

Cryptic biodiversity and phylogeographical patterns in a snapping shrimp species complex

LAUREN M. MATHEWS

Department of Biology, Worcester Polytechnic Institute, 100 Institute Road, Worcester, MA 01609, USA

Abstract

Recent investigations suggest that marine biodiversity may be much higher than earlier estimates, and an important hidden source of diversity in marine systems is the phenomenon of cryptic species complexes. Such complexes are informative models for research into the evolutionary processes that govern species compositions of marine fauna. The snapping shrimp genera *Alpheus* and *Synalpheus* are known to harbour large numbers of cryptic species; here, I characterize the genetic structure of the *Alpheus armillatus* species complex in the northern Caribbean, west Atlantic, and Gulf of Mexico using mitochondrial and nuclear sequence data. Over this geographical region, the complex harbours at least three lineages that are probable reproductively isolated species; all major lineages diverged subsequent to the close of the Isthmus of Panama. Only one lineage was present in the Gulf of Mexico, whereas outside the Gulf of Mexico there was no clear tendency for lineage dominance by geographical region, as most sites were populated by shrimp from at least two lineages. However, within each lineage, there was strong evidence of population genetic differentiation between geographical regions. All lineages showed strong signals of demographic expansion, and one lineage showed sharply reduced genetic diversity, suggestive of past population bottlenecks or recently founded populations with low gene flow from other sites. These results show that evolutionary processes leading to divergence and speciation have been common and recent in the snapping shrimp, and suggest that connectivity among shrimp populations may be limited.

Keywords: Caribbean, cryptic species, genetic structure, marine biodiversity, phylogeography, snapping shrimp

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Introduction

Many marine habitats, especially tropical shallow-water areas, have long been known to harbour particularly high levels of species richness. Early measures of marine biodiversity may have greatly underestimated the true number of species, as the occurrence of sibling species, or species that are difficult to distinguish morphologically (Mayr 1963), is common in marine groups (Knowlton 1993). The high biodiversity characteristic of many shallow warm-water marine habitats presents an interesting conundrum. Most marine habitats have traditionally been thought to be highly interconnected by gene flow, because of the absence of obvious barriers to migration and because

many marine taxa have pelagic larval stages that have been considered passive dispersers (Palumbi 1994). According to this paradigm, populations of all but the least dispersive taxa are predicted to be genetically homogeneous (Palumbi 1994), and most marine speciation should occur in association with long-term geographical barriers to dispersal, such as the formation of the Isthmus of Panama, or rare colonization events that found new populations. In contrast, high levels of biodiversity in marine systems suggest that genetic divergence and eventual speciation may be common and rapid in marine systems. Resolving this conflict, on a general or taxon-specific level, has important implications for management of at-risk marine populations; for example, a clearer understanding of connectivity among marine populations may result in more effective design for marine-protected areas and reserves (Palumbi 1993).

Correspondence: Lauren Mathews, Fax: 508-831-5936; E-mail: lmathews@wpi.edu

Increasingly sophisticated and accessible tools for generating and analysing molecular genetic information has resulted in a burst of empirical research that addresses this tension between high biodiversity and apparently high connectivity. The picture that emerges indicates that marine systems seem to be governed by no simple set of rules that dictate the connectedness of populations. Rather, interrelationships among marine populations are apparently the outcome of complex and dynamic interactions between the physical and biological environment and the physiology, behaviour, and life histories of individual taxa. A number of investigations have revealed that gene flow among marine populations may be largely restricted, resulting in moderate to high levels of genetic divergence among populations over a range of spatial scales (Hedgecock 1986; Palumbi 1994; Duffy 1996a, b; Palumbi *et al.* 1997; Benzie 1999; Barber *et al.* 2002; Gutiérrez-Rodríguez & Lasker 2004; Perrin *et al.* 2004; Baratti *et al.* 2005; Bilodeau *et al.* 2005). In some cases, the degree of differentiation is apparently related to the mode of larval development, suggesting that in many taxa larval stages may indeed disperse over longer distances than benthic juveniles (Doherty *et al.* 1995; Todd *et al.* 1998; Collin 2001; Riginos & Victor 2001; Dawson *et al.* 2002). However, other studies have found that larval duration is not a good predictor of genetic differentiation (Shulman & Bermingham 1995). In some cases, taxa with long larval life histories show substantial population genetic differentiation (Moberg & Burton 2000; Taylor & Hellberg 2003), and Sponer & Roy (2002) report evidence for moderate dispersal in a brittle star with crawling juveniles. Indeed, some empirical and theoretical analyses suggest that larvae long thought to be highly dispersive may be retained locally to some degree (Jones *et al.* 1999; Swearer *et al.* 1999; Cowen *et al.* 2000; Swearer *et al.* 2002; Warner & Cowen 2002; Taylor & Hellberg 2003), such that the realized dispersal distance of a larva may be on average much shorter than the theoretical maximum dispersal distance (assuming passive dispersal). In addition, it is clear that populations with pelagic larvae may be separated by isolation-by-distance effects (Palumbi *et al.* 1997; Johnson & Black 1998; Planes & Fauvelot 2002; Uthicke & Benzie 2003; Maier *et al.* 2005) or phylogeographical breaks (e.g. Indo-West Pacific: Benzie 1999; Lessios *et al.* 2001; Williams *et al.* 2002; Amazon River outflow: Lessios *et al.* 2001; Rocha *et al.* 2002; Florida Gulf/Atlantic coasts: Reeb & Avise 1990; Avise 1992; Felder & Staton 1994; Young *et al.* 2002; other locations: Dawson 2001; Nikula & Väinölä 2003; Bilodeau *et al.* 2005) that may correlate to past or present barriers to gene flow. In general, it seems that the dispersal capabilities of larvae may frequently not be high enough to maintain homogeneity among populations.

Phylogeographical studies have the power to yield insights into past as well as present evolutionary processes, and have been used to uncover information about the roles of

geographical history and ecology in generating marine species diversity (e.g. Hellberg 1998; Lessios *et al.* 2001; Jablonski & Roy 2003; Williams & Reid 2004; Taylor & Hellberg 2005). In addition, careful investigation of broadly distributed species has revealed the presence of several probable cryptic species in bonefishes (Colborn *et al.* 2001), copepods (Lee 2000; Rocha-Olivares *et al.* 2001; Goetze 2003), seastars (Howell *et al.* 2004), and sponges (Klautau *et al.* 1999), and other examinations have revealed the presence of cryptic species over smaller spatial scales (brittle stars: Sponer & Roy 2002; mussels: Lee & Ó Foighil 2004, 2005; snapping shrimp: Thompson *et al.* 2005). Indeed, cryptic genetic divergence with no associated morphological divergence may be common in marine taxa (Knowlton 1993). The occurrence of several to many cryptic or sibling species (Mayr 1963) within a taxon represents what Gittenberger (1991) termed a 'nonadaptive' radiation.

