Molecular Phylogenetics and Evolution 77 (2014) 116–125

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Phylogenetic relationships within the snapping shrimp genus *Synalpheus* (Decapoda: Alpheidae)

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ARTICLE INFO

Article history:
Received 20 July 2013
Revised 9 March 2014
Accepted 11 March 2014
Available online 28 March 2014

Keywords:
Synalpheus
Phylogeny
Alpheidae
Snapping shrimp
Cryptic species

ABSTRACT

The snapping shrimp genus *Synalpheus* (Alpheidae) is one of the most speciose decapod genera, with over 160 described species worldwide. Most species live in symbiotic relationships with other marine organisms, such as sponges, corals and crinoids, and some sponge-dwelling species have a highly organized, social structure. The present study is the first worldwide molecular phylogenetic analysis of *Synalpheus*, based on >2200 bp of sequence data from two mitochondrial (COI and 16S) and two nuclear (PEPCK and 18S) loci. Our molecular data show strong support for monophyly of three out of six traditionally recognized morphology-based species groups: the *S. brevicarpus*, *S. comatularum* and *S. gambarelloides* groups. The remaining three species groups (*S. paulsoni*, *S. neomeris* and *S. coutierei* groups) are non-monophyletic in their current composition and will need to be either abandoned or taxonomically redefined. We also identified potential cryptic species of *Synalpheus* in our dataset, using intraspecific and interspecific sequence variation in COI from the taxonomically well-studied *S. gambarelloides* group to establish a genetic divergence threshold. We then used both genetic divergence and tree-based criteria (reciprocal monophyly) to identify potential cryptic species in the remaining taxa of the genus. Our results suggest the presence of multiple cryptic lineages in *Synalpheus*, underlining the need for more integrative taxonomic studies—including morphological, ecological, molecular, and color pattern data—in this biologically interesting genus.

1. Introduction

Species-rich marine groups present a particular challenge for both taxonomy and systematics, as they often contain cryptic taxa that are difficult to identify using traditional, morphology-based criteria (Knowlton, 1986, 1993, 2000; Hebert et al., 2003; Witt et al., 2006; Barber and Boyce, 2006; Mathews, 2006). Diagnosing and defining species in these groups, and examining their phylogenetic relationships, is critical for our understanding of evolution and diversity of tropical marine invertebrate communities.

Shrimps of the family Alpheidae represent one of the most diverse groups of marine decapod crustaceans, with 45 genera and over 600 described species worldwide (Chace, 1972, 1988; Anker et al., 2006; De Grave and Fransen, 2011). Alpheid shrimps are among the most abundant decapods in tropical and subtropical, shallow-water habitats, in particular on coral reefs (Pearse, 1932; Ruetzler, 1976; Felder and Chaney, 1979). Many alpheids live in permanent association with a variety of other marine invertebrates and gobiid fishes (Banner and Banner, 1975; Bruce, 1976; Anker et al., 2005; Rios and Duffy, 2007; Karplus and Thompson, 2011). The genus *Synalpheus* Spence Bate, 1888 is the second-largest genus in the family, with over 160 described species worldwide (Banner and Banner, 1975; Chace, 1988; De Grave and Fransen, 2011). The ecological diversity of *Synalpheus*, which includes multiple instances of symbioses with sponges, echinoderms and cnidarians (Fig. 1), and several modes of social organization, has made this genus an attractive model system for studies of speciation, host specialization and evolution of sociality (Duffy, 1992, 1996; VandenSpiegel et al., 1998; Duffy and Macdonald, 2010; Hultgren and Duffy, 2012). However, most of the research in the genus *Synalpheus* has focused on a single clade of 45 described Caribbean sponge-dwelling species in the *Synalpheus gambarelloides* species group, and examining the

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http://dx.doi.org/10.1016/j.ympev.2014.03.008
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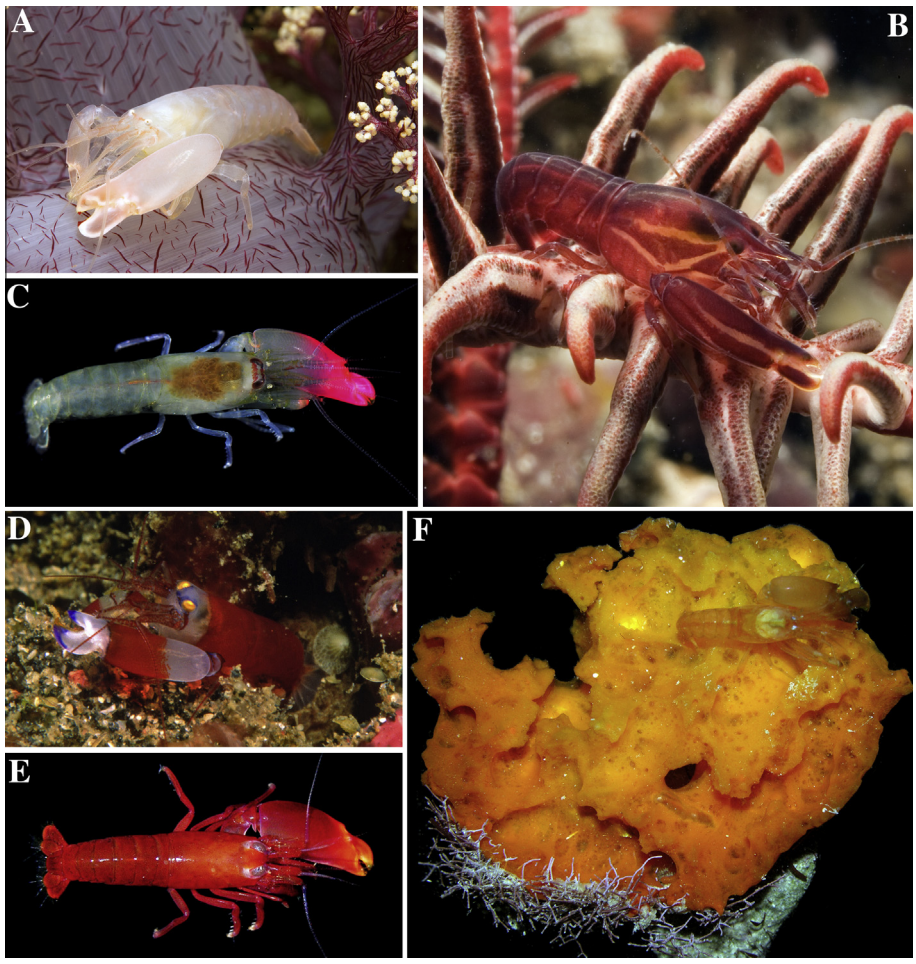

Fig. 1. Diversity of host ecology and coloration in the snapping shrimp genus *Synalpheus*: A – *S. neomeris*, an Indo-West Pacific species associated with soft corals (*Dendronephthya* sp.); B – *S. stimpsonii*, an Indo-West Pacific species complex associated with comasterid feather stars; C – *S. antillensis*, a West Atlantic species free-living in coral rubble; D – *S. modestus*, an Indo-West Pacific species, free-living or commensal of sessile invertebrates; E – *S. charon*, an Indo-Pacific species associated with pocilloporid corals; F – *S. dardeaui*, a West Atlantic sponge-dwelling species (here on partly cut-open *Lissodendoryx colombiensis*). Photographic credits: A, B, D – Ned Deloach; C, E, F – Arthur Anker.

evolution of specialized host use across the entire genus requires a robust phylogeny that spans the worldwide diversity of this group.

