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# A tree money grows on: the first inclusive molecular phylogeny of the economically important pink shrimp (Decapoda : *Farfantepenaeus*) reveals cryptic diversity

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**Abstract.** Species of *Farfantepenaeus* support economically important shrimp fisheries throughout the Western Hemisphere, necessitating proper fisheries management. To be effective, species management should be informed of the potential presence of cryptic species and of the evolutionary forces driving biodiversity. This is best accomplished through a robust phylogenetic framework and evidence-based species delimitation. This study represents the first comprehensive molecular phylogeny and species delimitation analyses of shrimps belonging to the genus *Farfantepenaeus*. Targeting three mitochondrial genes (12S, 16S, and COI), gene trees and a phylogeny for the genus were inferred using maximum likelihood and Bayesian approaches. In general, the phylogenetic relationships inferred here largely agree with those recovered from morphological data, including the most recent designation of *F. isabelae* as sister to *F. subtilis*. Molecular divergence was found between northern and southern populations of *F. brasiliensis*, suggesting the existence of unrecognised subspecies. However, previous recognition of *F. duorarum* and *F. notialis* as two distinct species was not supported by this study. The phylogeny inferred here also uncovers a phylogeographic signal of latitudinal speciation in the genus. The study presented here provides valuable insight into the evolutionary history of *Farfantepenaeus*, improving our ability to effectively manage these economically important species.

**Additional keywords:** fisheries management, genetics, penaeid.

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## Introduction

In 2015, almost 59 000 t of penaeid shrimp in the genus *Farfantepenaeus* (Burukovsky 1972, 1997), representing US$213.5 million in ex-vessel value, were fished from the Gulf of Mexico and Atlantic Ocean off the southern United States (compiled NMFS Landings query, 28 February 2018). The *Farfantepenaeus* spp. landings represented 59% of total USA penaeid shrimp landings in the combined Gulf of Mexico and Atlantic Ocean that year. With the occurrence of *Farfantepenaeus* spp. admixtures on various fishing grounds (e.g. Sheridan *et al*. 1987; Arreguín-Sanchez 1999; Arreguin-Sanchez *et al*. 2008; Charuau and Die 2000; Shepard and Ehrhardt 2000), effective fishery management is dependent on an understanding of the evolutionary forces driving biodiversity (Bernatchez 1995), which is greatly facilitated by a robust, comprehensive phylogenetic framework. Phylogenies can be critical to identifying evolutionarily significant units (ESUs) and determining whether certain units require unique management considerations (Ryder 1986). Much focus has been placed on identifying ESUs based on reproductive isolation (Waples 1991). However, when nothing less than reproductive isolation is accepted in distinguishing ESUs, it can overlook adaptive diversity (Crandall *et al*. 2000) within populations, which is critical to the evolutionary success of a species (Frankel 1974; Lande and Shannon 1996; Moritz 2002). In this, phylogenies are crucial: while distinct, historically isolated populations of a species may exist, they may not be reciprocally monophyletic. This means that these populations are the result of evolutionary processes *within* the ESU and the goal of management should be to maintain these processes (Crandall *et al*. 2000).

Proper classification, informed by an understanding of evolutionary relationships within the taxon of interest, is crucial to species conservation and management. Species divisions within *Farfantepenaeus* are based largely on morphology of external sexual structures (Fig. 1) (Pérez-Farfante 1967, 1969,

 

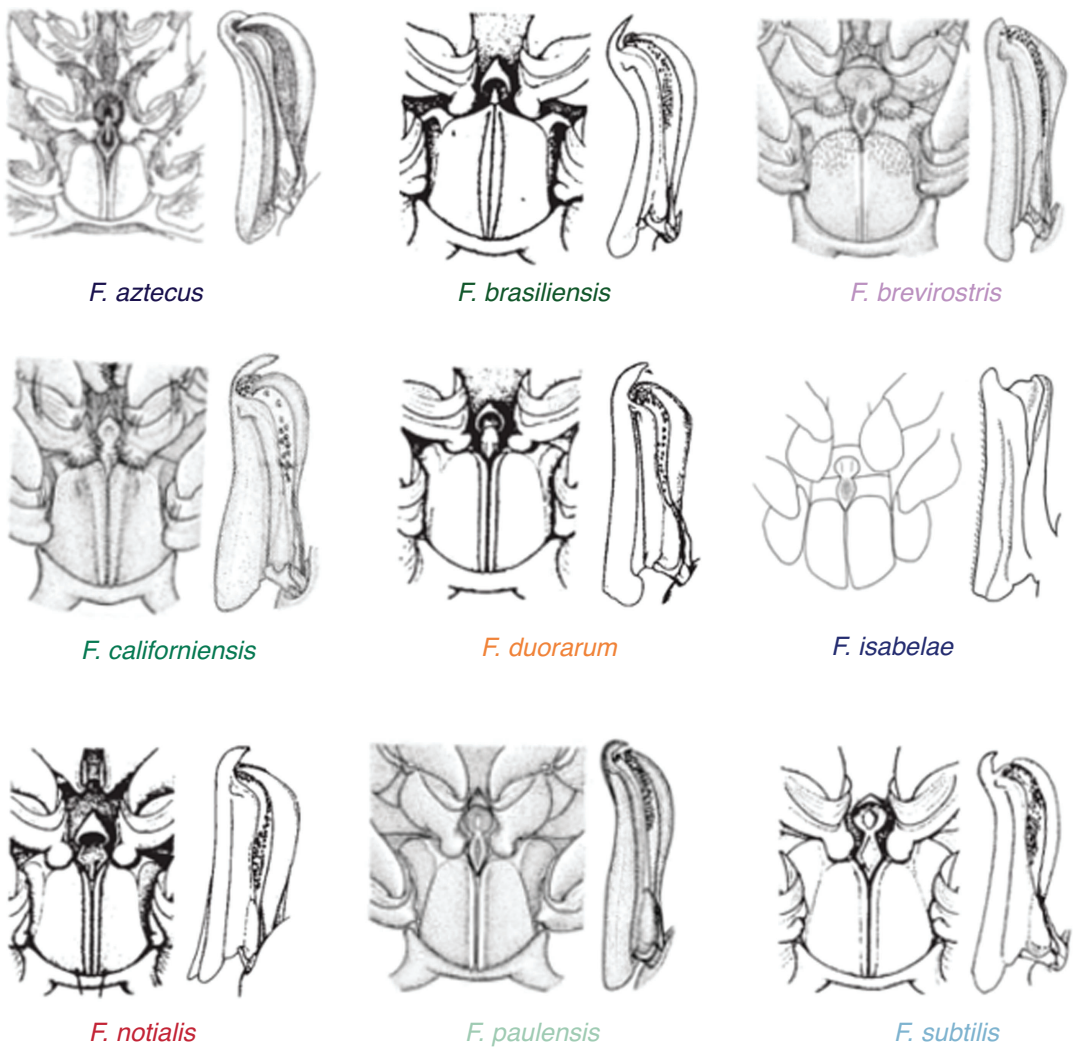


**Fig. 1.** For each species, the thelycum (left) and petasma (right) are shown. The colours of species' names correspond to the colours used on the gene trees, the distribution maps, and the phylogeny. Illustrations are adapted from the FAO key (Tavares 2002) and, in the case of *F. isabelae*, from Tavares and Gusmão (2016).