Alpheus and the closely related *Synalpheus* have served as excellent models for phylogeographical studies (Knowlton *et al.* 1993; Duffy 1996a, b; Knowlton & Weigt 1998; Williams *et al.* 2001; Morrison *et al.* 2004). Members of these genera, commonly called snapping shrimp, are a ubiquitous component of macrofaunal assemblages in tropical and subtropical shallow-water marine habitats. The snapping shrimp genera are highly speciose: Chace (1988) recognized ~220 species of *Alpheus* (24 in the Caribbean: Chace 1972) and ~115 species of *Synalpheus*. However, both snapping shrimp genera are commonly thought to conceal substantial biodiversity as cryptic species. Because of the extensive empirical evidence for the presence of many undescribed cryptic or sibling species (Knowlton & Keller 1985; McClure & Greenbaum 1994; Knowlton & Weigt 1998; Bruce 1999; Williams *et al.* 2001; Thompson *et al.* 2005; Macdonald *et al.* 2006), the species diversity estimates for these genera are probably low.

Previous studies suggest that ecological pressures play an important role in diversification of both *Alpheus* (Williams *et al.* 2001) and *Synalpheus* (Duffy 1996a, b). However, many sibling species of *Alpheus* show no clear differences in ecological specialization and often occur in sympatry (Knowlton & Keller 1985; Mathews *et al.* 2002). Therefore, sibling species complexes in *Alpheus* may represent recent nonadaptive radiations (Gittenberger 1991) that occurred either in sympatry or in allopatry followed by secondary contact. Such species complexes have great potential to provide insights into the roles of vicariant events and gene flow in generating both population genetic and species diversity (e.g. see Lee & Ó Foighil 2004, 2005).

This study focuses on phylogeographical relationships among members of the *Alpheus armillatus* species complex. This complex currently includes two described species, *A. armillatus* (Milne-Edwards 1837) and *Alpheus angulosus* (McClure 1995; 2002), which are sympatric in southern Florida, USA (Mathews *et al.* 2002), and an unknown

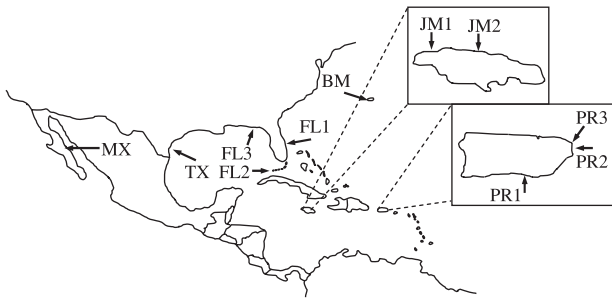


Fig. 1 Geographical locations of collecting sites and total numbers of *Alpheus* collected from each site. FL1 – Fort Pierce, Florida ($n = 154$); FL2 – Key West, Florida ($n = 162$); FL3 – Carrabelle, Florida (Florida State University Marine Laboratory) ($n = 10$); TX – Galveston and Port Aransas, Texas ($n = 4$); BM – Bermuda ($n = 19$); JM1 – Sandy Bay, Jamaica ($n = 67$); JM2 – Runaway Bay, Jamaica ($n = 58$); PR1 – Bosque Estatal de Aguirre, Puerto Rico ($n = 52$); PR2 – Bahía Demajagua, Puerto Rico ($n = 126$); PR3 – Las Croabas, Puerto Rico ($n = 45$); MX – Playa Santispac, Mexico ($n = 2$).

number of undescribed cryptic species. Chace (1972) originally described the range of *A. armillatus* as extending from North Carolina, USA to São Paulo, Brazil, including Bermuda and the Gulf of Mexico. Here, I used sequence data from one mitochondrial (16S ribosomal) and one nuclear (myosin heavy chain) gene to investigate evolutionary and population genetic relationships among *A. armillatus* complex populations in the northern Caribbean, west Atlantic, and Gulf of Mexico.

Materials and methods

Sample collection

Shrimp were collected from Florida in 1999 (FL3, Fig. 1) and 2004 (FL1 and FL2) and from Texas in 1999. Shrimp were collected from sites in Puerto Rico, Jamaica and Bermuda in 2005. All collecting was done by hand at low tides. The described species *Alpheus angulosus* and *Alpheus armillatus* are distinguishable by apparently consistent differences in colouration of the carapace (Mathews *et al.* 2002), despite morphological similarity, so it was possible that unknown sibling lineages would be characterized by colour differences. Therefore, at each site inhabited by different colour forms of *A. armillatus* complex shrimp, I collected ~50 or as many as possible of each colour form I detected.

Snapping shrimp are socially monogamous (Nolan & Salmon 1970; Knowlton 1980; Mathews 2002), and commonly occur in pairs in self-excavated burrows. For this study, shrimp were removed from their burrows by digging ~20 cm into the substrate and isolating pairs of shrimp in the holes. Wherever possible, both members of a pair were collected. Snapping shrimp are highly territorial,

defending their burrows from all nonpair conspecifics or heterospecifics (Mathews 2002). Therefore, I categorized shrimp as paired if they were found within 5 cm of one another, regardless of morphology, colouration, gender, or size. These data allow preliminary inferences on pre-mating isolation among lineages. In the field, shrimp were placed as pairs or individuals (as collected) in separate 50 mL centrifuge tubes (perforated for water exchange) within buckets of seawater, and were returned to laboratory holding facilities where available (Tropical Research Laboratory of Mote Marine Laboratory at FL2; Florida State University Marine Laboratory at FL3; Discovery Bay Marine Laboratory in Jamaica; Bermuda Biological Station for Research in Bermuda) or were housed in buckets with aeration until they could be processed. In either case, mortality before processing was minimal. At collection sites in the USA, shrimp were killed by freezing for ~10 min and were then placed in 70% ethanol or liquid nitrogen for return to the laboratory at Worcester Polytechnic Institute (WPI). At sites outside the USA, tissue samples were removed and placed in cell lysis solution from the Puregene DNA extraction kit (Gentra Systems) for easier transport to the laboratory at WPI. In the latter case, live specimens were also returned to the laboratory as vouchers.

DNA extraction, PCR, and genetic analysis

Genomic DNA was extracted from chelal or abdominal muscle tissue dissected from fresh, ethanol-preserved or frozen shrimp using the Puregene DNA extraction kit. Approximately 5–10 mg of tissue were used, and for abdominal tissue the gut was removed whole to avoid contamination by gut contents. Polymerase chain reaction (PCR) was performed on each DNA sample using the primers MyHC-1124 (5'-AAGCTCGAGTCTGACATCA-3') and MyHC-lottR (5'-CTTTCTCTGGTGACGGTG-3') (Williams *et al.* 2001) to amplify the nuclear MyHC gene, and the primers 16S-1472 (5'-AGATAGAAACCAACCTGG-3') (Schubart *et al.* 2000) and 16S-L2 (5'-TGCCTGTTTATCAAAAACAT-3') (Mathews *et al.* 2002) to amplify the 16S rRNA mitochondrial gene. For each gene, PCRs were carried out in 20 μ L volumes using 16 ng of genomic DNA, 0.75 μ M of each primer, 0.16 mM of each dNTP, 1 \times Thermopol buffer (New England Biolabs) and 1 U of *Taq* DNA polymerase (New England Biolabs). PCRs were performed with the following conditions: 95 $^{\circ}$ C for 2 min, followed by 40 cycles of 95 $^{\circ}$ C for 30 s, annealing temperature (48 $^{\circ}$ C for 16S, 60 $^{\circ}$ C for MyHC) for 30 s, and 72 $^{\circ}$ C for 60 s, followed by a final extension of 10 min at 72 $^{\circ}$ C. Reactions were sequenced directly in both directions on an Applied Biosystems 3100 automated sequencer at the University of Massachusetts Medical School Nucleic Acid Facility.