Due in part to the spectacular diversity of *Synalpheus* in tropical marine habitats, the taxonomy of many described species remains in an unsatisfying state. This is especially true for numerous Indo-West Pacific and East Pacific taxa, some with problematic synonyms, subspecies and varieties (De Man, 1888; Coutière, 1905, 1908, 1909, 1921; De Man, 1911). Furthermore, almost nothing is known about the phylogenetic structure within this genus worldwide (Banner and Banner, 1975; Anker and De Grave, 2008; Hermoso-Salazar et al., 2008). Coutière (1908, 1909) subdivided the genus into six informal species groups: *S. brevicarpus*, *S. biunguiculatus* (later changed to *S. coutierei*), *S. comatularum*, *S. laevimanus* (later changed to *S. gambarelloides*), *S. neomeris*, and *S. paulsoni* groups. Subsequent taxonomic treatments (Banner and Banner, 1975) concluded that only three of these species groups (*S. brevicarpus*, *S. comatularum*, and *S. gambarelloides* groups) had enough morphological support to be taxonomically useful (see also Hermoso-Salazar et al., 2008).

The *S. brevicarpus* group contains approximately a dozen species (both sponge-symbionts and non-symbiotic, Fig. 1C), some currently under description (Anker, unpublished data), distributed exclusively in the eastern Pacific and western Atlantic. The *S. comatularum* group includes at least 10 described species, all found in the tropical parts of the Indo-West Pacific; most (if not all) of its members are associates of crinoids (Fig. 1B). The very large *S. gambarelloides* species group (>70 described species) is distributed worldwide, although the vast majority of species occur in the tropical western Atlantic. It is by far the best-studied group of *Synalpheus*, in terms of ecology and phylogenetics. All members of the *S. gambarelloides* group are ecologically quite homogeneous, dwelling exclusively in the interior canals of sponges (Banner and Banner, 1975; Dardeau, 1984; Chace, 1988; Ríos and Duffy, 2007). Molecular studies have consistently indicated strong support for the monophyly of this group, although prior phylogenies sampled only a few taxa outside of the *S. gambarelloides* group (Duffy et al., 2000; Morrison et al., 2004; Hultgren and Duffy, 2011). Based on these data, Ríos and Duffy (2007) erected a new genus, *Zuzalpheus*, for the *S. gambarelloides* group. However, Anker and De Grave (2008) pointed out that *Zuzalpheus* was separated from *Synalpheus* based on minor and ambiguous morphological differences, and this rendered the rest of the genus *Synalpheus* paraphyletic, based on the phylogeny proposed by Morrison et al. (2004). The resolution of relationships within *Synalpheus*, and possible establishment of morphologically defined subgenera (including *Zuzalpheus*), requires a much more extensive and worldwide sampling of taxa from all six of Coutière's species groups worldwide.

Identifying and describing cryptic species complexes is a major challenge for assessing worldwide species diversity in *Synalpheus*. For example, since Dardeau's (1984) work on the western Atlantic

species of the *S. gambarelloides* group, many cryptic species complexes have been identified and split into several species; as a result, the total number of described species in the *S. gambarelloides* group has more than doubled over the last two decades, from 19 to currently 44 (summarized in Hultgren and Duffy, 2011). In several cases, it may be difficult to use morphological characters alone to accurately delimit species; in this group, molecular sequencing has assisted in discriminating between morphologically similar species (Hultgren et al., 2010), and has strongly supported the morphology-based species concepts (Morrison et al., 2004; Hultgren and Duffy, 2011). Overall, a more integrative approach is needed, which includes molecular data (e.g., COI barcoding gene), as well as color patterns, ecology (e.g., hosts for symbiotic species), and traditional morphological characters.

In many animal groups, molecular data are increasingly being used to identify potential cryptic species (Hebert et al., 2004; Witt et al., 2006; Barber and Boyce, 2006; Oliver et al., 2009), with the 650 bp 5′ region of the mitochondrial cytochrome oxidase I (COI) gene the generally accepted DNA barcode marker (Hebert et al., 2003; Goldstein and Desalle, 2010). Some workers have utilized a delimitation threshold of ten times greater than intraspecific distance (Hebert et al., 2004; Witt et al., 2006), and more recently the "gap" between intraspecific and congeneric heterospecific genetic distance in COI has been utilized as a threshold for species delimitation (Lefebure et al., 2006; Costa et al., 2007; Radulovici et al., 2009; Del-Prado et al., 2010; Goldstein and Desalle, 2010; Puillandre et al., 2012). Other workers advocate a tree-based approach, specifically using reciprocal monophyly as a criterion for species delimitation (reviewed in Goldstein and Desalle, 2010), and many studies use some combination of tree-based and genetic barcoding approaches to identify potential cryptic species (Barber and Boyce, 2006; Xavier et al., 2010; Murray et al., 2012). Ultimately, these potential cryptic species can be confirmed by future studies through traditional taxonomic and coalescent-based species tree inference methods.

