haplotypes were randomly shuffled between the two species. A P-value was estimated as the proportion of times the value for a randomization equalled or exceeded the observed difference between the species. For this test, α was set at 0.05.

Field data on ecological distribution

Populations of *A. angulatus* and *A. armillatus* were surveyed in the vicinity of Fort Pierce, FL to quantify differences in microhabitat use as possible evidence of ecological specialization. Because adults of these two species are apparently restricted to intertidal habitats with rocky, gravel or shell substrate (open sand or mud shores are not inhabited), *A. angulatus* and *A. armillatus* occur in small habitat patches or 'islands' at Fort Pierce, between which adult movement is probably restricted. We surveyed two such habitat patches to examine both within- and between-patch differences in distribution of these sibling species. The two patches were located in Fort Pierce Inlet and were separated by ≈ 1 km of open water. Within-patch variation in microhabitat structure seemed largely restricted to differences in depth (i.e. upper vs. lower intertidal). Between-patch variation was more pronounced. Patch 1 was located along the shore, and consisted of sand, mud and gravel substrate with scattered larger rocks serving as cover objects for shrimp and other invertebrates and fish. Patch 2 was located ≈ 100 m from shore and was surrounded on all sides by seagrass beds and soft-bottom areas in shallow subtidal waters. Substrates consisted of sand, gravel and shell rubble, with no large rocks serving as cover objects. The two patches were divided by visual inspection into lower, medium, and upper intertidal areas, and two 1-m² quadrats were selected haphazardly in each range for both of the two habitats (total of 12 quadrats). All shrimp in each quadrat were collected by removing upper substrates to a depth of ≈ 30 cm and gathering shrimp by hand as they were exposed. In the field, the species, sex, pairing status and position of all shrimp collected were recorded. Chi-square tests with α = 0.05 were used to analyse the data for differences in distribution between habitats and among tidal height ranges within each habitat.

Behavioural compatibility experiment

We conducted laboratory pairing and mating experiments to assess the level of reproductive isolation between individuals of *A. angulatus* and *A. armillatus* collected in the zone of sympatry. Members of the genus *Alpheus* are socially monogamous (Nolan & Salmon 1970; Schein 1975; Knowlton 1980), and for the two sibling species, ≈ 85% of individuals of *A. angulatus* and 82% of individuals of *A. armillatus* individuals in Fort Pierce populations were collected as heterosexual pairs (LMM personal observation). Pair-living alpheids display high levels of aggression toward nonmates (Schein 1975; Knowlton & Keller 1982),