Additional 16S sequences from a previous study (Mathews *et al.* 2002) were obtained from GenBank. These

were sequences from shrimp identified as *A. angulosus* collected from Gulf of Mexico sites TX and FL3 (TX: AF501632, AF501633, AF501634, AF501635; FL3: AF501630 & AF501637).

For each gene, sequences were first assembled and edited with the SEQMAN module of LASERGENE version 6.1 (DNASTAR). For MyHC, heterozygous sites were indicated by double peaks in both forward and reverse sequences. Sequences for each gene were then aligned using the CLUSTAL W program (Thompson *et al.* 1994) implemented in BIOEDIT 7.0.4.1 (Hall 1999) and refined by eye. Though pseudogenes occur for other mitochondrial genes in *Alpheus* (Williams & Knowlton 2001), I found no evidence for 16S pseudogenes (double peaks, consistent problems obtaining good quality sequence) in this data set.

Phylogenetic analyses of the 16S data set

Phylogenetic analyses were carried out on the mitochondrial data set using both Bayesian and maximum-likelihood (ML) methods. Sequence data from *Alpheus viridari*, another intertidal alpheid morphologically similar to the *A. armillatus* complex, were generated for use as an outgroup. In addition, sequence data for the 16S rRNA gene from *Alpheus tenuis* (collected in Baja California, Mexico: AF501648), a cross-Isthmus-of-Panama geminate species to the *A. armillatus* complex (Mathews *et al.* 2002), were downloaded from GenBank and included in phylogenetic analyses. Hierarchical likelihood ratio tests were used to determine the best-fit model of nucleotide substitution using the program MRMODELTEST 2.2 (Nylander 2004). For both data sets, a general time reversible model with a proportion of invariant sites and gamma distributed rate heterogeneity (GTR + I + Γ) was used.

Bayesian analyses were performed using the program MRBAYES 3.0b4 (Huelsenbeck & Ronquist 2001) with both unconstrained and clock-constrained branch lengths. For each condition, Markov chain Monte Carlo (MCMC) searches were run with four chains for 1×10^6 generations, sampling every 100 generations. The number of generations before stationarity of likelihood values (the burn-in) was estimated graphically as 300 for both analyses. Bayes factors were used to compare the unconstrained and clock-constrained models using the harmonic means of the likelihood values from the stationary phase of the run (Newton & Raftery 1994). The guidelines of Kass & Raftery (1995) were used in interpretation of Bayes factors.

Maximum-likelihood methods were implemented in PAUP* 4.0b10 (Swofford 2003) using the heuristic search option with tree-bisection-reconnection (TBR) branch swapping and random addition of sequences. Node support was inferred with bootstrap analysis (100 replications). As for the Bayesian analysis, the ML analysis was run with and without a clock constraint on branch lengths, and the

two models were evaluated with a likelihood ratio test. For computational efficiency, a reduced data set was used for the ML analyses, including three randomly chosen representatives of each of the four major clades identified by the Bayesian analysis, in addition to one of the haplotypes from Bermuda, sequence from *A. tenuis*, and sequence from *A. viridari*.

Estimation of divergence times among the major clades under the assumption of a molecular clock was based on a mutation rate for the 16S gene of 2% per million years (Myr). This mutation rate was derived from a re-analysis of data from Morrison *et al.* (2004) for four geminate species pairs in the closely related genus *Synalpheus*, including the Pacific species *S. bannerorum* (AY344737), *S. fritzmuelleri* (AY344750), *S. brevicarpus* (AY344741), *S. digueti* (AY344747), and their respective Atlantic geminates, *S. dominicensis* (AY344749), *S. fritzmuelleri* (AF230798), *S. brevicarpus* (AY344742), and *S. minus* (AY344761). Kimura 2-parameter (K2P) distances (calculated in MEGA 3.1) for these geminates ranged from 0.06 to 0.15. The K2P distances between the Pacific species *A. tenuis* and the Caribbean *A. armillatus* complex sequences generated in this study were similar, ranging from ~0.07–0.10. The lowest value from the synalpheid data set (0.06 for the geminates *S. digueti* and *S. minus*) was used to calculate a mutation rate of 2% per Myr. Geminates may show a range of genetic distances because some pairs began diverging some time prior to the close of the Isthmus of Panama (Knowlton & Weigt 1998); therefore, the divergences of pairs with the lowest genetic distances are probably more closely associated with the final close of the isthmus 3 million years ago (Mya; Coates & Obando 1996).

Analysis of MyHC data set

For the MyHC data set, the program PHASE 2.1.1 (Stephens *et al.* 2001) was used to reconstruct the haplotype phase for MyHC alleles for the MyHC data set as a whole. PHASE uses Bayesian inference to reconstruct haplotypes with comparatively low error rates, and provides estimates of uncertainty associated with each prediction (Stephens *et al.* 2001). Seven separate runs of 100 iterations each were carried out to ensure accurate haplotype estimation; all runs gave highly consistent haplotype frequency estimates. An a priori confidence limit of 60% was set for acceptance of allele calls, with any calls below this limit to be eliminated from the data set.

The reconstructed MyHC data set included only 13 parsimony-informative sites, and yielded trees (by both Bayesian and ML methods) with poor resolution (not shown). For the subset of MyHC data for which 16S data were also available from the same individuals, MyHC allele frequency distributions were examined for association with 16S haplotype clade. MyHC alleles were divided into

four groups corresponding to the four major 16S clades, and allele frequencies were compared by pairwise *G*-tests of independence with Yates's correction for continuity (Sokal & Rohlf 1995). Alleles that were present in fewer than five copies in the entire data set were excluded from *G*-tests. A Bonferroni correction of α was used to evaluate statistical significance.