1970*a*, 1970*b*, 1970*c*, 1988; Pérez-Farfante and Kensley 1997) and/or biogeography (Burukovsky 1972). When established, the subgenus *Farfantepenaeus* included six species: *F. duorarum* (Burkenroad, 1939), *F. brasiliensis* (Latreille, 1817), *F. aztecus* (Ives, 1891), *F. californiensis* (Holmes, 1900), *F. brevirostris* (Kingsley, 1878), and *F. paulensis* (Pérez-Farfante, 1967). *F. subtilis* (Pérez-Farfante, 1967) and *F. notialis* (Pérez-Farfante, 1967) were initially included as subspecies of *F. aztecus* and *F. duorarum*, respectively (Pérez-Farfante 1967). *Farfantepenaeus* was named as a subgenus of *Penaeus* in 1972 (Burukovsky 1972) and *F. brasiliensis* was designated the type species in a brief note in 1997 (Burukovsky 1997). In the same year, Pérez-Farfante and Kensley (1997) produced a seminal monograph that elevated several penaeid subgenera, including *Farfantepenaeus*, to the level of genus. In the same work, the subspecies *F. notialis* and *F. subtilis* were considered valid species (Pérez-Farfante and Kensley 1997). Since then, confusion has arisen concerning the taxonomic rank and placement of two morphotypes of *F. subtilis* described from the western Atlantic. This is discussed in greater detail in the Methods section, *Morphological identification of specimens*. Morphotype II (MII) has since been described as sister to/subclade of either *F. paulensis* or *F. subtilis* MI (Maggioni 1996; D'Incao *et al*. 1998; Gusmão *et al*. 2000; Maggioni *et al*. 2001). Most recently, MII has been named *F. subtilis sensu stricto* and *F. subtilis* MI has been formally described as *F. isabelae* (Tavares and Gusmão 2016). While phylogenetic relationships have been inferred between several species of *Farfantepenaeus* (Baldwin *et al*. 1998; Gusmão *et al*. 2000; Maggioni *et al*. 2001; Lavery *et al*. 2004; Voloch *et al*. 2005; Tavares and Gusmão 2016), these studies did not include all species within the genus, and frequently included, at most, two mitochondrial genes. Given the economic value of this group and the concomitant fishing pressures, a clear understanding of biodiversity and evolutionary relatedness is needed.

Traditionally, external sexual morphology has been used to define species within the genus. However, this has proved

convoluted because, although fully developed in adults, external reproductive structures are often absent or underdeveloped in juveniles and thus their utility for species identification is subjective and can be inconsistent (Ditty and Alvarado Bremer 2011; Teodoro *et al*. 2016). This difficulty has likely contributed to the lack of resolution within the genus. Previous studies have identified the existence of cryptic species within *Farfantepenaeus*, uncertainty of monophyly at the genus- and species-levels, and population genetic structure within *F. notialis*. Over the past three decades, population genetics studies of a wide variety of marine fauna occurring along the south-eastern coast of the United States have indicated significant genetic diversity between the Gulf of Mexico and other parts of the Atlantic (see review by Avise (1992) and Young *et al*. (2002) for a decapod-specific example). This suggests that species of *Farfantepenaeus* with large distributional ranges throughout the Gulf and into the Atlantic should be investigated for potential cryptic species (see Fig. 3 for species ranges). *F. brasiliensis*, whose range extends from North Carolina, USA, to the coasts of Brazil (including an extension into the Gulf of Mexico along the Yucatan coast), and *F. duorarum*, with a range from Chesapeake Bay, Virginia, USA, to the Yucatan, Mexico, seem likely candidates for cryptic diversity.

This study represents the first comprehensive phylogeny of the economically important shrimp genus *Farfantepenaeus*, with the inclusion of all nine currently recognised species of *Farfantepenaeus*. Phylogenetic relationships within *Farfantepenaeus* were recapitulated and the phylogeographic structure of mitochondrial haplotypes was examined to address three primary objectives: (1) test for monophyly of previously described subspecies, now elevated to species (*F. notialis* and *F. subtilis*, specifically), (2) examine evolutionary relationships within the genus and characterise them in a biogeographical framework, and (3) investigate cryptic diversification within the genus. Results from addressing all three objectives are needed to properly manage and conserve species within this heavily fished genus.

## Methods

### Specimen collection

In total, 171 postlarval, juvenile, and adult shrimp were collected for inclusion in the study. Most specimens were directly collected by the authors while others were donated by colleagues. Specimens were either collected aboard shrimp vessels and preserved on the ship or by field biologists and returned to the laboratory. Collected specimens were frozen at –20°C or directly stored in 70% ethanol. Every extant species of *Farfantepenaeus* was included, either as a collected specimen or through sequence data acquired from GenBank (Table 1 and Table S1, available as Supplementary Material to this paper). *Litopenaeus vannamei* (Boone, 1931), *L. stylirostris* (Stimpson, 1874), and *L. setiferus* (Linnaeus, 1767) were included as outgroups. Some individuals had morphological characters that matched *Farfantepenaeus notialis*, despite having been collected from outside of the described range of the species. These individuals were labelled '*F.* nr *notialis*' to distinguish them from specimens of the species collected from within range. This study also included representatives from both *F. subtilis* morphotypes: morphotype I (MI) and morphotype II (MII) were initially divided on the basis of the adrostral sulcus, rostral shape, 6th pleonite keel to sulcus (K/S) ratio, petasma, and thelycum (Pérez-Farfante 1969). For the purposes of clarity, in this paper individuals identified as *F. subtilis* MI will be designated as such, though they have now been reclassified as *F. isabelae*, and *Farfantepenaeus subtilis* s. str. will be referred to as '*F. subtilis* MII'.

**Table 1. Number of individuals included in the study, including the total number and the number of *de novo* sequences generated (reported in parentheses). Farfantepenaeus isabelae and F. subtilis MI are listed separately here to reflect how individuals were initially identified in the lab, prior to phylogenetic analysis**

| Species | 12S total (new) | 16S total (new) | COI total (new) | Concatenated |
|---|---|---|---|---|
| *F. aztecus* | 4 (3) | 11 (1) | 1 (1) | 4 |
| *F. brasiliensis* N | 20 (20) | 21 (21) | 21 (21) | 21 |
| *F. brasiliensis* S | 6 (6) | 10 (6) | 71 (6) | 6 |
| *F. brevirostris* | 3 (3) | 3 (3) | 0 (0) | 3 |
| *F. californiensis* | 4 (2) | 4 (2) | 2 (2) | 4 |
| *F. duorarum* | 22 (22) | 30 (21) | 21 (21) | 21 |
| *F. isabelae* | 2 (2) | 1 (1) | 2 (2) | 2 |
| *F. notialis* | 1 (0) | 1 (0) | 1 (0) | 2 |
| *F.* nr. *notialis* | 5 (5) | 5 (5) | 5 (5) | 5 |
| *F. paulensis* | 0 (0) | 0 (0) | 46 (0) | 5 |
| *F. subtilis* MI | 1 (1) | 9 (0) | 1 (1) | 9 |
| *F. subtilis* MII | 2 (2) | 10 (2) | 2 (2) | 10 |
| Total | 70 (66) | 105 (62) | 173 (61) | 92 |

### Morphological identification of specimens

Collected specimens were identified taxonomically in the Ecological Investigations Laboratory at the Southeast Fisheries Science Center in Miami, Florida (Pérez-Farfante 1967, 1969, 1970*a*, 1970*b*, 1970*c*, 1988; Pérez-Farfante and Kensley 1997) or identified by colleagues. Four morphological traits are commonly targeted to identify species within *Farfantepenaeus*: (1) adrostral sulcus, (2) keel height to sulcus width ratio (K/S) of the 6th pleonite (i.e. abdominal somite), and characteristics of the external genitalia of males and females: the (3) petasma and the (4) thelycum, respectively (Fig. 2). The adrostral sulcus (groove) and carina (ridge) flank the rostrum and postrostral crest. Adrostral sulci and carina that extend posteriorly beyond the epigastric tooth and usually to the dorsal posterior carapace margin are defining characteristics of *Farfantepenaeus*, known as the 'grooved shrimp' (Pérez-Farfante and Kensley 1997). Differences in adrostral sulci length and width may distinguish *Farfantepenaeus* species. The ratio of keel height to sulcus width refers to the dorsomedian keel and the dorsolateral sulcus of the 6th pleonite (abdominal segment). This ratio, measured at ~$^1/_3$ the somite length from the posterior margin of the 6th pleonite, may be useful to separate certain species in this genus, even in juvenile stages, which either exhibit incompletely developed external reproductive structures or lack them altogether.

In individuals whose carapace length exceeds 8–10 mm, external reproductive structures are sufficiently developed to assist with species identification (Pérez-Farfante 1970*b*, 1970*c*).