The main goal of the present study is to propose the first worldwide phylogeny of the genus *Synalpheus*, including representatives from all six informal species groups (Coutière, 1909; Banner and Banner, 1975) and from all four oceanic provinces (East Atlantic, West Atlantic, East Pacific, and Indo-West Pacific). We constructed phylogenetic trees using molecular sequence data from four loci: two nuclear loci and two mitochondrial loci, including the 5′ region of the mitochondrial COI gene used for genetic barcoding, to assess monophyly of each of the six species groups, and the relationships among these groups. Within the taxonomically well-studied *S. gambarelloides* group, we calculated intraspecific and interspecific divergence in COI, used these data to establish a genetic distance threshold, and used a combination of tree-based and genetic distance criteria to identify potential cryptic species in the remaining taxa of *Synalpheus*. This study provides the foundation for further taxonomic work exploring diversity and clarifying phylogenetic relationships within the genus *Synalpheus.*

2. Material and methods

2.1. Taxon sampling and species identification

Sequence data were generated from specimens of *Synalpheus* and *Alpheus* (latter used as outgroup) collected primarily over the last decade (2001–2010), including numerous specimens collected and processed by two of the authors (AA and KH, see Supplementary data 1). We also used 16S sequence data from prior studies (Morrison et al., 2004; Hultgren and Duffy, 2011) and sequence data (COI, 16S, and 18S) generated by the Smithsonian Institution for the Barcode of Life project (archived by the Barcode of Life Data Systems, or BOLD). Specimens were collected intertidally and subtidally (scuba diving or snorkeling), usually by breaking up coral rubble or examining interior of sponges; some were found under rocks or on crinoids and living corals. Most specimens were photographed alive in the field and preserved in 80–95% ethanol. Species identifications were made by AA and KH based on existing species descriptions (e.g., Banner and Banner, 1975; Ríos and Duffy, 2007; Anker and Tóth, 2008). Collection locations, host/habitat information, voucher locations, and additional taxonomic information are summarized in Supplementary data 1; information for selected species was also deposited in the Barcode of Life Data System website (BOLD, www.barcodinglife.org).

2.2. DNA/RNA extraction, sequencing, and alignment

For most specimens, RNA was extracted using the SV Total RNA Isolation System (Promega, Madison WI) using a modified protocol that preserved some genomic DNA (Regier, 2008). For a subset of specimens, we used genomic DNA that was extracted using the Qiagen tissue protocol on a Biosprint 96 workstation. Sequencing was done at the University of Miami core sequencing facility or through the Smithsonian's DNA barcoding project (Laboratories of Analytic Biology, hereafter LAB). We sequenced individuals for four loci: the 5′ end of the mitochondrial cytochrome oxidase I gene used for genetic barcoding (COI); partial sequences of the nuclear large ribosomal subunit 18S (18S); partial sequences of the mitochondrial rRNA molecule 16S (16S); and partial sequences of the nuclear phosphoenolpyruvate carboxykinase gene (PEPCK). 18S and 16S were amplified directly; for COI, we used both direct amplification using degenerate primers and in some cases a RT-PCR protocol, modified from Hurt et al. (2009), to reduce the risk of amplifying of nuclear pseudogenes (Williams and Knowlton, 2001); for interior and exterior primers, see Table 1. The RT-PCR protocol was also used to amplify the nuclear PEPCK locus in order to reduce the risk of amplifying non-functional copies of the gene and to allow us to predict the size of the desired amplicon. Briefly, we synthesized cDNA from RNA extractions using MuLV reverse transcriptase (Applied Biosystems), RNase inhibitor (Applied Biosystems), and a sequence-specific reverse primer. This cDNA was used as a template in a PCR reaction that utilized a forward primer and PCR/thermocycler conditions suggested by Regier (2008). PCR products of the correct size were excised from a 1% low-melt agarose gel, and extracted using the GELase protocol (Epicentre Biotechnologies), and sequenced on an ABI Prism 3130xl sequencer. Forward and reverse sequences from all loci were visually checked and trimmed using the program SEQUENCHER v4.8 (Gene Codes corporation).

Each individual locus was aligned using the default parameters on the program Muscle v3.8 (Edgar, 2004), and we used MacClade 4.08 (Maddison and Maddison, 2005) to translate coding loci (PEPCK and COI) into protein sequence to check for stop codons, which may indicate the presence of pseudogenes previously reported in Alpheus (Williams and Knowlton, 2001; Buhay, 2009). No stop codons were detected in any of the coding sequences. We also examined individual gene trees to check for unusual species placements that could indicate pseudogenes or multiple coding copies of the loci used in the study. In the case of PEPCK, we occasionally amplified multiple copies of the PEPCK locus (sometimes from the same individual) in individuals of *Synalpheus*. However, different loci formed well-supported clades that were distinct outgroups to the most commonly amplified locus, and were easily distinguished and removed. In most cases, sequences from the second locus did not contain stop codons, suggesting they may not necessarily be pseudogenes but possibly multiple functional copies. We trimmed sequences to exclude the trailing ends for COI (658 bp region utilized), 16S (603 bp), and PEPCK (519 bp). Although alignment

Table 1
Primers used in analysis, primer source, sequence generation, and amplification strategy; Smithsonian LAB indicates sequences generated at the Laboratory of Analytical Biology through the DNA barcoding project.

Locus/primer name		Sequence 5′–3′	Primer source	Amplification/location
COI	jgLCO1490	5′-GMA TAG TAG GMA CRG CYC TNA-3′		Direct PCR or RT-PCR (U. Miami)
	jgHCO2198	5′-YCC TGT GAA TAG GGG GAA TC-3′		
	jgHCO_2198	5′ TAI ACY TCI GGR TGI CCR AAR AAY CA 3′		Direct PCR (Smithsonian LAB)
	jgLCO_1490	5′ TIT CIA CIA AYC AYA ARG AYA TTG G 3′		
PEPCK	PEPCK-3F	5′-GCT ATG AAA ACC GTC CTT TCC-3′	*Alpheus/Synalpheus* Alignment	RT-PCR (U. Miami)
	PEPCK-454R	5′-TGC TGT AGG TAG TGG CCA AA-3′	*Alpheus/Synalpheus* Alignment	
18S	18S-Y	5′-CAG ACA AAT CGC TCC ACC AAC-3′	Apakupakul et al. (1999)	Direct PCR (U. Miami)
	18S-A	5′-AAC CTG GTT GAT CCT GCC AGT-3′	Medlin et al. (1988)	
	SR7_18Sr	5′-GTTCAACTACGAGCTTTTTAA-3′	Vilgalys and Bun (1994) PNAS	Direct PCR (EukF_18Sf/SR7_18Sr) (Smithsonian LAB)
	EukF_18Sf	5′-AACCTGGTTGATCCTGCCAGT-3′	Sands et a. BMC Ecol. 2008	
16S	16S-1472R	5′-AGA TAG AAA CCA ACC TGG-3′	Crandall and Fitzpatrick 1996	Direct PCR (16Sar/16S1472R or 16Sar/16Sbr) (U. Miami)
	16S-ar	5′-CGC CTG TTT ATC AAA AAC AT-3′	Palumbi et al. (1991)	Direct PCR (16Sar/16Sbr)(Smithsonian LAB)
	16Sbr	5′ -CCG GTC TGA ACT CAG ATC ACG T-3′		

of the 16S locus was fairly straightforward, some portions of the 18S locus were highly divergent among different described species and difficult to align. We used the program GBlocks v0.9 (Castresana, 2000; Talavera and Castresana, 2007) to exclude ambiguous areas of the alignment using relaxed gap selection criteria (allowed gap positions = all) suggested from simulation studies (Talavera and Castresana, 2007), yielding a 457-bp region for analysis near the 5′ end of our original sequences. As there are few well-tested methods for incorporating gap information into model-based phylogenetic methods (Phillips et al., 2000; Simmons et al., 2007), we treated all gaps as missing data. We used the program MrModeltest v.2.3 (Nylander, 2004) to assess different models of nucleotide substitution for each of the three loci, and selected the model of molecular evolution with the best fit to the data using the Akaike Information Criterion.