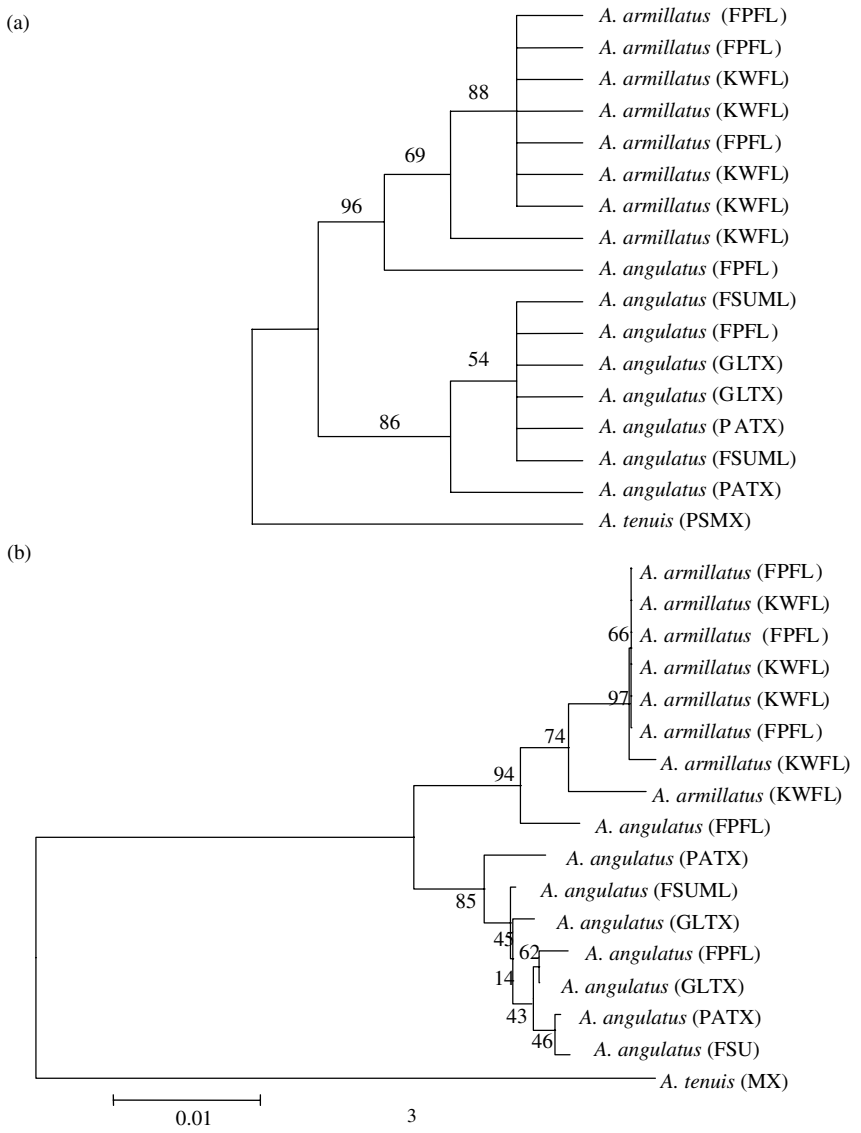

Fig. 3 Phylogenetic tree based on 16*S* rRNA mitochondrial gene for *Alpheus armillatus*, *A. angulatus* and *A. tenuis*. (a) MP analysis (2000 bootstrap replications); length = 60, consistency index (CI) = 0.867, retention index (RI) = 0.906. (b) NJ tree (Kimura 2-parameter, 2000 bootstrap replications). (See Fig. 1 for collecting location codes).

and intrasexual and interspecific pairings are rare or non-existent (LMM personal observation). Knowlton *et al*. (1993) used intersexual aggression in conspecific and heterospecific encounters as a measure of reproductive isolation between pairs of sister taxa from the Caribbean and eastern Pacific. In this study, we used two outcomes of behavioural interaction to detect reproductive isolation: first, the establishment of a heterosexual pair cohabiting a burrow, and second, the production of a fertile clutch of eggs by experimental females.

All shrimp used in reproductive isolation tests were collected from three different habitat patches in Fort Pierce Inlet, separated by ≈ 1 km of open water. In each trial, two opposite-sex shrimp size-matched to within 0.5 mm carapace length were placed in a ≈ 10 L test chamber with water, sand and a single artificial burrow (constructed by cutting two ≈ 2-cm diameter holes into the sides of ≈ 0.25-L plastic containers). Snapping shrimp readily accept artificial burrows and seem to treat them as they would natural burrows (see Mathews 2002). The positions of both test shrimp were noted daily until the female test shrimp moulted (mating occurs shortly after female moult: Nelson 1991). The duration of the experiment ranged from 8 to 22 days for each replicate. Test shrimp formed a 'pair' if both shrimp cohabited the artificial burrow for > 50% of all days sampled. On the first daily observation after the test female moulted, the shrimp were separated and the female was examined for eggs. Test females that had begun brooding a clutch of eggs were held for 3 days, at which time females were again examined for the presence of eggs (infertile eggs are usually abandoned after 1–2 days), and any remaining eggs were examined for development. In