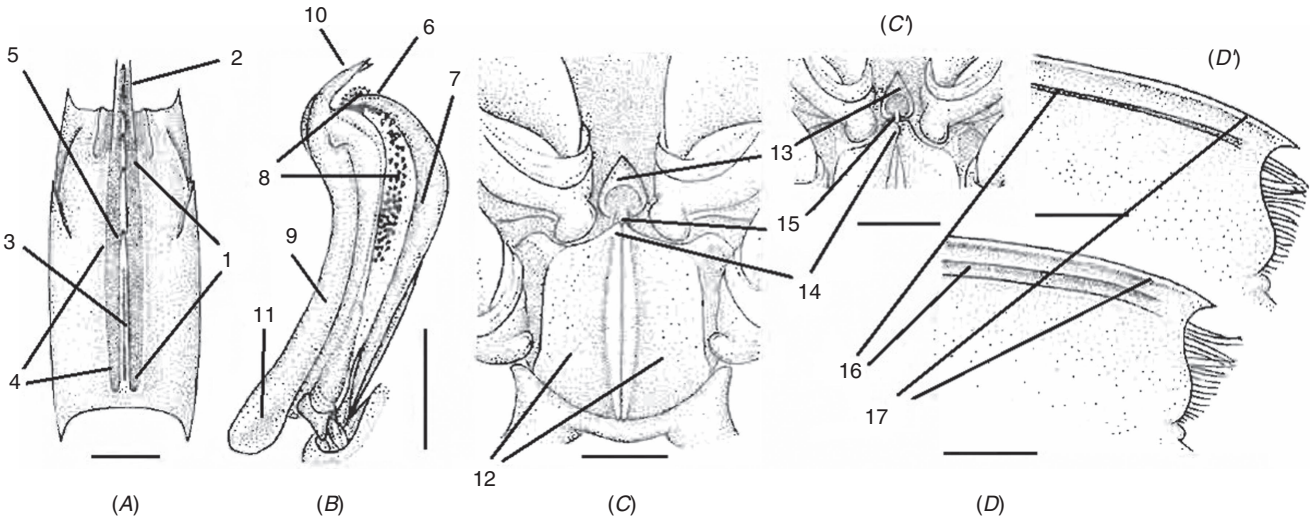


**Fig. 2.** Commonly used morphological features for identifying species of *Farfantepenaeus*: (*A*) overhead view of carapace dorsal surface, (*B*) lateral view of one-half of petasma, (*C*) overhead view of sternites XIII and XIV and thelycum (specimen from Little Bahama Bank), (*C′*) thelycum as viewed in *C* (specimen from Brazil), (*D*) lateral view posterior 6th pleonite (i.e. abdominal somite) posterior margin (specimen from Camocin, Brazil), and (*D′*) lateral view of 6th pleonite posterior (specimen from Saint Augustine, Florida, USA). Highlighted anatomical characters described in the text: 1, dexter adrostral sulcus; 2, rostrum; 3, postrostral crest; 4, adrostral carina; 5, epigastric tooth; 6, terminus of ventral costa; 7, ventrolateral lobe; 8, distomarginal spines; 9, median lobe; 10, distomeduab projection; 11, proxomedian projection of the median lobe; 12, lateral plates; 13, posterior process; 14, anterior process (hidden by lateral plates); 15, median carina; 16, dorsolateral sulcus; and 17, dorsomedian keel. Depicted specimen is *F. brasiliensis*, the selected type specimen for the genus (Burukovsky 1997). Illustrations are adapted from Pérez-Farfante (1988). Scales: A, 10 mm; B–D′, 3 mm.

Reproductive structure morphology is especially useful for identifying subadults and adults to species (Pérez-Farfante 1969, 1970*a*, 1970*b*, 1970*c*, 1988). In males, diagnostically useful specific features associated with the petasma include the shape of the ventral costa terminus on the ventrolateral lobule, the presence and pattern of distomarginal spines along the lateral lobe, and the shape of the distomedian projection of the median lobe. In practice, we also compare the shape and size of the proxomedian projection of the median lobe. In females, specific features of the thelycum that are diagnostically useful include the shape and curvature of the anteriomedian corners and median margins of lateral plates, which shield the seminal receptacle, as well as the shape and/or relative dimensions of the anterior process, posterior process, and median carina of the median protuberance.

### *DNA extraction, PCR, and sequencing*

Abdominal muscle tissue was plucked from individuals and DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit, following the manufacturer's instructions. After DNA extraction, three mitochondrial genes common to phylogenetic analysis (Cunningham *et al*. 1992; Gusmão *et al*. 2000; Lavery *et al*. 2004; Voloch *et al*. 2005) were sequenced in 170 specimens and several GenBank sequences were downloaded for inclusion in our dataset. The 5′ end of cytochrome oxidase subunit I (COI) and the two ribosomal structural genes, 12S and 16S, were targeted to infer interspecific relationships (as utilised in Schubart *et al*. 2000; Stillman and Reeb 2001). Primer combinations and annealing temperatures for each gene are included in Table S2.

PCR amplification reactions were performed in 26.75-μL volumes containing 2 μL of DNA template, 6.45 μL of sterile non-DEPC treated water, 5 μL of 5x combinatorial PCR enhancer solution (CES), 3 μL of 2 mM deoxyribonucleotide triphosphate mix (dNTPs), 2.5 μL of 10x PCR buffer, 2.3 μL of 5M betaine, 2 μL of each 10 μM forward and reverse primer, and 1.5 μL of 0.1 g $mL^{-1}$ bovine serum albumin (BSA). Unpurified PCR products were sent to Beckman Coulter Genomics (Danvers, MA, USA) for purification and sequencing on an Applied Biosystems PRISM 3730xl DNA Analyzer.

### *Phylogenetic analyses*

Sequences were assembled into contigs and cleaned in Sequencher 5.0.1 (GeneCodes, Ann Arbor, MI, USA). To prevent the inclusion of pseudogenes, COI sequences were visually inspected for indels and stop codons. After experts in shrimp taxonomy confirmed morphological identifications, sequences were queried against the GenBank (NCBI) database as a secondary means of identification. This assisted in diagnosing contamination and tentative misidentifications, both of which were removed from analysis. Using Geneious 8.1.3, sequences were cleaned and primers were removed. Cleaned sequences were aligned using MAFFT (Katoh and Standley 2013) and missing data were designated with a '?' for any incomplete sequences. Some species lacked data at a locus entirely, but, with the exception of *F. paulensis* (which could be represented only with COI data), all individuals included in the concatenated dataset had molecular data for at least two of the three targeted genes, as recommended by Wiens (2005, 2006). For this reason, as well as for the purpose of including as much data for as many taxa as possible, phylogenetic analyses were carried out on the single-gene alignments in addition to the concatenated dataset (12S+16S+COI). All sequences were uploaded to GenBank (Table S1).

To determine models of evolution for each individual gene, phylogenetic model averaging was used as implemented in jModelTest (Posada 2008). To identify partitioning across the concatenated data matrix, PartitionFinder 1.1.1 (Lanfear *et al*. 2012) was utilised. Results from jModelTest and PartitionFinder are presented as Supplementary Material in Tables S3–S6. Single-gene trees and the concatenated tree were constructed in RAxML 7.4.2 (Stamatakis 2006) and the RAxML bootstopping action was selected. Each tree had 1000 bootstrap replicates. This was completed on the CIPRES Science Gateway 3.1 (Miller *et al*. 2010). Bootstrap values were mapped onto the resulting topology using FigTree 1.4.2 (Rambaut 2012). Single-gene trees were inspected for potentially contaminated sequences and conflicting topologies. When contamination was found, these sequences were removed from the single-gene alignment(s) and the concatenated dataset and new maximum-likelihood trees were run.

Bayesian inference was conducted in MrBayes 3.2.6 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) for each gene and for the concatenated dataset of all genes. By analysing individual gene trees, as well as a concatenated tree, more representatives could be included across all species. Across datasets, the analysis was run with two simultaneous chains for 10 000 000 generations, or until the average standard deviation of split frequencies fell below 0.005, sampling every 1000 generations. The first 25% of trees were discarded as burn-in and a consensus tree was built from the remaining trees.

### *Genetic distance and species delimitation*

Genetic distances were calculated for each single-gene alignment in MEGA6 (Tamura *et al*. 2013) using maximum composite likelihood. Rates among sites were assumed to have a gamma distribution, and variance was estimated with 100 bootstrap replicates. We also investigated cryptic speciation using two species-delimitation methods: the '10X' method, as described by Hultgren *et al*. (2014), and the Automatic Barcode Gap Discovery (ABGD) method (Puillandre *et al*. 2012). Using the 10X method, we utilised the mean intraspecific distance to determine the interspecific distance threshold to delineate sister species. The ABGD method infers a model-based, one-sided confidence limit for intraspecific divergence from the data and uses this to delineate species. We performed the ABGD with uncorrected pairwise *p*-distances calculated from the COI dataset (as this is the most complete dataset) in MEGA6. Prior intraspecific divergence was set to range from 0.001 to 0.1 and *X* (the relative gap width) was set to 0.5. The analysis was run using a range of steps (20–1000).