Within-clade analyses of the 16S data set

Median-joining networks were created for each of the four major clades identified by Bayesian and ML analyses with the program NETWORK 4.1.1.2 (Bandelt *et al.* 1999) with equal weights for variable sites. For each clade, haplotype (Nei 1987) and nucleotide (Tajima 1983) diversities were calculated using the program ARLEQUIN 3.01 (Excoffier *et al.* 2005). An analysis of molecular variance (AMOVA; Excoffier *et al.* 1992) was used to examine overall genetic structure within each clade. Pairwise F_{ST} values were calculated for each clade by pooling samples into geographical units that were likely to be biologically meaningful, as follows. All three collecting sites in Puerto Rico are located within ~100 km of coastline from one another, so within-clade sequences from PR1, PR2, and PR3 were grouped together. Likewise, within-clade sequences from the two collecting sites in Jamaica (~75 km apart) were pooled. Clade 1 contained sequences from both FL1 ($n = 11$) and from FL2 ($n = 2$), which were not pooled because these sites are ~450 km apart. Therefore, sequences from FL2 were excluded from the analysis because of small sample size. For clade 3, AMOVAs were carried out between sequences from FL3 and FL1, and sequences from TX were not considered because of small sample size ($n = 4$). The significance of F_{ST} measures was assessed using the permutation procedure of Excoffier *et al.* (1992) with 1000 permutations. Because phylogenetic and network analyses (Fig. 2) and the AMOVA (Table 2) all indicated strong separation between two subclades within clade 4, all other within-clade analyses were performed on subclades 4a and 4b separately.

Demographic analyses were carried out separately for each of the clades detected in Bayesian and ML analyses. Tajima's (1989) *D* statistic was calculated in ARLEQUIN 3.01. Past population expansions result in large negative values for Tajima's *D* (Aris-Brosou & Excoffier 1996). Mismatch analysis, which considers the distribution of pairwise differences between haplotypes, was also carried out in ARLEQUIN 3.01. This program uses a least-squares approach to estimate parameters of demographic expansion, including the mutational expansion time τ . The expansion model was tested with 1000 bootstrap replications comparing the sum of square deviations between observed and expected mismatches. Timing of expansion events was estimated by the relationships $\tau = 2ut$, where t is the time since expansion in generations and u is the mutation rate for the entire

region, and $u = 2\mu k$, where μ is the per-nucleotide mutation rate and k is the number of nucleotides in the sequence (Rogers & Harpending 1992).

I used the program FLUCTUATE (Kuhner *et al.* 1998) to estimate the maximum likelihood of the population parameters Θ (equal to $2N_e\mu$ for mitochondrial data, where N_e is the effective population size of females and μ is the per-site mutation rate) and g (the exponential growth parameter). For each clade, I ran initial Markov chain Monte Carlo searches with a range of chain numbers and lengths. Final searches were run five times each with 10 short chains of length 5000 and 4 long chains with length 100 000 with sampling every 20 steps and a transition to transversion ratio of 7.8 (estimated in PAUP*). Mean values for all five runs are reported. As calculations of the parameter g tend to be biased upwards (Kuhner *et al.* 1998), estimates were considered to be significant only if they exceeded three times the standard deviation of g (Lessa *et al.* 2003).

Results

Sequence data for a 551-bp region of the 16S rRNA gene were obtained from 192 shrimp that keyed to *Alpheus armillatus* and one shrimp that keyed to *Alpheus viridari* according to Abele & Kim (1986); no sample for which sequencing was attempted failed to yield usable 16S sequence in both directions. This data set contained a total of 72 unique haplotypes (GenBank Accession nos DQ682849–DQ682920). The Bayesian analyses indicated strong support for the clock-constrained model (clock: $M_1 = -1958.38$; no clock: $M_0 = -2051.52$; $2 \log_e(B_{10}) = 186.28$). A likelihood ratio test comparing unconstrained- and clock-constrained ML trees failed to reject the null (clock-constrained) model ($\chi^2 = 21.7$, d.f. = 13, $P > 0.05$). Bayesian and ML analyses (Fig. 2) revealed four major clades in the 16S data set, with two clades (clades 1 and 2) broadly distributed across the sampled geographical region. Clade 3 includes all haplotypes from the Gulf of Mexico ($n = 13$) and a subset of haplotypes from site FL1 (Atlantic coast). Clade 4 includes haplotypes only from sites FL2 and from Puerto Rico. Median-joining networks for each clade suggest that haplotypes are not distributed homogeneously among geographical locations for clades 2–4 (Fig. 2). Haplotype and nucleotide diversities for the 16S gene were similar for clades 1–3, but were sharply reduced in clade 4 (Table 1), in which a single common haplotype dominated each subclade (Fig. 2). Two haplotypes ($n = 3$ individuals) collected from Bermuda were placed outside the clade formed by all other *A. armillatus* (clades 1–4) and the geminate species *Alpheus tenuis*. The Bayesian analysis placed the Bermuda shrimp in a clade with the outgroup *A. viridari*, another intertidal alpheid with morphology similar to that of the described *A. armillatus*. The unrooted ML analysis, however, placed the Bermuda shrimp in a clade