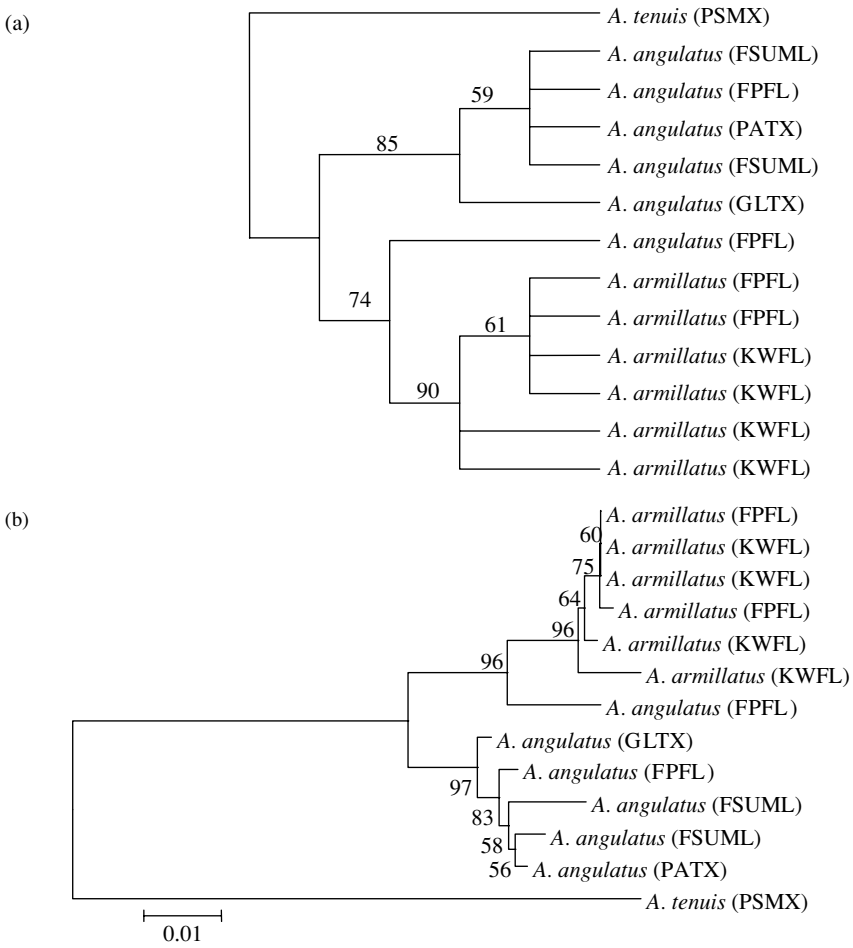

Fig. 4 Phylogenetic tree based on the cytochrome oxidase I (*COI*) mitochondrial gene for *Alpheus armillatus*, *A. angulatus* and *A. tenuis*. (a) MP analysis (2000 bootstrap replications); length = 127, consistency index (CI) = 0.882, retention index (RI) = 0.858. (b) NJ tree (Kimura 2-parameter, 2000 bootstrap replications). (See Fig. 1 for collecting location codes).

Table 2 Treatments and results of reproductive incompatibility experiment for two species of *Alpheus*

			Trials in which pair formed		
Female	Male	Total trials	Fertile clutches	Infertile clutches	Trials in which no pair formed*
A. armillatus	*A. armillatus*	14	12	0	2
A. armillatus	*A. angulatus*	11	0	2	9
A. angulatus	*A. armillatus*	10	0	0	10
A. angulatus	*A. angulatus*	16	16	0	0

*No females produced clutches (fertile or infertile).

this experiment, there were four treatments with different intra- and interspecific pairings of males and females (Table 2).

For replicates of the interspecific treatments in which test animals did not form a pair, we looked at the outcome of the trial as a rough measure of 'competitive ability'. Because snapping shrimp compete over 'ownership' of burrows (Mathews 2002), we judged that the shrimp residing inside the burrow in these trials was the 'winner' of an agonistic contest between the two shrimp. We used chi-square tests to analyse the data for both species and sex differences in the outcome of contests.