## Results

Across all species and all genes, 253 sequences were included in the analyses, including 193 *de novo* sequences. These *de novo* sequences have been uploaded to GenBank (MG000981–MG001172; see Table S1) and are also publicly available through the Gulf of Mexico Research Initiative Information and Data Cooperative (GRIIDC) under doi 10.7266/n7-9yq3-3177. Twenty of the *de novo* sequences were removed after preliminary trees indicated that individuals were misidentified or the DNA template was contaminated. To investigate cryptic speciation within *Farfantepenaues brasiliensis* and *F. duorarum*, 143 sequences and 73 sequences were included of each species, respectively. Overall, four major clades were recovered in the concatenated tree (Fig. 3): Clade 1 contains *Farfantepenaeus brevirostris*, sister to all the remaining *Farfantepenaeus* species; Clade 2 consists of *F. duorarum*, *F. notialis*/*F.* nr *notialis*; Clade 3 consists of *F. paulensis*, *F. aztecus*, *F. isabelae*/*F. subtilis* MI, and *F. subtilis* MII; and Clade 4 comprises *F. californiensis* and *F. brasiliensis*.

### *Concatenated analysis (12S+16S+COI)*

The concatenated data matrix included 70 individuals. In total, 189 new sequences were generated, including 66 new 12S sequences (369 bps), 62 new 16S sequences (501 bps), and 61 new COI sequences (659 bps). Every species was represented in the concatenated analyses ('Concatenated' in Table 1 and Table S1). The results from PartitionFinder partitioned 12S+16S together under the Hasegawa–Kishino–Yano model with invariable sites and gamma distribution (HKY+I+G). COI was partitioned by codon: Position 1 was best fit by the Felsenstein 81 model (F81); Position 2 by Tamura–Nei with invariable sites (TrN+I); and Position 3 by Tamura–Nei with equal base frequencies and invariable sites (TrNef+I).

With the exception of *Farfantepenaeus notialis*, all currently recognised species have high nodal support (> 0.99 posterior probability and >94 bootstrap support) (Fig. 3). Individuals of *F. brasiliensis* fall out into two highly supported subclades associated with collection locality.

Clade 1, containing *F. brevirostris*, is confidently recovered as sister to the remaining *Farfantepenaeus* spp. (1.0/100). Clade 2 consists of a polytomy including representatives of *F. notialis*/*F.* nr *notialis* and *F. duorarum* (1.0/100). *F. isabelae*/*F. subtilis* MI (1.0/94) is recovered as sister to *F. subtilis* MII and this clade exists as a polytomy with *F. aztecus* and *F. paulensis* in Clade 3. Nodal support for the polytomy is high (0.99/100). Clade 4 reveals strong population structure within *F. brasiliensis*: individuals fall into two strongly supported subclades divided by collection locality: *F. brasiliensis* N collected from the Gulf of Mexico and Florida Peninsula (1.0/77) and *F. brasiliensis* S collected off the east coast of Central and South America (from Nicaragua to Brazil) (1.0/99). The *F. brasiliensis* clade is confidently recovered as sister to *F. californiensis* (1.0/100).

### *Single-gene trees (12S, 16S, COI)*

Results from PartitionFinder specified the Jukes–Cantor (JC69) model for the 12S and 16S datasets. COI was partitioned by codon position: all three positions were best approximated by Tamura–Nei; Position 3 was best fit by additionally including fixed equal base frequencies and gamma distribution across sites (1: TrN; 2: TrN; 3: TrNef+G).

The 12S RAxML and Bayesian trees (Fig. 4) differ slightly from the concatenated tree. *F. brasiliensis* does not fall as two distinct clades in the 12S tree, instead forming a polytomy of *F. brasiliensis* N, *F. brasiliensis* S, and two representatives of *F. brasiliensis* N. *F. paulensis* is not included in the 12S alignment, so the branch containing sisters *F. isabelae*/*F. subtilis* MI and *F. subtilis* MII falls as sister to all other species except *F. brevirostris*. Clade 3 is fractured, resulting in

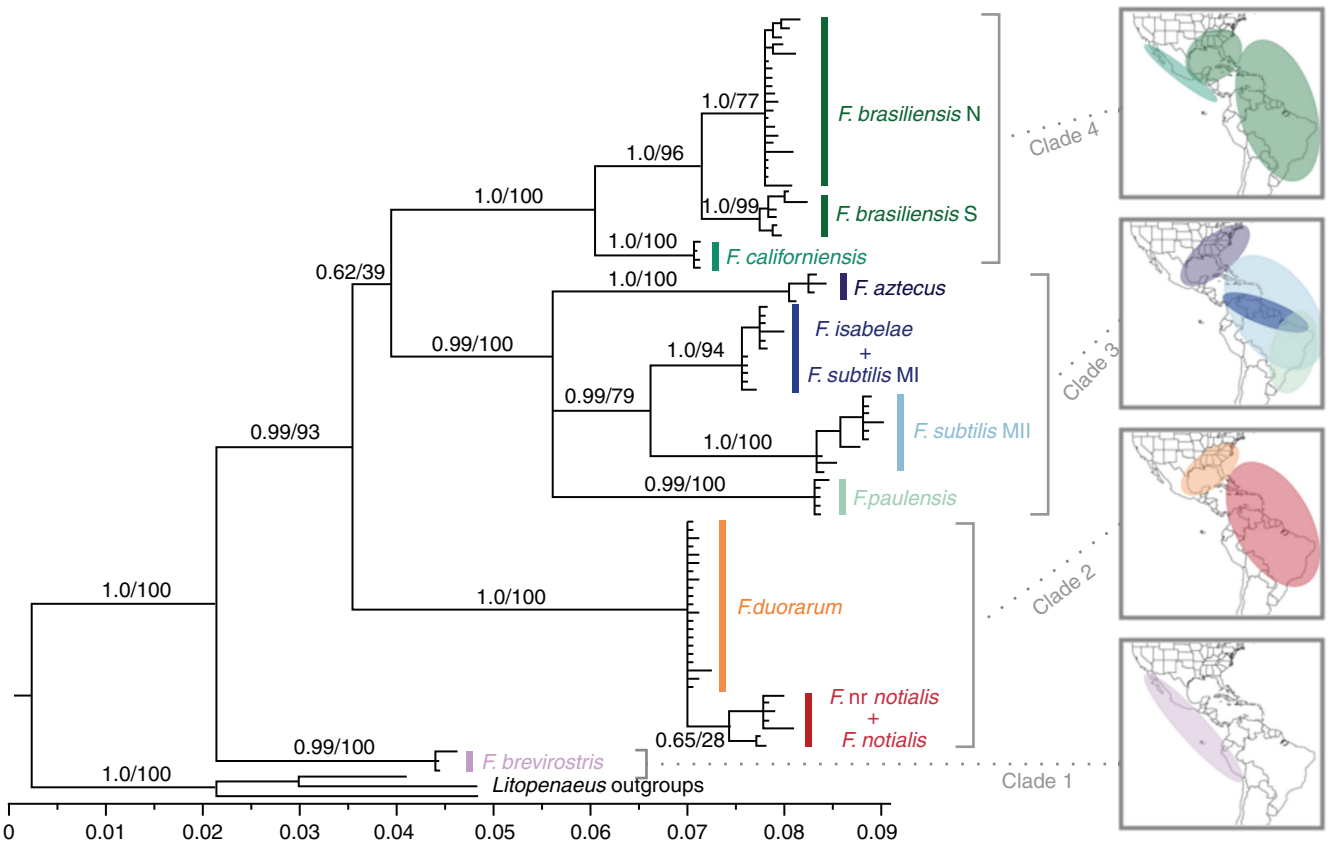


**Fig. 3.** Bayesian phylogram based on concatenated molecular data (12S+16S+COI). Vertical coloured bars represent species and the black vertical bar denotes outgroups. Clades are designated by gray brackets, which connect to colour-coded distribution maps. Support values (Bayesian posterior probabilities/maximum-likelihood bootstrap) are noted above each branch.