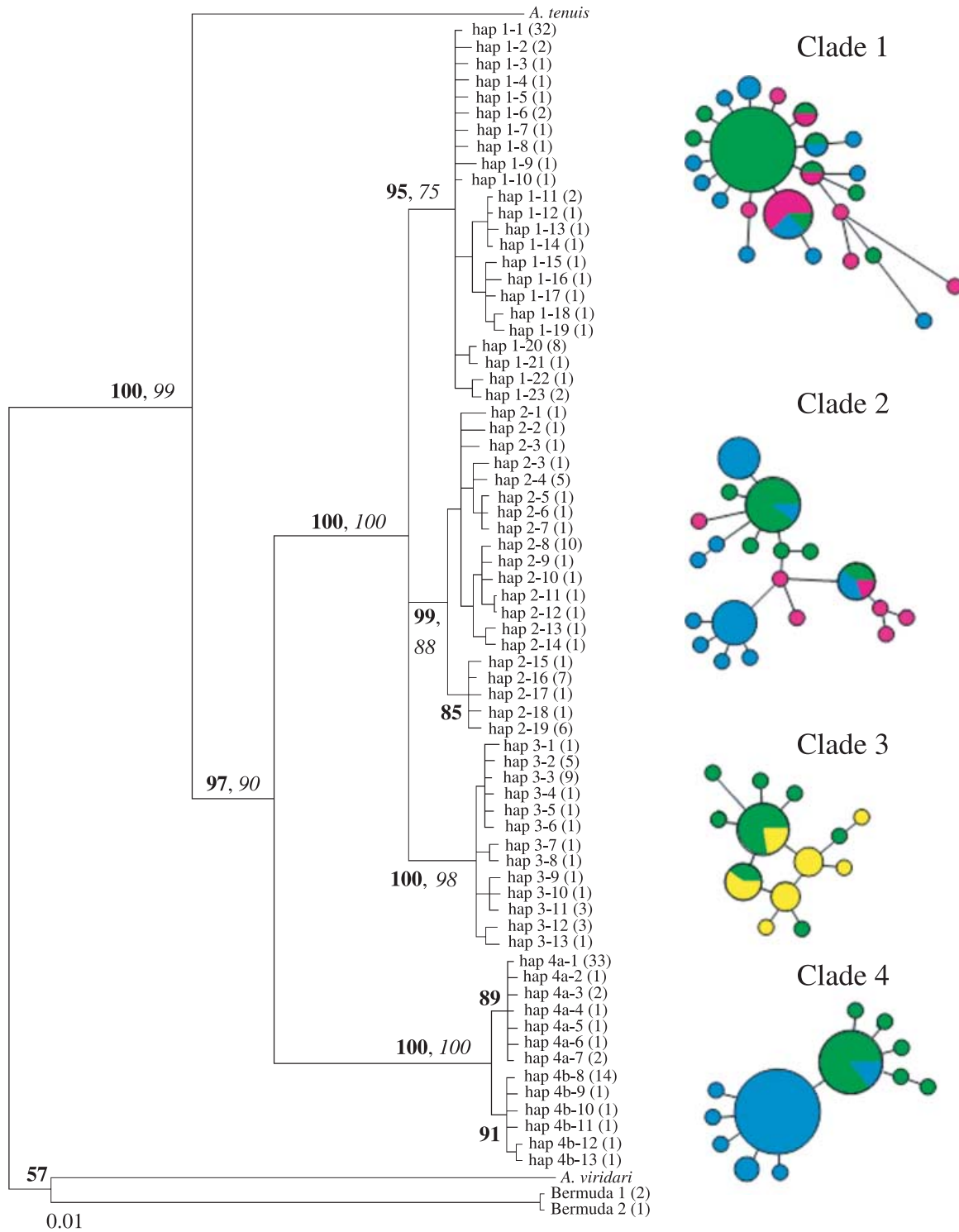


Fig. 2 Bayesian tree of unique 16S rRNA haplotypes, and corresponding median-joining networks for each of the 4 major clades. Numbers next to nodes indicate Bayesian posterior probabilities (in bold) or bootstrap support (in italics) from the ML analysis. For some nodes, no bootstrap number is shown because the ML analysis was performed on a reduced data set (see Methods) only to elucidate relationships among the four major clades. In addition, no bootstrap value is shown for the clade including *Alpheus viridari* and the Bermuda shrimp, because the unrooted ML analysis placed the Bermuda shrimp in a clade with the *Alpheus armillatus* complex (bootstrap = 97). Numbers in parentheses next to haplotype names represent the frequency of that haplotype in the data set. In networks, circles are colour coded by general location (green, Florida; blue, Puerto Rico; pink, Jamaica; yellow, Gulf of Mexico) and are sized according to each haplotype's relative abundance. Lengths of the lines connecting haplotype circles are proportional to the number of nucleotide differences between those haplotypes.

Haplotype group	N	Collection sites	Haplotype diversity	Nucleotide diversity
Clade 1	66	FL1, FL2, JM1, JM2, PR2, PR3	0.745 (0.057)	0.0027 (0.0019)
Clade 2	44	FL1, FL2, JM2, PR2	0.898 (0.026)	0.0062 (0.0036)
Clade 3	29	FL1, FL3, TX	0.872 (0.045)	0.0032 (0.0021)
Clade 4a	19	FL2, PR1	0.468 (0.140)	0.0012 (0.0011)
Clade 4b	38	PR1, PR2	0.248 (0.091)	0.0005 (0.0006)

Table 1 Samples sizes, collection sites, and haplotype and nucleotide diversities for major 16S mitochondrial DNA clades. See Fig. 1 for collection site codes. Numbers in parentheses are standard deviations

with *A. tenuis* and the *A. armillatus* complex, separated from *A. viridari* with bootstrap support of 98 (not shown).

No obvious morphological differences were noted among individuals whose 16S haplotypes clustered into clades 1–4 and three individuals collected from Bermuda, and all specimens keyed to *A. armillatus* or *Alpheus angulosus* using the descriptions of Abele & Kim (1986) and McClure (1995). However, there were differences in gross colour patterns among clades. Most (64 of a total $n = 66$) individuals in clade 1 and all ($n = 29$) individuals in clade 3 were pale brown to olive green with no banding patterns or spots on carapace or claws. All individuals whose haplotypes fell into clade 2 ($n = 44$) had dark transverse bands across the abdomen, distinct speckling patterns on the chelae, and two pairs of dark spots on the second and fourth abdominal segments. All individuals whose haplotypes fell into clade 4 ($n = 57$) were pale brown with no banding patterns or speckling, but possessed the two pairs of abdominal spots. This colouration was also characteristic of two individuals whose haplotypes fell into clade 1, and both of these individuals were collected as unpaired shrimp. Of the sequenced individuals whose haplotypes fell into clades 1–4 ($n = 195$), ~76% of them were collected as heterosexual pairs, and all heterosexual pairs consisted of individuals with the same colour patterns.

Analysis of MyHC sequence data

Sequence data for a 301-bp region of the MyHC gene were obtained from 156 individuals, including *A. tenuis* and the three individuals collected in Bermuda. Amplifications of MyHC failed more often than amplifications of 16S, resulting in a lower sample size for this gene, but there was no clear pattern for amplification failure (i.e. no one population or colour form was clearly more or less likely to amplify MyHC successfully than any other population or colour form). Allele phase was inferred by PHASE 2.1.1 (Stephens *et al.* 2001) with > 60% confidence in all 156 individuals, and only 7 out of 107 total calls had confidence values < 80%. Therefore, no MyHC haplotypes were eliminated from the data set. Because of small sample size, sequences from both *A. tenuis* and the Bermuda shrimp were excluded from further analysis, leaving a total of

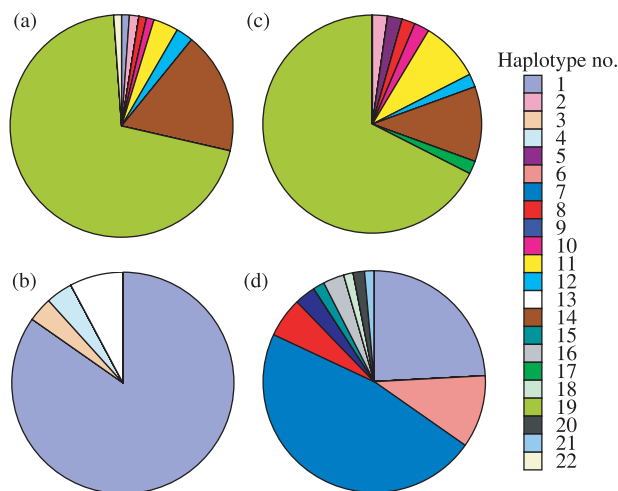


Fig. 3 Distribution of reconstructed nuclear MyHC alleles by major 16S rRNA mtDNA haplotype clade. (a) clade 1, $N = 84$; (b) clade 2, $N = 52$; (c) clade 3, $N = 46$; (d) clade 4, $N = 66$. Arbitrary haplotype numbers here were used in GenBank accession definitions.

22 unique MyHC haplotypes (GenBank Accession nos DQ682827–DQ682848) among shrimp from Puerto Rico, Jamaica, Florida, and the Gulf of Mexico.