Results

Genetic analysis

We obtained sequence data for the 16*S* rRNA gene from eight individuals each of *Alpheus armillatus* and *A.*

angulatus, and one individual each of *A. tenuis*, *A. heterochaelis*, *A. estuariensis* and *Automate gardineri* (GenBank Accession nos. AF501630–AF501649). The primer combinations COIa and COIf or COI-H4 amplified the *COI* gene in six individuals each of *A. armillatus* and *A. angulatus*, and the individual of *A. tenuis* (GenBank Accession nos. AF501650–AF501662). The single heuristic search using a tree-bisection-reconnection (TBR) branch swapping algorithm yielded: (i) trees of length 238 for species-level comparisons of 16*S* rRNA (544 positions: 173 variable and 75 parsimony-informative positions, Fig. 2a); (ii) trees of length 60 for population-level comparisons of 16*S* rRNA (545 positions: 49 variable and 15 parsimony-informative positions, Fig. 3a); and (iii) trees of length 127 for population-level comparisons of *COI* (640 positions: 104 variable and 29 parsimony-informative positions, Fig. 4a). Combined (16*S* and *COI*) trees showed similar topologies with bootstrap values of 93/74 (neighbour joining/maximum parsimony) for the monophyletic *A. armillatus* sequences, and 95/94 for the monophyletic group including all *A. armillatus* sequences and one *A. angulatus* sequence.

Analysis of mtDNA sequence data indicates that *A. armillatus* and *A. angulatus* are sister taxa, which is consistent with strong morphological similarity between these species (Fig. 2). The data also indicate that *A. armillatus* and *A. angulatus* are relatively distant in relation to *A. heterochaelis* and *A. estuariensis*, two species with which *A. angulatus* has been confused by some workers because of some similarities in coloration (McClure & Greenbaum 1994; McClure 1995). *A. armillatus* and *A. angulatus* formed a clade with *A. tenuis*, a Pacific species that may be considered a transisthmian 'sister' taxon to the Caribbean and Atlantic armillatus-angulatus group (see Knowlton & Weigt 1998). Divergence between the sister taxa *A. armillatus* and *A. angulatus* appears to postdate their divergence from *A. tenuis*. Divergence values measured with the Kimura 2-parameter model between *A. angulatus* and *A. armillatus* sequences ranged from 1 to 3% for 16*S* and 2 to 5% for *COI*. Divergence between *A. tenuis* and *A. armillatus* for 16*S* was 9% and for *COI* was 15–17%, whereas divergence between *A. tenuis* and *A. angulatus* for 16*S* was 8–9% and for *COI* was 14–16%.

Our 16*S* sequence data included eight unique haplotypes of eight individuals of *A. angulatus* and three unique haplotypes of eight individuals of *A. armillatus*. The difference in haplotype diversity was statistically significant at $P = 0.007$.

Each set of mtDNA sequences (16*S* and *COI*) was resolved into two distinct lineages that corresponded to *A. armillatus* and *A. angulatus*, except for the sequences from one individual of *A. angulatus*. Both maximum parsimony and neighbour-joining analyses placed this sequence in sister relationships to all of the sequences from *A. armillatus* (Figs 3 and 4). Kimura 2-parameter divergence values between this individual and all *A. angulatus* sequences were 2–3% for 16*S* and 3–4% for *COI*. Kimura 2-parameter values between this individual and all *A. armillatus* sequences were 1% for 16*S* and 2–3% for *COI*. This specimen, which was collected singly (not as a member of a heterosexual pair), had typical *A. angulatus* coloration. Our data show no evidence for phylogeographical subdivision within either *A. angulatus* or *A. armillatus* (Figs 3 and 4).