*F. aztecus* falling as sister to Clade 4 (0.96). In this tree, *F. duorarum* and *F. notialis*/*F.* nr *notialis* fall out in a polytomy.

The 16S RAxML and Bayesian trees (Fig. 4) are very similar to the concatenated tree; however, in the 16S trees, Clade 2 falls as sister to Clade 3 (0.51/46) instead of being sister to Clades 3 and 4 (0.99/93), as seen in the concatenated tree. The relationships within Clade 3 differ due to a lack of *F. paulensis* sequences in the 16S alignment. In the 16S trees, *F. isabelae*/*F. subtilis* MI and *F. subtilis* MII form a highly supported clade (1.0/86), sister to *F. aztecus* (1.0/92).

COI sequences were included for *F. paulensis*, but no COI sequence data were obtained for *F. brevirostris*. Because of this, only Clades 2–4 were recovered (Fig. 4). The COI trees differ from the concatenated tree in two respects only: first, *F. brasiliensis* forms a single clade, with *F. brasiliensis* N falling out as a highly supported subclade alongside the comb-like terminal nodes of *F. brasiliensis* S; second, the relationships within Clade 3 are very different. In the RAxML tree, *F. isabelae*/*F. subtilis* MI and *F. subtilis* MII form a poorly supported clade (37), sister to *F. aztecus*. This clade, which also lacks strong support (24), is recovered as sister to *F. paulensis* (96). The Bayesian tree recovers a well-supported clade containing *F. aztecus* and *F. paulensis* (0.95), sister to *F. subtilis* MII (0.79). *F. isabelae*/*F. subtilis* MI is strongly supported as sister to this clade (1.0). In this tree, *F. duorarum* and *F. notialis*/*F.* nr *notialis* form two reciprocally monophyletic clades.

### *Genetic distances between species and species delimitation*

Genetic distances were measured between species in MEGA by grouping individuals by species identification (unidentified individuals were not included) and performing between-group calculations. Two analyses were run: a ‘lumped’ analysis on species and a ‘split’ analysis in which designation was made between *F. brasiliensis* N (North; collected from the Gulf of Mexico and the Florida Peninsula) and *F. brasiliensis* S (South; collected off the east coast of Central and South America). Similar results were seen across each single-gene analysis, but here only the COI values are discussed because this is the only marker for which data were available for all *Farfantepenaeus* species with the exception of *F. brevirostris* (Table 2). Distances ranged from 1.2% between *F. notialis*/*F.* nr *notialis* and *F. duorarum* to 21.5% between *F. aztecus* and the outgroup *Litopenaeus vannamei*.

Species-delimitation results showed general agreement across analyses. Using the 10X method, we determined a relatively high interspecific distance value of 6.1% (under the Kimura 2-parameter model, mean intraspecific distance in the COI data was calculated to be 0.61%). Comparing this threshold value to

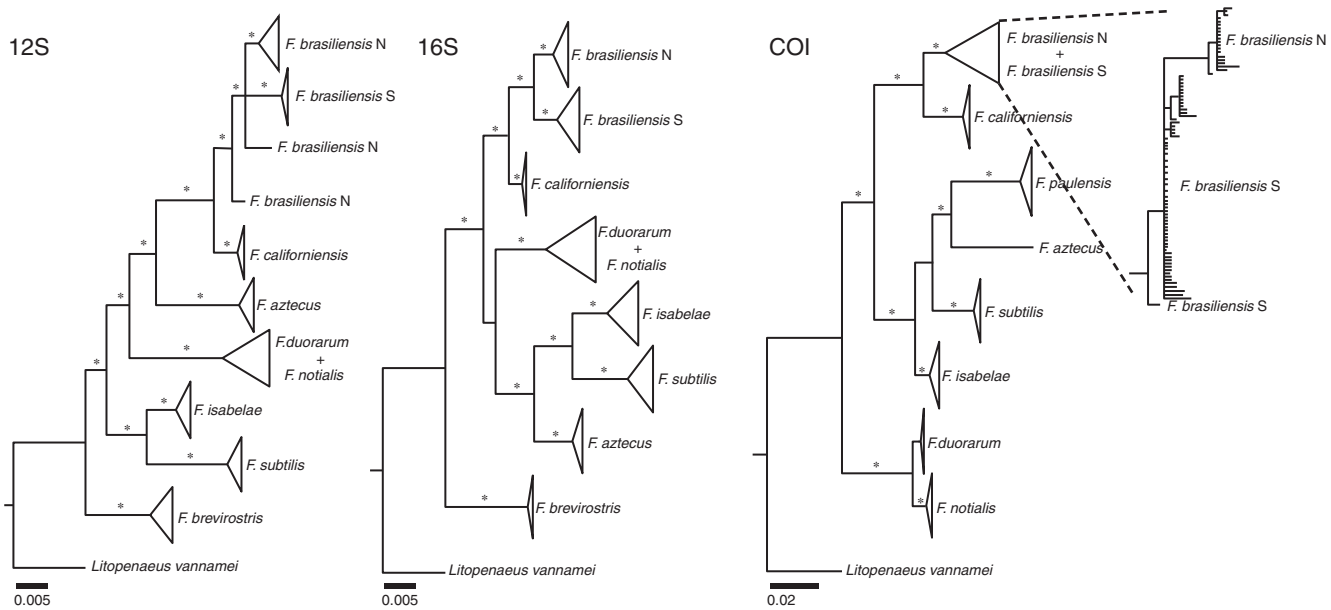


**Fig. 4.** From left to right: single-gene phylograms for 12S, 16S and COI, including an expanded view of the *Farfantepenaeus brasiliensis* N and S clades from the COI tree. Nodes supported by Bayesian posterior probabilities >0.9 and bootstrap support >70 are denoted with an asterisk above each branch.

**Table 2. Genetic distances between species are presented for a "lumped" analysis (below the diagonal), in which F. brasiliensis is analyzed as a single species, and a "split" analysis (above the diagonal), in which F. brasiliensis is divided into the two subclades suggested by the concatenated phylogram**
Values are from COI data. Values below the 10X threshold (6.1%) are indicated with *

| | *1* | *2* | *3* | *4* | *5* | *6* | *7* | *8* | *9* | *10* |
|---|---|---|---|---|---|---|---|---|---|---|
| 1. *F. aztecus* | – | 11.8% | 11.0% | 13.4% | 14.6% | 9.0% | 15.3% | 11.9% | 10.0% | 21.5% |
| 2. *F. brasiliensis* N | 11.2% | – | 2.3%* | 5.1%* | 13.6% | 9.1% | 13.6% | 11.2% | 10.0% | 21.1% |
| 3. *F. brasiliensis* S | | – | | 4.2%* | 12.6% | 8.3% | 12.6% | 10.9% | 9.9% | 20.0% |
| 4. *F. californiensis* | 13.4% | 4.4%* | | – | 12.7% | 10.3% | 12.6% | 12.5% | 11.0% | 19.9% |
| 5. *F. duorarum* | 14.6% | 12.9% | | 12.7% | – | 11.0% | 1.2%* | 14.3% | 11.0% | 17.8% |
| 6. *F. isabelae*/*F. subtilis* MI | 9.0% | 8.5% | | 10.3% | 11.0% | – | 11.5% | 7.8% | 3.3%* | 17.6% |
| 7. *F. notialis*/*F.* nr. *notialis* | 15.3% | 12.8% | | 12.6% | 1.2%* | 11.5% | – | 14.9% | 11.4% | 18.2% |
| 8. *F. paulensis* | 11.9% | 11.0% | | 12.5% | 14.3% | 7.8% | 14.9% | – | 9.0% | 20.2% |
| 9. *F. subtilis* MII | 10.0% | 9.9% | | 11.0% | 11.0% | 3.3%* | 11.4% | 9.0% | – | 17.8% |
| 10. Outgroup | 21.5% | 20.3% | | 19.9% | 17.8% | 17.6% | 18.2% | 20.2% | 17.8% | – |