2.3. Tree construction

We constructed both single-locus gene trees and combined data trees from the sequence data. We first constructed individual gene trees to examine congruence among the four genetic loci we used (COI, 16S, PEPCK, and 18S). Next, we constructed a combined-data tree using only individuals that were sequenced for COI, PEPCK, and 18S (the three loci sampled most frequently); this "complete data" tree maximized sequence coverage. However, as extraction of nuclear loci using the RT-PCR method was difficult from older samples (i.e., preserved in ethanol for >5 years), portions of molecular datasets were missing for some taxa; for example, none of the *S. comatularum* complex (and only one species from the *S. coutierei* complex) were included in the complete-data tree. As simulation studies suggest that utilization of taxa with at least $\geqslant$50% of the data matrix present can improve the accuracy of trees (Wiens, 2005, 2006), we also constructed a tree that maximized taxon sampling by including all specimens sequenced for COI mtDNA and at least one other locus. This "complete-taxa" tree included specimens sequenced for 2-4 loci, excluding individuals with <50% of the data matrix sequenced. *Alpheus percyi* was used as the outgroup for all tree reconstructions; both morphological and molecular data show that *Alpheus* is clearly distinct from *Synalpheus* (Morrison et al., 2004; Anker et al., 2006; Bracken et al., 2009).

Bayesian and maximum likelihood methods were used to construct trees, because of their ability to incorporate different models of evolution for each individual locus and to correct for multiple substitutions (Huelsenbeck et al., 2002). We ran partitioned Bayesian analyses on MrBayes v.3.1.2 (Ronquist and Hulsenbeck, 2003; Nylander et al., 2004), and coded the general model of evolution for each locus calculated by MrModeltest (e.g., shape of rate distributions) while allowing MrBayes to estimate other model parameters for each locus (base frequencies, nucleotide substitution rates, proportion invariable sites). We ran Markov Chain Monte Carlo (MCMC) searches with four chains and two runs (sampling the tree every 1000 generations) for our concatenated datasets (complete-data tree, 3×10^7 generations; complete-taxa tree, 6×10^7 generations) and our individual genetrees (16S, 6×10^7 generations; COI & 18S, 2×10^7 generations; PEPCK, 3×10^7 generations). These numbers of generations insured that runs had converged to a stationary distribution, i.e., standard distribution of split frequencies $\ll$0.01. Convergence of each run to a stationary distribution was also assessed by visualizing trace plots of log-likelihood values across all generations in Tracer 1.5 (Rambaut and Drummond, 2007); we discarded the first 25% of the samples as the burn-in. We estimated support for nodes on trees using Bayesian posterior probabilities (bpp). For maximum likelihood analyses, we ran combined-data trees using the program GARLI-part v.2.1 (Zwickl, 2006, 2010), using model parameters calculated by MrModeltest set different models of evolution for each data partition, and used 1000 bootstrap replicates (bs) to estimate support.

2.4. Cryptic species delimitation

We used two criteria—genetic distance and reciprocal monophyly—to identify potential cryptic species in our taxa set. First, we developed a sequence threshold criterion using pairwise species divergence in the COI gene for the *Synalpheus gambarelloides* group, which is taxonomically and phylogenetically the most well-studied species group in this genus (Morrison et al., 2004; Rios and Duffy, 2007; Anker and Toth, 2008; Hultgren and Duffy, 2011); all species are described or actively under description, with multiple morphological characters distinguishing them. Specifically, within the *S. gambarelloides* group, we compared mean sequence divergence in the COI sequence data from our study (1) between different species ("interspecific"), (2) between sister species identified on our multi-locus complete-taxa tree ("sister species"), and (3) within a species ("intraspecific"), for species with >2 individuals sampled. We used MEGA v.4 (Tamura et al., 2007) to calculate nucleotide divergences using the Kimura two-parameter (K2P) distance model (Kimura, 1980). Interspecific distances were calculated to compare to published crustacean congeneric distance values for COI (Lefebure et al., 2006; Costa et al., 2007), and sister species comparisons were used to calculate minimum interspecific genetic distance (Meier et al, 2008). Mean sister species divergence was 8.1% (n = 7 pairs), while mean intraspecific divergence was 1.02% (n = 16; see Results). We used two criteria to identify

potential cryptic species in the remaining species groups of *Synalpheus*. First, genetic divergence in COI between a potential cryptic species and its closest sister species taxa or group had to exceed 10.2%, i.e. ten times mean intraspecific divergence. This percentage also exceeded minimum interspecific distance—i.e., mean sister-species divergence (8.1%)—in the *S. gambarelloides* group. Second, multiple individuals of a potential cryptic species had to be reciprocally monophyletic in both combined-data (complete-taxa tree) and COI gene trees. In this study, the four undescribed species in the *S. gambarelloides* group are distinguishable by several morphological characters and are currently under taxonomic study.

3. Results

3.1. Alignment and sequence data

We obtained mitochondrial COI sequence data from 196 individuals representing 93 different species (here including both described and potential cryptic species), which is about 60% of the current described species diversity of *Synalpheus*. For PEPCK, we obtained sequence data from 91 individuals (67 species); for 16S, we obtained sequence data from 176 individuals (87 species); for 18S we obtained sequence data from 105 individuals (60 species). The complete-data tree (Fig. 2) consisted of 63 total individuals representing 48 species, while the complete-taxa tree (Fig. 3) consisted of 133 individuals representing 88 species (30 species with 2 loci, 54 species with 3 loci, 26 species with 4 loci). Best-fit nucleotide substitution models calculated from MrModeltest were as follows: COI, 18S, and 16S: GTR + I + G; PEPCK: HKY + I + G.