Field data on ecological distribution

Individuals of both *A. angulatus* and *A. armillatus* were collected from patches 1 and 2. Most individuals of both species were collected as heterosexual pairs (89% of *A. angulatus*, 94% of *A. armillatus*); no interspecific pairs were collected in the field. *A. armillatus* was significantly more common at patch 1 ($\chi^2 = 25.00$, 1 df, $P < 0.0001$), and *A. angulatus* was significantly more common at patch 2 ($\chi^2 = 312.71$, 1 df, $P < 0.0001$, Fig. 5). There were no significant differences in the distributions of either species with respect to tidal height at either patch (*A. armillatus*: patch 1, $\chi^2 = 1.20$, 2 df, $P = 0.5488$; patch 2, $\chi^2 = 3.00$, 2 df, $P = 0.0833$; *A. angulatus*: patch 1, $\chi^2 = 5.43$, 2 df, $P = 0.0663$; patch 2, $\chi^2 = 4.94$, 2 df, $P = 0.0844$).

Behavioural compatibility experiment

Of 21 trials, a total of 2 interspecific pairs formed (Table 2). In both cases, these interspecific pairs consisted of a male of

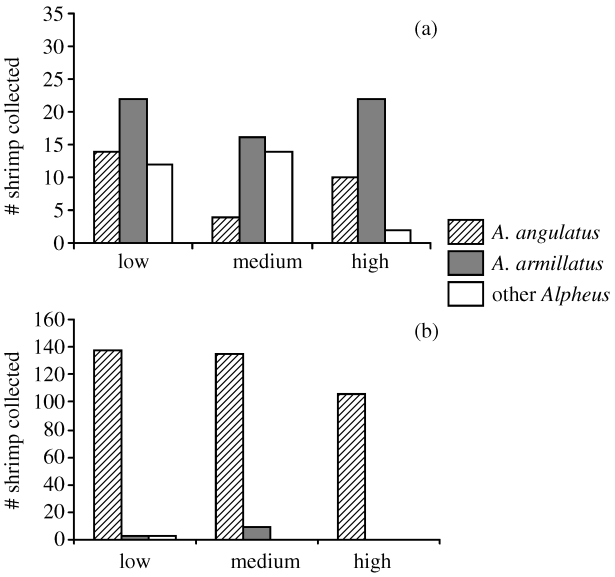

Fig. 5 Total numbers of *Alpheus angulatus* and *A. armillatus* collected in two 1-m² plots in each of three habitat types (low, medium and high intertidal) of (a) site 1 and (b) site 2.

A. angulatus and a female of *A. armillatus*, and in both cases, the female produced an infertile clutch after moulting. In all other interspecific trials, test shrimp were never found cohabiting the burrow for any portion of the trial period. Most intraspecific trials resulted in the formation of cohabiting pairs (Table 2), and in all cases in which an intraspecific pair formed, females produced a fertile clutch of eggs after moulting. For interspecific trials in which no pair formed, there was no significant difference in the tendency for individuals of *A. angulatus* or *A. armillatus* to control access to the burrow (*A. angulatus*: $n = 8$; *A. armillatus*: $n = 11$; $\chi^2 = 0.47$, 1 df, $P = 0.4913$).

Discussion

The mitochondrial sequence data resolve phylogenetic relationships among *Alpheus armillatus*, *A. angulatus* and other members of the genus. *A. angulatus* is most closely related to *A. armillatus*, its sibling species, and is relatively distant in relationship to *A. heterochaelis* and *A. estuariensis*, two species with which *A. angulatus* has on occasion been confused in some collections (McClure & Greenbaum 1994; McClure 1995), despite distinct morphological differences. Furthermore, *A. angulatus* and *A. armillatus* are more closely related to *A. tenuis*, a Pacific species, than to the other Atlantic species we collected, indicating a relatively old split between the armillatus-angulatus group (including at least *A. armillatus*, *A. angulatus* and *A. tenuis*) and the heterochaelis-estuariensis clade.