the interspecific distances measured, we found support for six taxa: (1) the outgroup, *Litopenaeus vannamei*, (2) *F. aztecus*, (3) *F. brasiliensis* (N and S) and *F. californiensis*, (4) *F. duorarum* and *F. notialis*/*F.* nr *notialis*, (5) *F. isabelae*/*F. subtilis* MI and *F. subtilis* MII, and (6) *F. paulensis*. The two clades of *F. brasiliensis* do not exhibit sufficiently large genetic distance (2.3%) to qualify as sister species using the 10X method (≥6.1%), nor do *F. duorarum* and *F. notialis* (1.2%). We also noted a lack of support for a sister-species relationship between *F. isabelae*/*F. subtilis* MI and *F. subtilis* MII (3.3%). Surprisingly, the genetic distance measured between *F. brasiliensis* and *F. californiensis* (4.4%) also falls below the 6.1% threshold. However, both the *F. brasiliensis*-*F. californiensis* and the *F. isabelae*/*F. subtilis* MI-*F. subtilis* MII groups were confidently differentiated in phylogenetic reconstruction, as well as in ABGD analysis. Results from ABGD support eight stable groups (initial partition, threshold range: 0.21–1.83%) (Fig. 5), including the outgroup (1) *L. vannamei* and seven groups of *Farfantepenaeus*: (2) *F. aztecus*, (3) *F. brasiliensis* (both N and S), (4) *F. californiensis*, (5) a group containing all representatives of *F. duorarum* and *F. notialis*/*F.* nr *notialis*, (6) *F. isabelae*/*F. subtilis* MI, (7) *F. paulensis*, and (8) *F. subtilis* MII.

## Discussion

This study represents the first comprehensive phylogeny of the genus *Farfantepenaeus* and utilises more molecular markers than any previous study. Although previous studies lacked representatives of *F. brevirostris* and typically did not include representatives of both *F. isabelae*/*F. subtilis* MI and *F. subtilis* MII, the phylogenetic relationships recovered through concatenated data analysis recover the same three clades (Clades 2–4) as did previous molecular studies (Lavery *et al.* 2004; Voloch *et al.* 2005). However, in investigating cryptic

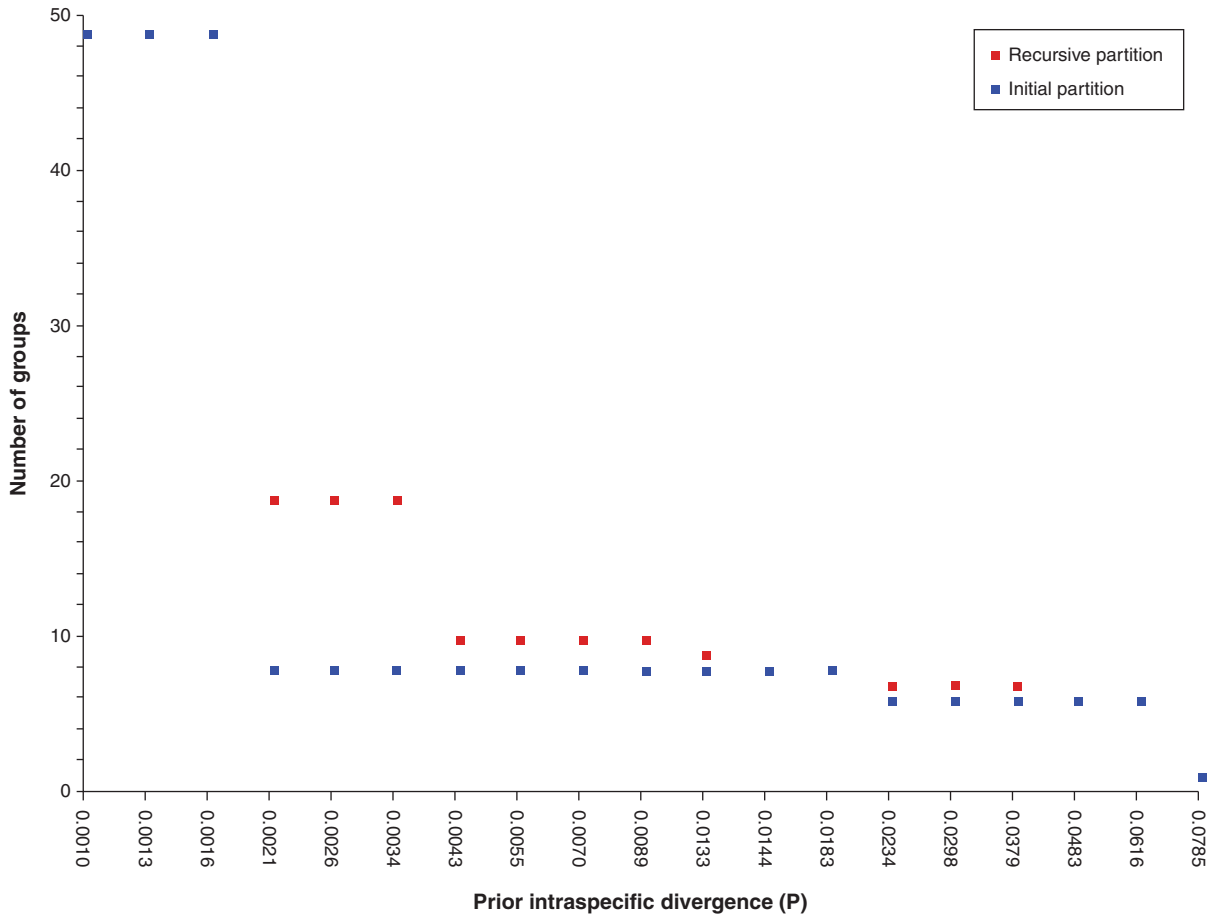


**Fig. 5.** Automatic Barcode Gap Discovery (ABGD) analysis of uncorrected pairwise p-distances calculated from the COI dataset in MEGA6. Prior intraspecific divergence ranged from 0.001 to 0.1. The relative gap width (X) was set to 0.5 and run between 20 and 1000 steps (figure generated from analysis run for 20 steps). The number of groups (*y*-axis) is stable at 8 in the initial partition across prior intraspecific divergence (0.21%–1.83%).

speciation, our results uncovered evidence for previously undescribed population structure in *F. brasiliensis*, lack of evidence for species status of *F. notialis*, and strong molecular support for *F. isabelae*, previously described as *F. subtilis* morphotype I, as sister to *F. subtilis* morphotype II.

### *Phylogenetic relationships and morphological considerations*

The concatenated tree recovers *F. brevirostris*, previously not included in molecular phylogenies, as sister to the remaining species. *F. brevirostris* and *F. californiensis* are both Pacific species, but are differentiated by the detailed structure of the gastrofrontal carina (anteriorly indistinct or well defined, respectively), gastro-orbital carina (short or long, respectively), adrostral sulcus (mesially directed towards posterior or almost straight, respectively), distomedian projection of the petasma (short and apically blunt with 1–4 teeth or long and apically pointed with teeth absent, respectively), anterior setae of the thelycum (present or absent, respectively), and the auricle (absent or present and relatively large, respectively) (Pérez-Farfante 1988). Interestingly, *F. brevirostris* is distantly related to *F. californiensis*, despite both having distributions restricted to the Pacific Ocean.

The concatenated tree recovers a clade containing *F. notialis* and *F.* nr *notialis* nested within *F. duorarum*, and only a small genetic distance was recovered between these taxa (1.2%). This differs from previous molecular phylogenies, which confidently separate *F. duorarum* and *F. notialis* (Lavery *et al*. 2004; Voloch *et al*. 2005), including molecular analyses with low resolution at deeper nodes (Maggioni *et al*. 2001). Previous topologies may be a result of data recycling since both Lavery *et al*. (2004) and Voloch *et al*. (2005) include *F. notialis* as a single GenBank sequence collected from Cuba (X84350: García-Machado *et al*. 1999). The analysis presented here also included this sequence, as well as five sequences of *F.* nr *notialis* (collected from multiple sites within Biscayne Bay on the southeast coast of Florida, USA). The specimens that were identified as *F.* nr *notialis* were all collected outside the current distributional range but grouped with the *F. notialis* GenBank sequence from within the described range (Cuba). Nodal support for this clade was low (0.65/28). However, as we have included only mitochondrial sequence data in this study, the lack of resolution between *F. duorarum* and *F. notialis* may

be the result of incomplete lineage sorting at the mitochondrial level, rather than a lack of reciprocal monophyly between these species.