3.2. Phylogenetic trees

Although we constructed separate gene trees for each molecular locus to examine congruence among all four datasets, only combined-data trees provided resolution of phylogenetic relationships among clades with high Bayesian posterior probabilities or bootstrap values. Although ML and Bayesian trees resolved the same major clades (Table 2), Bayesian trees provided more information about relationships among these clades. Therefore, we present the Bayesian topologies with Bayesian posterior probabilities (bpp), along with ML bootstrap (bs) values on clades resolved with both methods (all support values are converted to percentages). Bayesian gene trees for COI 16S, 18S, and PEPCK are presented in in Supplementary Figures 2–5, respectively.

In general, four major clades of *Synalpheus* were identified in both combined-data trees, but the complete-data tree tended to provide higher support for these clades (Table 2, Figs. 2 and 3). First, monophyly of the *S. brevicarpus* group was strongly supported in both combined-data trees using all methods (bpp, bs = 100), and the *S. gambarelloides* group was supported in combined Bayesian analyses (bpp = 100), but was not well supported in ML analyses (bs = 28–60). Second, there was strong support in both combined-data trees (bpp, bs $\geqslant$ 97) for "Clade I," which consisted of eight species (described and potential cryptic species) from the *S. paulsoni* group, primarily from the eastern Pacific (six species), with one species each from the western Atlantic and the eastern Atlantic. This clade was also supported in all individual gene trees (bpp $\geqslant$ 97) except 18S, and was recovered as a sister clade to the *S. comatularum* clade in the complete-taxa tree. Third, there was also strong support in both combined-data trees (bpp = 100, bs = 80–98) for "Clade II," which consisted of species (sampled worldwide) currently assigned to the *S. coutierei*, *S. paulsoni*, and *S. neomeris* groups (Coutière, 1909; De Man, 1911; Banner and Banner, 1975). Finally, the *S. comatularum* clade had strong support in the complete-taxa tree (bpp = 99); this group was not included in the complete-data tree due to insufficient genetic sampling. Combined-data trees and the aligned data matrix are available on Treebase: (http://purl.org/phylo/treebase/phylows/study/TB2:S14730).

3.3. Cryptic species delimitation

In the *S. gambarelloides* group, mean intraspecific divergence (mean ± standard error) was 1.02 ± 0.92% (n = 16); mean sister-species divergence was 8.14 ± 1.41% (n = 7); mean interspecific divergence was 19.57 ± 0.21% (n = 496 comparisons). Potential cryptic species were identified using a COI sequence divergence threshold of 10.2% (10× intraspecific divergence), and all potential cryptic species (with >1 individual sampled) were reciprocally monophyletic in both complete-taxa (Fig. 3) and COI (Supplementary Fig. 2) trees. Using these criteria, we detected potential cryptic species in the *S. comatularum* clade (2 species), Clade I (3 species), the *S. brevicarpus* clade (5 species), and Clade II (10 species). The species coverage across the six species groups, expressed as % of currently known diversity (Anker, 2001a; De Grave and Fransen, 2011), was ~17% for the *S. coutierei* group, 25% (*S. comatularum*), 65% (*S. gambarelloides*), 68% (*S. neomeris*), 57% (*S. paulsoni*), and 100% (*S. brevicarpus*).

4. Discussion

The first worldwide phylogenetic study of the diverse snapping shrimp genus *Synalpheus* demonstrates strong support for the monophyly of three out of six species groups originally established by Coutière (1909): the *S. gambarelloides*, *S. brevicarpus*, and *S. comatularum* groups. However, the phylogenetic relationships among these groups, and the other two major clades recovered in the combined-data trees, were generally less resolved.

4.1. Monophyly of Coutière's species groups

The *S. gambarelloides*, *S. brevicarpus*, and *S. comatularum* groups were the only three species groups consistently recovered as monophyletic in combined-data trees (Figs. 2 and 3, Table 2) and in COI and 16S gene trees (Supplementary Figs. 2–3). These data suggest a strong congruence between our molecular topologies and morphology-based taxonomic hypotheses, as these are the only three species groups deemed taxonomically coherent by Banner and Banner (1975). Importantly, two of these groups are also characterized by obligate symbiotic associations: all of the members of the *S. gambarelloides* group dwell exclusively in sponges (Ríos and Duffy, 2007; Fig. 1F), whereas members of the *S. comatularum* group are thought to be primarily (if not exclusively) associated with crinoids (Banner and Banner, 1975; Fig. 1B).

As predicted by Banner and Banner (1975), there was no support in our study for monophyly of the *S. neomeris*, *S. paulsoni* and *S. coutierei* groups, as defined by Coutière (1909) and De Man (1911). In our study, members from these groups are distributed among Clades I and II (Figs. 2 and 3). The smaller Clade I is formed by several eastern Pacific and Atlantic species that are more or less closely related to *S. apioceros*, a species originally assigned to the *S. paulsoni* group by Coutière (1909). The much larger Clade II is a morphologically, ecologically and biogeographically heterogeneous assemblage of members of the *S. neomeris* and *S. coutierei* groups, as well as the remaining members of the *S. paulsoni* group *sensu* Coutière (1909). However, Clade II contains several biogeographically constrained sub-clades with species from the Indo-West Pacific (*S. neptunus* / *S. coutierei* complex, *S. neomeris*