Both 16S and MyHC data were available for 128 individuals, and for these individuals, MyHC alleles were not distributed randomly with respect to 16S clade (Fig. 3). G-tests of independence with a Bonferroni-corrected α of 0.008 were performed after excluding 15 rare alleles (5 or fewer copies) from the data set. MyHC allele frequencies were not significantly different between 16S clades 1 and 3 ($G = 2.1$, $P > 0.05$) but were significantly different in every other pairwise combination (clades 1 and 2: $G = 68.4$, $P < 0.001$; clades 1 and 4: $G = 74.8$, $P < 0.001$; clades 2 and 3: $G = 49.6$, $P < 0.001$; clades 2 and 4: $G = 28.8$; clades 3 and 4: $G = 32.9$, $P < 0.001$; d.f. = 6 for all contrasts).

Analysis of molecular variance

The analysis of molecular variance indicated no geographical structure in clade 1, with ~100% of the variation found

Table 2 Analysis of molecular variance (AMOVA) of 16S mitochondrial DNA data. Grouping of samples into geographical region is discussed in the text. See Fig. 1 for site locations

Haplotype group	Geographical contrast	Variation among populations (%)	Variation within populations (%)	F_{ST}	
				Overall	Paired
Clade 1	FL1 vs. JM	0.65	99.35	0.007	0.012
	FL1 vs. PR				0.004
	JM vs. PR				0.004
Clade 2	FL2 vs. JM	23.61	76.39	0.236*	0.414*
	FL2 vs. PR				0.200*
	JM vs. PR				0.195*
Clade 3	FL1 vs. FL3	18.58	81.42	0.186*	—
Clade 4	FL2 vs. PR	76.37	23.63	0.763*	—

* $P < 0.01$.**Table 3** Estimates of Tajima's D statistic and results of mismatch distribution analysis under the demographic expansion model. SS , sum of squared deviation; t , expansion time in years, assuming a generation time of 1 year

Clade	Tajima's D	τ (95% CI)	SS_D (P)	θ_1 (95% CI)	θ_0 (95% CI)	t
Clade 1	-2.16**	1.32 (0.00–3.15)	0.002 (0.236)	21.85 (0.78–917.16)	0.00 (0.00–1.43)	30000
Clade 2	-1.00	4.31 (1.56–8.82)	0.013 (0.208)	73.53 (1.23–1364.93)	0.00 (0.00–1.91)	98000
Clade 3	-1.27	1.89 (0.00–3.78)	0.003 (0.487)	12.29 (4.95–459.00)	0.00 (0.00–1.70)	42000
Clade 4a	-1.76*	0.68 (0.00–3.60)	0.000 (0.759)	6.04 (0.00–147.19)	0.00 (0.00–1.23)	16000
Clade 4b	-1.76*	3.00 (0.37–7.44)	0.003 (0.274)	0.00 (0.00–1.96)	0.35 (0.00–113.38)	68000

* $P < 0.05$, ** $P < 0.01$.

within populations (Table 2). However, the AMOVA revealed significant genetic structure in clades 2–4. For clade 2, all contrasts among three geographical locations (Puerto Rico, Jamaica, and Florida) had significant F_{ST} values, and a substantial amount of the variation occurred among populations. For clade 3, the analysis indicated that Atlantic and Gulf of Mexico populations are genetically differentiated. Within clade 4, Florida and Puerto Rico populations formed two subclades with almost no geographical overlap (Fig. 2) and AMOVA confirmed that ~76% of the variance occurred between geographical locations.

Demographic history

Median-joining networks for the four main mtDNA clades (Fig. 2) showed evidence of star-like phylogenies, suggestive of past demographic expansions (Slatkin & Hudson 1991). This is supported by values for Tajima's D statistic (Table 3), which was negative for all clades, though significantly so only for clades 1, 4a, and 4b. All five clades (considering 4a and 4b independently) showed a single peak of pairwise differences among sequences (Fig. 4), and the model of sudden demographic expansion was not rejected for any clade (Table 3, $SS_D P > 0.05$). The timing of these expansions

differed among clades, and ranged from ~100 000 years ago (clade 3) to ~15 000 years ago (clade 4a) under the assumption of a generation time of one year. Maximum-likelihood estimates of g also indicate that all five clades are undergoing significant population growth (Table 4).

Discussion

Interclade comparisons

Recent phylogeographical investigations have revealed surprising levels of previously hidden marine biodiversity, casting doubt on the long-held paradigm that marine systems are largely open to movement among populations. This study reports evidence of several lineages within the *Alpheus armillatus* complex that occur in a small geographical area, and with broad range overlaps. Comparison of the reconstructed MyHC allele frequencies supports the separation of mtDNA sequences into at least four major clades, though there was a strong overlap in MyHC allele frequencies between the mtDNA clades 1 and 3 (Fig. 2). While other studies (Knowlton & Weigt 1998; Morrison *et al.* 2004) report older (pre-Isthmian) divergences for cryptic snapping shrimp lineages, the *A. armillatus* complex

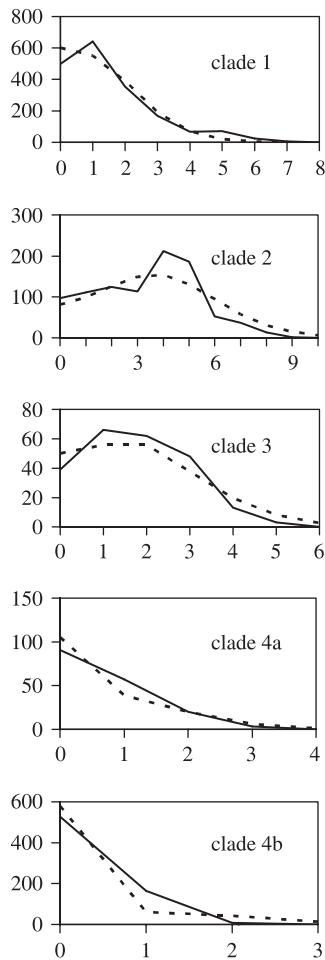


Fig. 4 Results of mismatch analysis. Solid lines represent actual distribution of pairwise differences, and dotted lines represent theoretical distribution under the hypothesis of sudden expansion.

seems to have radiated recently, following the close of the Isthmus of Panama.