Morphologically, there is little to differentiate between *F. duorarum* and *F. notialis*. The primary distinguishing characteristic for adults is difference in K/S (<3 or >3, respectively) (Pérez-Farfante 1988). The initial separation of *F. subtilis* as a subspecies of *F. aztecus* was also by means of difference in K/S (Pérez-Farfante 1967), but the morphological difference here may have been more pronounced: modal K/S ratio was 3.5 for *F. subtilis* versus 1.25 for *F. aztecus*. At the time, Pérez-Farfante (1967) suggested that this difference in K/S observed between populations of *F. subtilis* could have been due to environmental factors. The variability Pérez-Farfante viewed may have been due to looking at *F. subtilis* intermingled with what later was described as *F. isabelae*, as suggested by Tavares and Gusmão (2016) in the description of *F. isabelae*. Teodoro *et al*. (2016) reported difficulty in discriminating between *Farfantepenaeus* species using morphological features: only 38% of taxonomically identified *F. paulensis* and *F. brasiliensis* juveniles had their identity confirmed with molecular methods. Our results suggest that another morphologic characteristic commonly used in *Farfantepenaeus* taxonomy, adrostral sulci condition, may not be diagnostic. Additional molecular data, especially the inclusion of nuclear genes, are needed to resolve the relationship between *F. notialis* and *F. duorarum*.

*Farfantepenaeus aztecus*, *F. paulensis*, *F. isabelae*/*F. subtilis* MI, and *F. subtilis* MII form a clade. In previous studies, wherein *F. subtilis* was included only as MI, all three possible arrangements have been recovered (Maggioni *et al*. 2001; Lavery *et al*. 2004; Voloch *et al*. 2005). The analysis conducted here recovered a clade of *F. isabelae*/*F. subtilis* MI and *F. subtilis* MII, sister to *F. aztecus* and *F. paulensis*, in an unresolved polytomy. These four taxa are differentiated morphologically by the adrostral sulcus (long in *F. aztecus* and *F. paulensis*; short, shallow, and posteriorly narrow in *F. isabelae*/*F. subtilis* MI; and short and of equal width along its entire length in *F. subtilis* MII), median sulcus (long and deep in *F. aztecus*; short, shallow, and rarely continuous in *F. paulensis*), dorsolateral sulcus (broad in *F. aztecus*, narrow in *F. paulensis*), and K/S (less than 3 in *F. aztecus*, greater than 3 in *F. paulensis*). Additionally, reproductive morphology can be used to distinguish between these four taxa, specifically: the distal part of the ventral costa of the petasma (tapered to a point and armed with a patch of tightly grouped small teeth in *F. aztecus*, blunt and straight with irregular teeth around the border in *F. paulensis*, or unarmed with a narrow patch of small teeth irregularly occurring around the border in *F. subtilis* MI and MII) and thelycum processes (both broad in *F. aztecus*, both narrow in *F. paulensis*, anterior process sharply pointed and posterior process diamond-shaped in *F. isabelae*/*F. subtilis* MI, or anterior process rounded and posterior process foliaceous in *F. subtilis* MII) (Pérez-Farfante 1988). Our results support the species status of *F. isabelae*, specifically as *F. subtilis* MI, and find a relatively large genetic distance between *F. isabelae*/*F. subtilis* MI and its sister, *F. subtilis* MII. Despite the polytomy at the deeper node, the reciprocally monophyletic sister relationship between *F. isabelae*/*F. subtilis* MI and *F. subtilis* MII, when considered alongside the genetic distances and branch lengths separating the species in this clade, suggests that *F. subtilis* MII does not represent the northernmost population of *F. paulensis*, as has been posited in previous research (D'Incao *et al*. 1998).

*Farfantepenaeus brasiliensis* and *F. californiensis* are consistently recovered as a clade, in agreement with previous molecular studies analysing 16S and COI data (Lavery *et al*. 2004; Voloch *et al*. 2005). Both species bear a long distomedian petasma projection that folds distally to form a large, inwardly protruding auricle (Pérez-Farfante 1988). The two species differ in their distributions: as their names suggest, *F. brasiliensis* occurs in the Atlantic and *F. californiensis* occupies a Pacific range. Additionally, *F. brasiliensis* is typically distinguished from other species of *Farfantepenaeus* by the dark red spot that occurs at the juncture of the 3rd and 4th abdominal segments, although this feature is also present in *F. duorarum* and *F. notialis* (Pérez-Farfante 1988), albeit less consistently.

The presence of polytomies within the phylogenetic tree indicates a need for additional molecular data. The addition of nuclear genes would likely clarify these relationships and may resolve the tree. Unfortunately, we were unable to include these in this study, largely due to a lack of voucher specimens. *F. notialis* and *F. paulensis* are included here only as GenBank Accessions as we were unable to obtain samples of these species. Without taxonomically identified samples in hand, we are unable to confidently or responsibly include additional loci for *F. notialis* or *F. paulensis*. As such, we interpret our results cautiously, aware of the limitations of this study.

### Phylogeographic patterns

Interpreting the phylogeny as a whole, an intriguing phylogeographic signal is revealed: latitudinal speciation supporting a biogeographic break between the coasts of North America and Central/South America. Clade 1 contains the Pacific species *F. brevirostris* and is recovered as sister to the rest of the *Farfantepenaeus* species. This agrees with previous work suggesting that the genus originated in the Indo-Pacific (Dall *et al*. 1990; Baldwin *et al*. 1998; Lavery *et al*. 2004). The relationships between the remaining species exhibit a latitudinal trend within each clade.

*Farfantepenaeus duorarum* and *F. notialis*/*F.* nr *notialis* form Clade 2. These species currently have described ranges that reflect this latitudinal biogeographic break: *F. duorarum* has been reported along the east coast of the USA and along the Gulf coast through Mexico, and *F. notialis* is found in the Caribbean, along the coast of Brazil (Tavares 2002), and in the southern Gulf of Mexico in Mexican estuaries (Pérez-Castañeda and Defeo 2000; May-Kú and Ordóñez-López 2006). However, the molecular results suggest that this may not be a true break: low genetic distance and intermixed terminal nodes of *F. notialis*/*F.* nr *notialis* and *F. duorarum* bring the validity of *F. notialis* as a species into question. The genetic homogeneity seen between *F. duorarum* and *F. notialis* could be attributed to oceanographic currents, especially the Gulf Loop Current, which would mix individuals of *F. duorarum* and *F. notialis* near the limits of their respective southern and northern ranges in the Gulf of Mexico. Indeed, *F. duorarum* and *F. notialis*, along with *F. brasiliensis*

and *F. aztecus*, have been reported as co-occurring in estuaries in the southern Gulf of Mexico (Pérez-Castañeda and Defeo 2000; May-Kú and Ordóñez-López 2006).

All species within Clade 3, *F.aztecus*, *F. isabelae*/*F. subtilis* MI, *F. paulensis*, and *F. subtilis* MII, occur along the western Atlantic at slightly overlapping latitudes: *F. aztecus* occupies the northern shores, along the east coast of the USA and in the Gulf of Mexico (Tavares 2002); *F. isabelae*/*F. subtilis* MI has a described range in the Caribbean, ranging from Cuba to northern Brazil, which entirely overlaps with the range of its sister *F. subtilis* MII (Tavares 2002; Tavares and Gusmão 2016). The range of *F. paulensis* also overlaps *F. subtilis* MII to a large degree, with a described range from northern Brazil to Rio de La Plata (Heemstra and Randall 1993), *F. paulensis* co-occurs with *F. subtilis* MII from northern Brazil to Rio de Janeiro. In general, it appears that *F. aztecus* occupies territory north of the Equator, *F. isabelae*/*F. subtilis* MI and *F. subtilis* MII are distributed across the Equator, and *F. paulensis* occurs south of the Equator. Such phylogeographic structure has been associated with historical low sea levels (Dall *et al*. 1990): hypothetically, populations of a species could have become separated and formed new species when low sea levels geographically isolated basins.