Because the Caribbean and Atlantic species *A. armillatus* and *A. angulatus*, and the Pacific species *A. tenuis*, are all species that occur primarily in intertidal and shallow subtidal areas (Chace 1972; Kim & Abele 1988), the separation between the Pacific species *A. tenuis* and Atlantic species *A. armillatus* and *A. angulatus* must predate the closing of the Isthmus of Panama (Knowlton & Weigt 1998), which occurred ≈ 3 mya (Coates *et al*. 1992). Based on the estimate of 2.2–2.6% divergence per million years for *COI* (Knowlton *et al*. 1993), the Pacific species *A. tenuis* diverged from the Atlantic species *A. armillatus* and *A. angulatus* ≈ 6–8 mya, whereas *A. angulatus* and *A. armillatus* diverged from one another 1–2.5 mya. For the 16*S* sequence data, based on the estimate of 0.88% divergence per million years (Schubart *et al*. 2000), separation between *A. tenuis* and the Atlantic species *A. angulatus* and *A. armillatus* occurred 9–10 mya, and separation between *A. angulatus* and *A. armillatus* occurred 1–3.5 mya. These estimates show that divergence between *A. angulatus* and *A. armillatus* occurred more recently than the divergences previously reported by Knowlton & Weigt (1998) for *Alpheus* sibling species pairs that occur sympatrically in the Caribbean or Pacific coasts of Panama; divergences between those intraoceanic species pairs all predated divergences between transisthmian pairs (Knowlton & Weigt 1998). The separation between *A. armillatus* and *A. angulatus* may have occurred in association with transient allopatry resulting from late Pliocene or early to mid-Pleistocene climate changes and associated range expansions and/or contractions. These data provide more empirical support to the growing body of evidence (Hellberg 1998; Marko 1998; Barber *et al*. 2000) suggesting that strong or semi-permanent geographical barriers (i.e. ocean basins, Isthmus of Panama) are not required for population differentiation and eventual speciation, even in marine taxa with potentially dispersive larvae.

In this study, sequences from individuals of *A. angulatus* were paraphyletic. The sequence data from *A. angulatus* and *A. armillatus* fell into two distinct clades (Figs 3 and 4), with the exception of one individual, which was identified morphologically (by coloration and the shape of the ventral carapace margin: McClure 1995) as *A. angulatus*, but grouped with the clade containing sequences from individuals identified morphologically as *A. armillatus*. Data from this individual may suggest that colour patterns in this clade are either not consistent or have not been adequately described to serve as identifying characters (i.e. the individual was of *A. armillatus* but was misidentified as *A. angulatus*). However, in a collection totalling 467 shrimp, no individuals were collected that appeared to be intermediate in coloration, i.e. that could not be clearly categorized as either *A. armillatus* (transverse bands across abdomen) or *A. angulatus* (solid coloration with no banding). There are a number of alternative explanations for the paraphyly of the *A. angulatus* sequences. First, the unusual sequences (16*S* and *COI* from one individual) may be the result of introgression between the two gene pools following secondary contact. Second, the unusual sequences may indicate that we sequenced a pseudogene from this individual rather than functional mtDNA sequence. Williams & Knowlton (2001) report that mitochondrial pseudogenes may be common in snapping shrimp species; although they investigated only possible duplications of the *COI* gene, duplication events may transfer segments much larger than a single gene. This possibility warrants further investigation, perhaps by analysis of sequence from the nuclear genome. However, we found no evidence for the amplification of multiple sequences from this individual; sequences were of similar quality to 16*S* or *COI* sequences obtained from other individuals, and we did not observe double signals. Furthermore, it is unclear why the same primer pairs and reaction conditions would have amplified a pseudogene in one individual but not in any others of the same species. As a third possible explanation, in such recently diverged species, paraphyly is also likely to be the result of the persistence in both species of mtDNA lineages that predate the divergence between the two species (e.g. see Schneider-Broussard *et al*. 1998). Neigel & Avise (1986) modelled the dynamics of mtDNA lineage sorting during