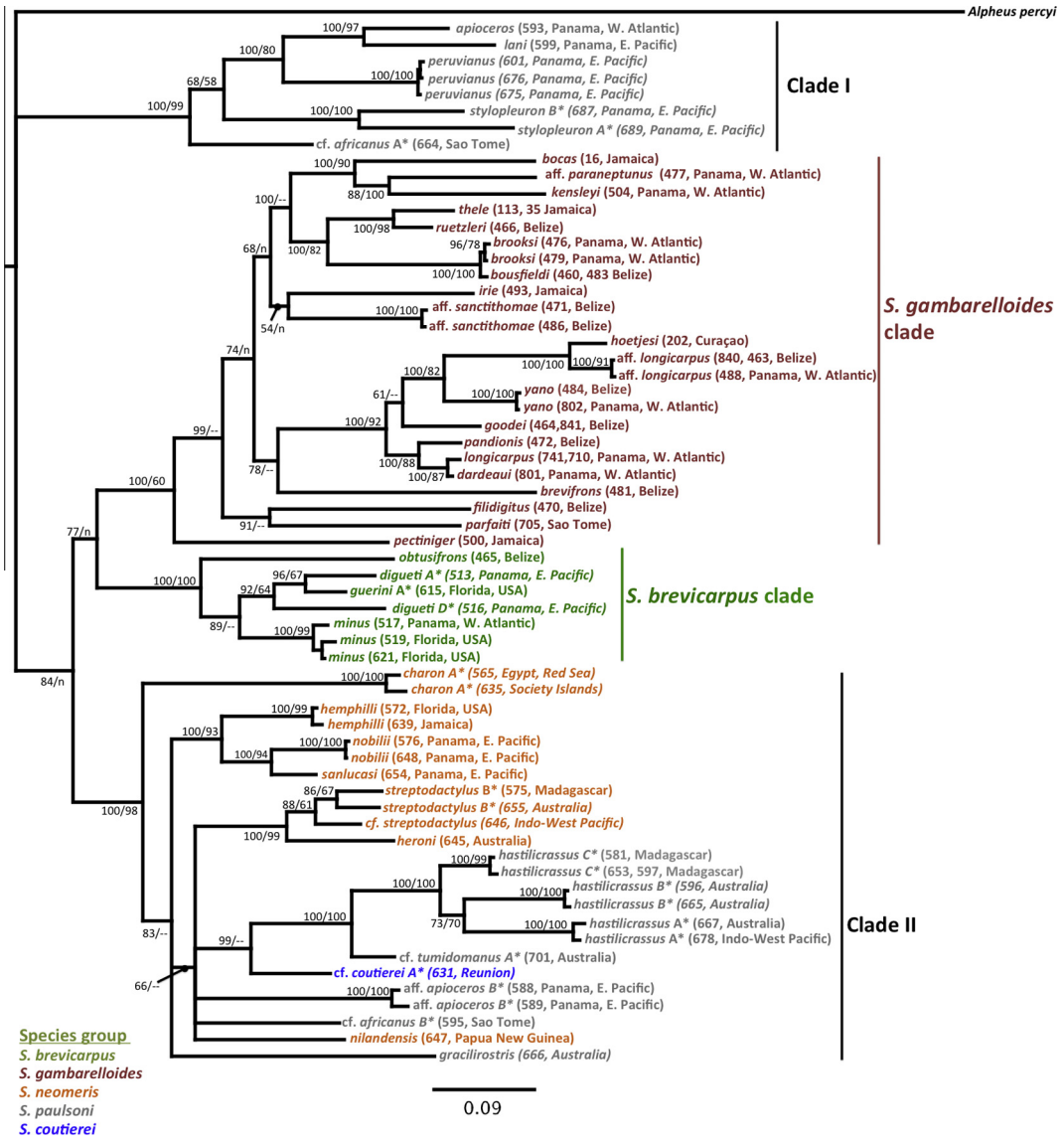

Fig. 2. Bayesian consensus tree of all individuals sequenced for 18S, PEPCK, and COI (complete-data tree); specimen number and collection locality are included. *Synalpheus* species groups are indicated by different colors; major clades indicated by vertical bars. Numbers by each node indicate Bayesian posterior probability values (bpp)/bootstrap support from maximum likelihood analyses (bs), expressed as percentages. A "–" indicates that the clade is present but bs or pp values < 50; "*n*" indicates clade was not present in ML trees. Values below the level of species are omitted. A "*" indicates potential cryptic species identified using COI sequence divergence data. Scale bar indicates number of substitutions/site in the Bayesian analysis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

complex, Fig. 1A), Indo-Pacific (*S. charon* complex, Fig. 1E), or West Atlantic – East Pacific (*S. fritzmuelleri* complex).

Prior to our study, the only formal phylogenetic analysis of the genus *Synalpheus* was a study focused on the American members of the *S. paulsoni* group, using a morphological dataset of 51 characters (Hermoso-Salazar et al., 2008). The authors of this study concluded that only two species groups should be recognized in *Synalpheus*: the *S. paulsoni* group and the *S. gambarelloides* group. However, their taxa set was heavily biased towards American species, especially those assigned to the *S. paulsoni* group. The large *S. gambarelloides* group was represented only by two East Pacific taxa, *S. occidentalis* and *S. mulegensis*. The *S. paulsoni* group *sensu lato*, as recovered as monophyletic by Hermoso-Salazar et al. (2008), included an array of morphologically different species, such as *S. fritzmuelleri*, *S. apioceros*, *S. charon* and *S. digueti* (the latter is a member of the *S. brevicarpus* group). In contrast, our molecular analysis provided no support for a monophyletic *S. paulsoni* group. For instance, *S. charon* and *S. fritzmuelleri* were formed two distinct lineages within Clade II, and *S. apioceros* clustered with several other species in Clade I. Both our molecular study and the morphological study by Hermoso-Salazar et al. (2008) recovered a clade that included *S. fritzmuelleri*, *S. nobilii*, *S. sanlucasi*, and *S. hemphilli* (within Clade II in our trees). However, Hermoso-Salazar et al. (2008) also placed *S. bannerorum* in this clade, while previous molecular work (Morrison et al., 2004) and our study recovered *S. bannerorum* and *S. dominicensis* as transithsmian sister taxa most closely related to the *S. gambarelloides* group. The remaining topology of the *S. paulsoni* group in Hermoso-Salazar et al. (2008) was mostly not supported by our molecular analysis, with *S. townsendi* and *S. spinifrons* recovered in Clade II, and *S. lani*, *S. stylopleuron*, *S. sanjosei*, *S. apioceros*, and *S. peruvianus* recovered in Clade I. The phylogenetic analysis of Hermoso-Salazar et al. (2008) underlines the enormous difficulties of purely morphological species-level analyses in rapidly evolving groups.