Clade 4 comprises *F. californiensis* and *F. brasiliensis*, a Pacific and Atlantic species, respectively. Expanding from an Indo-Pacific origin, *Farfantepenaeus* is hypothesised to have migrated eastward and westward (Dall *et al*. 1990; Baldwin *et al*. 1998; Lavery *et al*. 2004). The eastward expansion, combined with oscillating sea levels beginning in the Pliocene, would have allowed transisthmus migration into the Atlantic Ocean and subsequently impeded back-migration (Baldwin *et al*. 1998; Lavery *et al*. 2004). Clade 4 does not exhibit the latitudinal speciation pattern seen in Clade 3, as *F. brasiliensis* extends along the coasts of both North and South America (Tavares 2002). However, the strongly supported northern and southern subclades of *F. brasiliensis* do lend support to the biogeographic break between the coasts of North America and those of Central/South America (Avise 1992; Young *et al*. 2002; Cowen *et al*. 2006).

The phylogeographic patterns indicated in our results are intriguing, providing tentative evidence of the biogeographic role of oceanographic currents in the evolutionary history of species of *Farfantepenaeus*. Our results prompt further inquiry into the effects of the major current systems of the western North Atlantic, Caribbean, and Gulf of Mexico as driver and succor of speciation in the genus.

### *Investigation of cryptic diversification within pink shrimp, and economic implications*

Early allozyme studies of genetic diversity within the genus indicated very small genetic distances between species (Mulley and Latter 1980; Nelson and Hedgecock 1980; Redfield *et al*. 1980; Salini 1987; Sunden and Davis 1991; Tam and Chu 1993), causing researchers to posit that these shrimps were very slow-evolving (Dall *et al*. 1990). More recent studies of diversity between the species of *Farfantepenaeus* found 8–24% distance in COI alone (Baldwin *et al*. 1998). The results of the present study agree with these recent studies: except for *F. notialis*/*F.* nr *notialis*–*F. duorarum*, *F. isabelae*/*F. subtilis* MI–*F. subtilis* MII, and *F. brasiliensis*-*F. californiensis,* all interspecific distances were greater than 6.1% (7.8–21.5%). Genetic distance between *F. notialis*/*F.* nr *notialis* and *F. duorarum* was 1.2%, which is more than 50% higher than the previous measure of 0.7% (Gusmão *et al*. 2000). This may be due to the collection of *F. notialis* from outside the described species range. The results indicate substantial genetic distance between the northern and southern representatives of *F. brasiliensis* (2.3%), perhaps even representing distinct ESUs.

Pérez-Farfante (1967) established *notialis* as a subspecies of *duorarum*, even before the genus *Farfantepenaeus* was established. The two taxa were primarily distinguished by variation in adrostral sulcus condition. Described petasmas and thelycums were very similar in these two species (see Fig. 1; Pérez-Farfante 1970*a*, 1970*c*). In previous molecular phylogenies, *F. notialis* is treated, and supported, as the sister species to *F. duorarum* (Maggioni *et al*. 2001; Lavery *et al*. 2004; Voloch *et al*. 2005; Tavares and Gusmão 2016). However, the phylogenetic trees and calculated genetic distances presented here do not support *F. notialis* as a species distinct from *F. duorarum*. Indeed, the small genetic distance between the two is about one-sixth of the threshold value of 6.1% determined using the 10X method. Comparatively, our calculated threshold value is more conservative than those calculated in the amphipod genus *Hyalella* (3.75%: Witt *et al*. 2006) and birds (2.7%: Hebert *et al*. 2004), but lower than that calculated for the snapping shrimp *Synalpheus* (10.2%: Hultgren *et al*. 2014). It is also important to note that the 10X method is a relatively simple, descriptive (non-statistical) approach. Because of this, when results from the 10X method and ABGD analysis disagree, we tend to have more confidence in the ABGD results. Moreover, species identified using ABGD analysis were also frequently recovered as reciprocally monophyletic in phylogenetic analysis (as in the case of *F. brasiliensis*-*F. californiensis*).

While caution must be taken when analysing species delimitation based solely on mitochondrial data (Lee 2004; Moritz and Cicero 2004), COI sequence data can identify potential cryptic species that warrant further taxonomic investigation (Desalle 2006). Due to the limited sampling from within the currently recognised distributional range of *F. notialis*, the findings should be interpreted critically; however, *F. duorarum* and *F. notialis* do not appear to represent separate ESUs. While this may be the case, the phylogenetic analyses indicate that *F. notialis* adds structure within the clade, which is otherwise fairly homogeneous. The genetic diversity represented by this structure must be preserved, so in this respect, treating the two as distinct ESUs may be beneficial to prevent over-harvesting of *F. notialis*, whose larger distribution makes it an economic target for a greater number of nations. It is critical that future phylogenies include representatives of *F. duorarum* and *F. notialis* throughout their currently described distributional ranges, nuclear data, and, ideally, the holotypes in order to validate or refute the results we present here.

Individuals of *F. brasiliensis* fall into two subclades, strongly suggesting two distinct ESUs. Indeed, Peréz-Farfante noted two geographically separated populations of *F. brasiliensis*, differing in K/S (Pérez-Farfante 1970*c*, fig. 5, p. 168; Pérez-Farfante 1988, fig. 13, p. 10, and reproduced here in Fig. 2D and 2D′). Although

the northern (Barbuda and Saint Augustine, Florida, USA: Pérez-Farfante 1970*a* and 1988, respectively) and southern (Camocin, Brazil and Rio de Janeiro, Brazil: Pérez-Farfante 1970*c* and 1988, respectively) populations described by Peréz-Farfante do not align with the northern and southern geography we find, a latitudinal pattern is supported. While genetic distance alone is not enough to warrant new species status, revealing population structure across the distributional range is important to fishery management. Varying fishing pressure may be experienced across the distribution of this species. In the southern part of its range, *F. brasiliensis* is a major component of the Brazilian 'pink shrimp' fishery (Leite and Petrere 2006), whereas in the north *F. brasiliensis* may be a lesser, and generally unrecognised, component of commercial *Farfantepenaeus* landings. Given the immense importance of genetic diversity to species health, such uneven fishing pressure may be threatening diversity unique to *F. brasiliensis* S while unintentionally applying positive selection pressure to *F. brasiliensis* N. A summary of evidence for and against separating *F. notialis* from *F. duorarum* and *F. brasiliensis* N from *F. brasiliensis* S is presented in Table S7.

## Conclusions

The work we present here agrees well with previous molecular work in many respects, while also furthering our understanding of taxonomy and evolutionary relationships within *Farfantepeaneus*. In including *F. brevirostris* for the first time, we identify it as sister to the remaining species in the genus. Additionally, we provide evidence establishing *F. subtilis* MII as sister to *F. isabelae*/*F. subtilis* MI, contradicting a previous hypothesis that *F. subtilis* MII represented a population of *F. paulensis*. However, our results call into question whether accepted diagnostic characters (K/S and adrostral sulci condition) are taxonomically informative. Our concatenated phylogeny does not separate *F. notialis* and *F. duorarum* into separate species, though this may be an artefact of the sequence data used, and more individuals that span the distributional range of these species need to be included. We also uncovered structure within *F. brasiliensis*, indicating the existence of two populations. Our study also identifies a previously undescribed phylogeographic signal of latitudinal speciation in the genus. Overall, this work provides an inclusive, robust phylogeny that contributes to our knowledge of *Farfantepenaeus*.

## Future work

Future efforts to clarify the evolutionary relationships within *Farfantepenaeus* should focus on increasing the number and genetic source of molecular markers (e.g. nuclear, as per Timm and Bracken-Grissom 2015), as well as on the discovery and inclusion of diagnostic morphological characters. Additionally, more thorough sampling along species' ranges would better elucidate the biogeographic factors facilitating speciation in the genus (Ayre *et al*. 2009). Inclusion of representatives within each species across its range would improve estimates of intra- and inter-specific genetic diversity and likely clarify phylogenetic relationships. The population structure we find is unexpected and may inform us about the role of oceanographic features in marine speciation processes. To investigate population structure in more species of *Farfantepenaeus*, a population genetics/genomics-level study should be conducted, focusing on the species along the described distribution. Research efforts in the realm of *Farfantepenaeus* evolution should focus on contextualising phylogeographic patterns in terms of environmental factors (e.g. currents, habitats of juveniles and adults, and geological events) and economic pressures (e.g. fishing pressures and active species management efforts).

## Conflicts of interest

The authors declare that they have no conflicts of interest.

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