speciation, and found that recently diverged daughter species may be paraphyletic with respect to mtDNA lineages for some time after speciation. However, estimating the likelihood of paraphyly for this particular data set would require more extensive information on the population dynamics both during and after speciation (e.g. numbers of founders, effective population sizes, number of generations since divergence). Finally, the individual that yielded the unusual 16*S* and *COI* sequences may represent a third, as yet unidentified, taxon. The genus *Alpheus* has long posed taxonomic challenges because it presumably harbours large numbers of cryptic species groups (Knowlton & Keller 1983; Knowlton *et al*. 1993; Knowlton & Weigt 1998; Williams *et al*. 2001; DLF personal observation). More extensive sampling of similar habitats in the north-eastern Atlantic and the Caribbean, coupled with genetic and morphological examinations, may provide further insights into the taxonomic status of the individuals included in this study.

We found no evidence that populations of *A. angulatus* in the Gulf of Mexico and along the Atlantic coast of Florida are phylogeographically differentiated over the limited range from which we sampled. Because most members of the genus have relatively long free-living larval life histories (Knowlton 1973; Yang & Kim 1999), larvae may travel large distances before settling and thus there may be extensive gene flow among populations. Furthermore, suitable habitats appear to exist throughout southern Florida, without disjuncture.

We detected significantly higher 16*S* haplotype diversity for *A. angulatus* (eight haplotypes for eight individuals) than for *A. armillatus* (three haplotypes for eight individuals). This difference may be due to stochastic effects in these lineage's evolutionary histories. Alternatively, the difference in haplotype diversity may be a result of sampling over different geographical scales. We sampled more populations over a broader geographical scale for the more genetically diverse taxon, *A. angulatus*, than for *A. armillatus* (Table 1). However, we found no evidence for phylogeographical differentiation in either species, and infer that populations may be connected by high levels of gene flow. Finally, the difference in haplotype diversity is consistent with the transient geographical isolation of a small, genetically homogeneous subset (the lineage leading to *A. armillatus*) of a large ancestral population (the lineage leading to *A. angulatus*).

Our field data suggest that these two species may be weakly isolated ecologically where their ranges overlap. Where we sampled, both species occur in the same habitat patches, such that interspecific contact probably occurs regularly in the field. We found no evidence that these two species are isolated to different depth ranges within patches. However, one of the patches we sampled was heavily dominated by *A. angulatus*, though it is located < 1 km from areas (including site 1) where *A. armillatus* is more common. Data from the reproductive compatibility experiment showed no difference in interference competitive ability between these two species: in interspecific contests that did not result in pairing, neither species was markedly more likely to gain ownership of the burrow. Variation in local distribution of the two species may reflect differences in habitat preferences between these two species, or may result from interspecific exploitative competition or selective larval settlement patterns. However, our ecological data are limited to a small number of sampling locations; broader and more extensive sampling should yield stronger inferences into the ecological relationships of these two species.

Our field and laboratory data suggest strong reproductive isolation between these two sibling species. Among 467 individuals collected in Ft. Pierce, we found no interspecific pairs, even though (i) most of the populations of both species occurred in heterosexual pairs, and (ii) individuals of the two species often occurred in close proximity (i.e. < 10 cm) to heterospecific individuals. In our behavioural compatibility experiment, only 2 interspecific pairs formed in 21 trials, whereas intraspecific pairs formed in 28 of 30 trials, all resulting in the production of fertile clutches. Neither interspecific pairing resulted in fertile clutches, although in both cases, the female did release and brood infertile eggs for 1–2 days, suggesting that mating may have occurred (females in other interspecific trials in which no pair formed did not release and brood eggs after moulting). Our data are in contrast to those of Knowlton *et al*. (1993), who found that heterospecific individuals in transisthmian sister species pairs showed some level of behavioural compatibility in laboratory experiments and a low frequency of fertile clutches. The behavioural incompatibility that separates individuals of *A. armillatus* and *A. angulatus* may be the result of strong selection for reproductive isolation resulting from transient allopatry followed by secondary contact over a broad ecological range.

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