In contrast to many other marine taxa, recently diverged snapping shrimp lineages are likely to provide clues about reproductive isolation. Though all members of the complex reported in this study are morphologically similar, there are consistent differences in colouration of the exoskeleton that distinguish some clades. For example, clades 2 and 4 are each characterized by unique (within this study) colouration, while clades 1 and 3 share similar colouration. Other studies (Knowlton & Keller 1983; Knowlton & Mills 1992) suggest that newly identified lineages in other alpheid complexes are also distinguishable by subtle differences in external markings, indicating that such characteristics evolve quickly in this taxon (though, because snapping shrimp apparently are nocturnal and have poor vision, it is unlikely that these differences function in mate recognition). Because snapping shrimp are socially

Table 4 Maximum-likelihood estimates of Θ ($= 2N_e m$) and the exponential growth rate g

Clade	Θ_{ML} (SD)	g (SD)
Clade 1	0.207 (0.019)	2132.03 (86.309)
Clade 2	0.065 (0.007)	953.634 (71.012)
Clade 3	0.152 (0.025)	3000.112 (156.449)
Clade 4a	0.209 (0.064)	6826.307 (584.254)
Clade 4b	0.044 (0.006)	4442.229 (256.434)

monogamous (Nolan & Salmon 1970; Knowlton 1980; Mathews 2002), accurate pair identification in the field can yield preliminary insight into premating isolation. Snapping shrimp are strongly territorial, and burrows almost always house one or two (heterosexual and size-matched) shrimp; in this study, I categorized two shrimp as 'paired' if they were within 5 cm of one another, regardless of colouration.

No colour-mismatched pairs were collected, out of 277 pairs of shrimp (82% of total sample) collected from sites in Florida, Puerto Rico, and Jamaica, though multiple colour forms were collected at most sites (Table 1). Though it is difficult to estimate the rate at which I made incorrect pairing assignments, false negatives (shrimp collected singly that had actually existed as a pair) are more likely than false positives (shrimp categorized as paired when they had actually existed singly or paired with other shrimp), because snapping shrimp are highly aggressive to all conspecifics and heterospecifics other than their territorial 'partners' (Mathews 2002). In addition, no same-sex shrimp were ever categorized as a pair, suggesting that shrimp categorized as paired were in actuality socially monogamous. Therefore, these pairing data suggest reproductive isolation between all pairs of clades except for clades 1 and 3.

Because they do not differ in colour, no inference can be made regarding reproductive isolation between clades 1 and 3 from the field data. However, for 11 pairs collected from site FL1 (the only location where individuals from both clades 1 and 3 were collected), haplotype data from both pair members were available. For eight pairs, haplotypes from both pair members fell into the same clade (four into clade 1 and four into clade 3), and for the remaining three pairs, haplotypes fell into different clades. Interestingly, gamma-corrected Kimura 2-parameter (K2P, calculated in MEGA 3.1) distances indicate that clades 1 and 3 (with no colour differences; K2P distance = 0.021) are more divergent than are clades 1 and 2 (with strong, consistent colour differences; K2P distance = 0.012). Under the assumption of a uniform substitution rate of 2% per Myr among all lineages, K2P distances suggest that the oldest divergence between the group of clades 1, 2, and 3 and clade 4 (K2P = 0.055) occurred around the time of the closure of the Isthmus of Panama, ~3 Mya (Coates & Obando 1996).

Though phylogenetic analyses failed to resolve the order of branching among clades 1–3, K2P distances suggest that clade 3 diverged first (K2P = 0.021), ~1 Mya, and put the split between clades 1 and 2 (K2P = 0.012) at ~500 000 years ago.

Though the 16S data showed clades 1 and 3 to be comparatively divergent (relative to clades 1 and 2), there was no difference in nuclear MyHC haplotypes between those two clades (Fig. 3). There are at least two possible explanations for this conflict between nuclear and mtDNA data. Williams & Knowlton (2001) reported a frequent occurrence of mitochondrial cytochrome oxidase I (COI) pseudogenes in several other species of *Alpheus*. Therefore, the sequences contained in either clade 1 or clade 3 could represent a 16S pseudogene. This explanation is unlikely, because sequences from both clades were consistently long and clean with no double peaks that would indicate the presence of multiple gene copies, and also because separate amplifications of the COI gene from a subset of individuals belonging to 16S clades 1 and 3 yielded sequences that showed the same clustering of individuals into separate clades (data not shown). Alternatively, the mitochondrial clades 1 and 3 may be the result of genetic differentiation between historically separated populations, followed by introgression during secondary contact, which would homogenize the recombining nuclear gene but could retain separate clades of the nonrecombining mitochondrial genome. Such cases of 'mitochondrial capture' have been reported in many hybridizing species (Avice 2004). This second explanation is supported by the apparent geographical distributions of the two clades, as clade 1 seems to dominate in the Caribbean, and clade 3 in the Gulf of Mexico, with the Atlantic coast of Florida potentially representing a zone of secondary contact. Similar patterns of mtDNA differentiation between Florida Atlantic coast and Gulf of Mexico populations have been reported for a number of other species (Avice 1992; Young *et al.* 2002; Lee & Foighil 2004), and the presence of a biogeographical break around the Florida peninsula has been attributed to sea level changes during the Pleistocene (Avice 1992; but see Lee & Foighil 2005).

In phylogeographical studies, the sampling strategy may have substantial impacts on the representation of lineages in the data set. In this study, shrimp were collected by hand at extreme low tides from the intertidal and shallow subtidal zones, and likely collecting spots were identified as intertidal or shallow subtidal areas with a substrate consisting of mixed sand and/or mud, and small rock and shell rubble. At all sites, both estuarine and fully marine habitats were investigated for the presence of snapping shrimp, because the recently described *Alpheus angulosus* commonly occurs in estuarine areas in the Gulf of Mexico (McClure 1995). This sampling design would fail to obtain samples of species or lineages that are restricted to waters

deeper than shallow subtidal areas. *A. armillatus* and *A. angulosus* are described as intertidal species and subtidal habitats are inhabited by other, apparently more distantly related, species of alpheids (such as *A. floridanus*-Chace 1972), so it is likely that members of the complex are entirely or partially intertidal. However, it is possible that some cryptic lineages in this geographical region went undetected because they exploit a habitat that was not sampled, for example a deeper subtidal region. This may explain the rarity of *A. armillatus* complex individuals in Bermuda, where only three individuals keying to *A. armillatus* were collected. The intertidal zone of Bermuda seems dominated instead by the morphologically similar *Alpheus viridari*, and competition between these two lineages may have resulted in an ecological shift by the *A. armillatus* complex lineage to deeper waters.

The genetic relationships between the *A. armillatus* complex and the three individuals from Bermuda that keyed to *A. armillatus* remain unresolved. These three shrimp represent a lineage that is more distantly related to the complex than are clades 1–4 from one another (Fig. 2). Bayesian analyses grouped 16S sequences from these three individuals with *A. viridari* with low statistical support (Fig. 2), but unrooted ML analyses placed the Bermuda sequences with the *A. armillatus* complex with bootstrap support of 98 (not shown), suggesting that it may represent a member of the *A. armillatus* complex that diverged prior to the close of the Isthmus of Panama. All three individuals from this lineage were characterized by the same colouration, which differed from all other *A. armillatus* complex lineages in this study, and it is likely to be endemic to Bermuda.