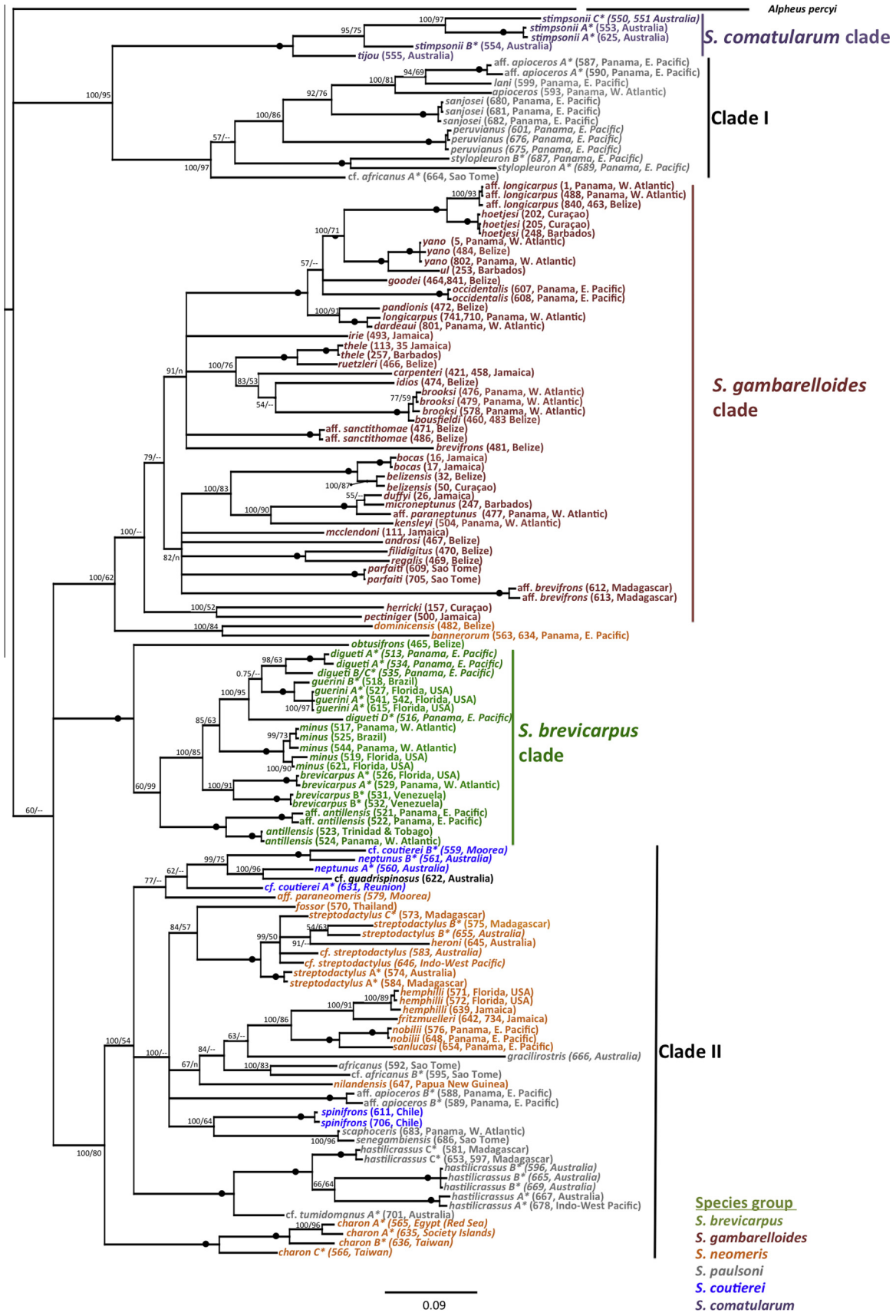

Fig. 3. Bayesian consensus tree (complete-taxa tree), constructed with 18S, PEPCK, 16S, and COI sequence data. Major species groups, major clades, scale bar, and other abbreviations as in Fig. 2. Numbers by each node indicate either Bayesian posterior probability values (bpp) or bootstrap support (bs) from ML analyses, expressed as percentages; bpp/bs (bpp and bs $\geqslant$ 98 are indicated by a dot).

Table 2
Phylogenetic support for different species groups in *Synalpheus*, for Bayesian (BY) and maximum likelihood (ML) trees constructed from different character sets (gene trees) and taxon sets (combined trees).

	COI (Supp. Fig. 2)	16S (Supp. Fig. 3)	18S (Supp. Fig. 4)	PEPCK (Supp. Fig. 5)	Complete-data tree (Fig. 2)		Complete-taxa tree (Fig. 3)	
	BY	BY	BY	BY	BY	ML	BY	ML
brevicarpus	98	100	–	99	100	100	100	100
comatularum	97	100	–	n/a	n/a	n/a	100	100
coutierei	–	–	–	–	–	–	–	–
gambarelloides	–	–	–	–	100	60	100	<50
neomeris	–	–	–	–	–	–	–	–
paulsoni	–	–	–	–	–	–	–	–
Clade I	97	100	–	100	100	99	100	97
Clade II	–	–	–	–	100	98	100	80

Clade confidence values are given as posterior probabilities (BY analyses) or bootstrap confidence values (ML analyses), converted to percentages; dashes "–" indicates clade was not supported; "n/a" indicates hypothesis was not tested in that tree (the *S. comatularum* group was not included in the complete-data tree).

The monophyly of the *S. gambarelloides* group was previously demonstrated by Morrison et al. (2004), although one East Atlantic taxon (*S. parfaiti*) and four Indo-Pacific taxa (*S. mulegensis*, *S. crosnieri*, *S. sladeni*, *S. spongicola*, see Ríos and Duffy, 2007) were not included in their analysis. The present study corroborates the monophyly of the *S. gambarelloides* group, at least in Bayesian trees (support was minimal in maximum likelihood trees), and clearly places *S. parfaiti* within this group, close to the West Atlantic *S. filidigitus* (Fig. 3).

4.2. Phylogenetic relationships among major clades

While both combined-data trees showed strong support for four major clades in *Synalpheus* (*S. gambarelloides*, *S. brevicarpus*, Clade I, and Clade II), the exact relationships among these clades remain less clear. In both combined-data trees, there were several polytomies within Clade II, but both trees placed the *S. charon* complex as a sister to the rest of Clade II. In the complete-data tree (Fig. 3), Clade I and the *S. comatularum* clade formed a well-supported clade (bpp = 100, bs = 95; members of the *S. comatularum* clade were not included in the complete-taxa tree). Difficulty in resolving the basal branching relationships within major clades in *Synalpheus* could be due, in part, to rapid speciation events in some groups of *Synalpheus* that result in shorter basal/deep branches, and longer terminal branches; for example, Morrison et al. (2004) found evidence of a fairly rapid radiation of the *S. gambarelloides* group approximately 5–7 Mya. In our study, the unresolved relationships between the major clades and the difficulty of finding diagnostic morphological characters to support Clades I and II make a more formal subgeneric subdivison unfeasible at this stage.

Conversely, each of the three monophyletic species groups supported in our study (*S. brevicarpus*, *S. gambarelloides*, *S. comatularum*) can be characterized by a diagnostic set of morphological characters (Banner and Banner, 1975), although some characters will need to be taxonomically re-evaluated. For instance, a setal row very similar to the gambarelloid setae characteristic of all members of the *S. gambarelloides* group is also found in some Indo-West Pacific species of Clade II (Banner and Banner 1975; A. Anker, pers. obs.). In addition, *S. gambarelloides* and *S. brevicarpus* groups are nested within a larger clade, which also includes the heterogeneous Clade II (Figs. 2 and 3). These results further support the conclusions reached by Anker and De Grave (2008), who argued that the recognition of *Zuzalpheus* as a separate genus for the *S. gambarelloides* group, and more generally splitting of *Synalpheus* into several genera, would be taxonomically and phylogenetically problematic. In addition, the presence of gambarelloid-type setae outside the *S. gambarelloides* group (Banner and Banner, 1975) renders this group's most diagnostic character rather ambiguous. *Synalpheus* may need to be internally subdivided into subgenera, perhaps making use of the subgeneric name *Zuzalpheus* for the *S. gambarelloides* group. However, subgeneric subdivison of the genus should await a concerted taxonomic effort to find diagnostic morphological characters supporting various subgeneric clades and additional sequencing of a greater range of taxa.

Although our study did not include enough alpheid outgroups to robustly test the monophyly of the genus *Synalpheus*, only the COI gene tree resolved *Synalpheus* as a monophyletic group (bpp = 100, Supplementary Fig. 2). In previous studies, a monophyletic *Synalpheus* was supported by COI and morphological analyses (Morrison et al., 2004), by combined molecular analyses (18S + 16S; Bracken et al., 2009), and by several morphological synapomorphies (Anker et al., 2006). However, these species included few outgroups to *Synalpheus* (Morrison et al., 2004), or only a few representative ingroup species of *Synalpheus* (Anker et al., 2006; Bracken et al., 2009). Rigorous examination of the monophyly of *Synalpheus* and other major genera within the Alpheidae will require adequate taxon sampling of diversity within *Synalpheus* as well as other major alpheid groups.

4.3. Cryptic species delimitation

Although DNA barcoding is increasingly being used as a tool to aid in delimiting crustacean species (summarized in Goldstein and DeSalle, 2010), we recognize that use of mtDNA sequences as a sole criterion for species delimitation is controversial, due to the low correspondence between coalescence of mtDNA and divergence of nuclear genes, and the potential for barcoding to overlook recently diverged species or closely related sister species (Lee, 2004; Moritz and Cicero, 2004). Nonetheless, we feel that in alpheid shrimps and in particular in the genus *Synalpheus*, COI sequence data can help to detect potential cryptic species that subsequently should be examined and confirmed using traditional taxonomic methods (DeSalle, 2006; Puillandre et al., 2012). Our genetic distance threshold for potential cryptic species was 10.2% (10X intraspecific divergence), more conservative than 10X distance thresholds used in previous studies on amphipods (3.75%; Witt et al., 2006), or birds (2.7%; Hebert et al., 2004). Mean interspecific genetic distance (19.57% for the *S. gambarelloides* group) was comparable to mean (17.16%, Costa et al., 2007) or median (25.1%, Lefebure et al., 2006) congeneric interspecific distances reported in other crustacean studies.

In our study, we detected 20 potential cryptic species across several different clades (Supplementary data 1, Figs. 2 and 3). It is crucial to note here that not all of these potential cryptic taxa may ultimately merit separate species status, upon further examination of morphology and additional specimens. Furthermore, some potential cryptic species may correspond to previously described taxa, either to inadequately described and thus poorly known species, or to species placed in synonymy by earlier workers. For

instance, Banner and Banner (1975) invalidated a number of species, subspecies and varieties described by Coutière, De Man, and even by the Banners themselves.

Interestingly, several potential cryptic species complexes detected with molecular data include wide-ranging species associated with larger hosts: crinoids, corals, and sponges. For example, *S. stimpsonii* (Fig. 1B) appears to be a complex of at least three potential cryptic species, separated from each other by ~15% COI sequence divergence, rather than a "single but highly variable" species as asserted by Banner and Banner (1975). Anker (2001a) noted that *S. stimpsonii* may be a species complex because of important differences in morphology, color patterns and association with a multitude of different crinoid hosts, and VandenSpiegel et al. (1998) found a certain degree of host specificity in *S. stimpsonii* both in the field and during laboratory experiments. Our COI sequence data confirm that *S. stimpsonii* indeed appears to be a species complex in need of a taxonomic revision, which will certainly include revalidation of some nominal species currently under synonymy of *S. stimpsonii* (Banner and Banner, 1975). Similarly, *S. charon* (Fig. 1E), a wide-ranging species associated exclusively with pocilloporid corals, appears to be a species complex with at least three distinct cryptic species, including *S. charon s. str.* and *S. charon obscurus* placed in synonymy of *S. charon* (Banner, 1956; Banner and Banner, 1975). Whether these cryptic species are also host-specific remains to be shown.

Although we stress that these potential cryptic species complexes await confirmation by morphological examination, the magnitude of cryptic speciation in our present sample mirrors patterns generally observed in alpheid shrimps (Knowlton, 1986, 1993; Anker, 2001b). For instance, the number of described species in the *S. gambarelloides* species group has more than doubled in the last two decades due to intense taxonomic work (summarized in Hultgren and Duffy, 2011). Integrative taxonomy has been extensively used in the genus *Alpheus*, helping to resolve several cryptic species complexes, including the large *A. armillatus* complex (Mathews and Anker, 2009; Anker, 2012).

4.4. Future directions

In conclusion, the first worldwide molecular analysis of the genus *Synalpheus* provides a phylogenetic framework for examining topics such as the evolution of specialized host use, as well as a set of phylogenetic and taxonomic hypotheses to test with additional morphological and ecological data. We hope to expand the current taxa set in future combined molecular and morphological studies, in particular by adding more Indo-West Pacific taxa from the Coral Triangle (Singapore, Indonesia, Philippines, Papua New Guinea), Japan, Hawaii, and the Red Sea. This will enable (i) formal taxonomic treatment of major clades of *Synalpheus*, leading to the establishment of subgenera; (ii) taxonomic revisions of several species complexes of *Synalpheus*, especially in the Indo-West Pacific taxa; and (iii) additional analyses of the evolution of morphological characters (e.g., gambarelloid-type setae), color patterns (e.g., markings on the major chela), and ecological traits (host use and specificity).

Acknowledgments

Funding for this study, including support of KH and CH, was provided by Wolcott funds at the Smithsonian Institution. This study could not have been done without the support of Nancy Knowlton (National Museum of Natural History, Smithsonian Institution). This study also benefited greatly from the taxonomic expertise and collections of J. Emmett Duffy (Virginia Institute of Marine Studies). Dr. Amy Driskell (Smithsonian Laboratory of Analytical Biology) provided assistance with barcoding and database management. Early drafts of the manuscript were improved by the comments of two anonymous reviewers. Most of the Indo-West Pacific material used in this study was collected during BIOTAS (Madagascar, Reunion Island) and BIOCODE (French Polynesia) expeditions co-organized by Gustav Paulay (Florida Museum of Natural History). The Panamanian material was collected with support from the Smithsonian Tropical Research Institute (STRI); collection permits were issued by the ANAM. The São Tomean material was collected with permits from the Fisheries Department (Direccão das Pescas) of São Tomé and Principe.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2014.03.008.

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