Recent investigations into the phylogeography of another Caribbean marine invertebrate complex, the scorched mussel *Brachiodontes exustus*, uncovered similar numbers of cryptic lineages (Lee & Ó Foighil 2004, 2005). In this complex, cryptic lineages are distributed primarily allopatrically, with sister lineages frequently having adjacent ranges, suggesting a history of allopatric speciation followed by range expansions. In contrast, for the *A. armillatus* complex, there was strong geographical overlap among the four clades, with all of them represented on the Atlantic coast of Florida (Table 1), and two (clades 1 and 2) showing broadly overlapping distributions throughout the entire sampled region (except for Bermuda). Thompson *et al.* (2005) report similar geographical sympatry between two cryptic lineages of *Alpheus djeddensis* in the South Pacific. In this study, clade 3 was the sole representative in samples from the coastal Gulf of Mexico, which is the only sampled region with grossly different ecological characteristics (lower salinity and strong salinity fluctuations in estuarine areas), suggesting a possible role for ecological selection in the evolutionary history of this clade. With the exception of the Gulf of Mexico sites, there is no clear pattern of ecological dominance by geographical region. However, individual

sites often appeared to be dominated by one of the four clades. For example, at site FL2, collections included 17 individuals of clade 4 and 138 individuals with the colouration characteristic of clade 2; at site JM2, collections included eight individuals of clade 2 and 32 individuals with the colouration characteristic of clades 1 and 3. Three sites (JM1, PR1 and PR3) were apparently only inhabited by members of one clade. Only at one site, PR2, were individuals of multiple clades collected in similar proportions (clade 1, $n = 47$; clade 2, $n = 44$; clade 4, $n = 35$, determined by colour pattern), with no sign of dominance by one clade over another, or of microhabitat segregation between clades. Therefore, though the complex shows a pattern of regional sympatry, individual populations are typically allopatric or heavily dominated by one lineage. Mechanisms regulating local population dynamics of snapping shrimp lineages may be elucidated by analyses over finer geographical scales with highly variable markers such as microsatellites.

Intraclade analyses

Within the Caribbean region, the direction of current flow is predominantly southeast to northwest through the West Indies, veering to the north along the southeastern USA. Current flow into the Gulf of Mexico is via the Gulf Loop, which flows in through the Straits of Yucatan and out through the Straits of Florida (Roberts 1997). Assuming passive dispersal of larvae, Roberts (1997) made predictions of gene flow (for taxa with 1- or 2-month larval durations) that imply high connectivity among Caribbean and West Atlantic populations. Empirical studies have yielded equivocal results. While Shulman & Bermingham (1995) found no evidence for differentiation among populations in several species of reef fish, other studies have found evidence for phylogeographical breaks in this region (Avice 1992; Lee & Foighil 2004, 2005). In addition, the assumption of passive dispersal may not hold for many marine larvae (Doherty *et al.* 1995; Cowen *et al.* 2000; Taylor & Hellberg 2003), and local retention of larvae could lead to genetic divergence among populations. Larval durations and the dispersal distances of larvae in the *A. armillatus* complex remain unknown. Knowlton (1973) suggested that *Alpheus* larvae may spend anywhere from 4 days to 2–3 weeks as pelagic larvae before settlement, depending on species, and embryos of *A. armillatus* complex lineages hatch into floating or swimming zoea, rather than as crawling juveniles (personal observation), suggesting a pelagic larval stage during which dispersal among populations might occur, thereby leading to genetic homogenization.

However, this study showed strong evidence for population genetic differentiation within three of the four major mtDNA clades. Clade 2 showed strong separation among

Jamaican, Puerto Rican, and Floridian populations in both network (Fig. 2) and AMOVA (Table 2) analyses, and phylogenetic and network analyses revealed the presence of a mtDNA subclade (supported by 85% posterior probability in Fig. 2) that was detected only in Puerto Rico. Conversely, clade 1, which was also detected in the same three geographical locations, showed no population genetic divergence in any pairwise comparison (Table 2). This strongly conflicting pattern between such closely related lineages argues against low dispersal rates as the sole isolating mechanism. Though different larval development patterns have been reported in other sister alpheid lineages (Wehrmann & Albornoz 2002), I did not note any disparity in egg size or number that might indicate developmental differences between clades 1 and 2. Rather, the disagreement in population genetic patterns between clades 1 and 2 may involve disparate evolutionary histories of range and/or demographic changes since the lineages diverged. Mismatch analysis indicates that, while both clades 1 and 2 may have undergone demographic expansions, the expansion of clade 2 occurred earlier (~100 000 years ago, Table 3) than that of clade 1 (~25 000 years ago).

The range of clade 3 is apparently restricted to the Atlantic coast of North America and to the Gulf of Mexico and shows evidence of genetic differentiation between Gulf and Atlantic populations (Table 2). Similar patterns have been reported for a number of other marine species, and these phylogeographical breaks are commonly attributed to sea-level changes during the Pleistocene (Avice 1992), though Lee & Ó Foighil (2004) report an older Pliocene separation between Atlantic and Gulf populations of mussels. For clade 3, genetic differences between the two geographical regions may be a remnant of past geographical isolation during sea-level changes followed by introgression during secondary contact of the two populations. Alternatively, the Gulf/Atlantic separation may be the result of low gene flow between Atlantic and Gulf populations, and may be just one of several genetic breaks in this clade; Lee & Ó Foighil (2004) report such a pattern for the mussel *Brachidontes exustus*.

Both phylogenetic and AMOVA analyses indicate that clades 4a and 4b (corresponding to Florida and Puerto Rico populations, respectively) are strongly divergent, with K2P distances suggesting a divergence time of ~150 000 years, and these two populations may represent reproductively isolated species. However, I did not note any colour differences or other distinguishing characteristics between them, and their geographical isolation precludes any initial inferences into reproductive isolation. These two subclades were also characterized by substantially lower genetic diversity than the other clades (Table 1), as each population was largely dominated by a one common haplotype, suggesting that clade 4 has undergone at least one population bottleneck. Additionally, significant Tajima's

D statistics and mismatch analyses provide strong signals of past demographic expansion.

In summary, over the northeastern part of its described range, the *A. armillatus* species complex shows strong genetic heterogeneity at both the species and population levels. Probable cryptic species show extensive geographical overlap; however, at the intraclade level, strong genetic differentiation among populations correlates with geographical location. Range overlap between cryptic species, then, may be the result of range shifts and secondary contact after allopatric speciation. Signals of genetic differentiation among populations within the same lineage may be remnants of past vicariant events that isolated the populations, but also indicate that shrimp populations may be poorly connected by gene flow. Further examination of this complex over the rest of its described range (to São Paulo, Brazil: Chace 1972), as well as similar investigations into other snapping shrimp species complexes, will provide deeper insights into the evolutionary history of these marine invertebrates.

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Lauren Mathews (<http://www.wpi.edu/Academics/Depts/Bio/People/Mathews/>) is currently an assistant professor of biology at Worcester Polytechnic Institute, where her research interests are in the evolutionary ecology of marine and freshwater macro-invertebrates, particularly the processes that generate biodiversity and the evolution of social behavior.
