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First worldwide molecular phylogeny of the morphologically and ecologically hyperdiversified snapping shrimp genus *Alpheus* (Malacostraca: Decapoda)

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ABSTRACT

Hyperdiverse animal groups raise intriguing questions regarding the factors that generate and maintain their diversity. The snapping shrimp genus *Alpheus* (with >300 described species) is a spectacularly diversified group of decapod crustaceans that serves as an exemplary system for addressing evolutionary questions regarding morphological adaptations, symbiosis, cryptic diversity and molecular divergence. A lack of information regarding evolutionary relationships among species has limited investigations into the mechanisms that drive the diversification of *Alpheus*. Previous phylogenetic studies of *Alpheus* have been restricted in scope, while molecular datasets used for phylogenetic reconstructions have been based solely on mitochondrial and a handful of nuclear markers. Here we use an anchored hybrid enrichment (AHE) approach to resolve phylogenetic relationships among species of *Alpheus*. The AHE method generated sequence data for 240 loci (>72,000 bp) for 65 terminal species that span the geographic, ecological and taxonomic diversity of *Alpheus*. Our resulting, well-supported phylogeny demonstrates a lack of monophyly for five out of seven morphologically defined species groups that have traditionally been used as a framework in *Alpheus* taxonomy. Our results also suggest that symbiotic associations with a variety of other animals have evolved independently in at least seven lineages in this genus. Our AHE phylogeny represents the most comprehensive phylogenetic treatment of *Alpheus* to date and will provide a useful evolutionary framework to further investigate questions, such as various modifications of the snapping claw and the role of habitat specialization and symbiosis in promoting speciation.

Running head: PHYLOGENY OF THE SNAPPING SHRIMP GENUS *ALPHEUS*.

1. Introduction

The genus *Alpheus* is the largest among the caridean shrimp family Alpheidae and one of the most species-rich genera within the malacostracan order Decapoda, with >300 species described to date (De Grave and Fransen, 2011; Scioli and Anker 2020). Shrimps of this genus are popularly known as snapping or pistol shrimps, for they possess two enlarged, asymmetrical claws, with the larger (major) claw capable of producing a loud snapping sound. In *Alpheus*, the snapping claw is primarily used for defense, but may also be used for predation, burrowing in soft sediments, boring into hard substrates (basaltic rocks, dead and living corals), and for visual and possibly sound-mediated communication (Schein, 1977; Hughes, 1996; Karplus and Thompson, 2011; Hughes et al., 2014). This wide range of functions, combined with an impressive range of habitats occupied by *Alpheus* and the relative ancient origin of the group, with pulses of diversifications occurring at least since the Oligocene (Hyžný et al., 2017), underpin the extensive morphological variation in the form of the snapping claw (Anker et al., 2006; Kaji et al., 2018) (Fig. 1).

Most species of *Alpheus* are known from shallow (intertidal to about 50 m) tropical and subtropical marine waters. Snapping shrimps, especially *Alpheus* spp., are among the most diverse and abundant crustaceans found on coral reefs worldwide (Fig. 1). However, many tropical species are found in other habitats such as rocky shores, mud flats, sand flats, sea grass beds, mangroves and estuaries (Banner and Banner, 1982; Anker and De Grave, 2016), and at least two South-East Asian species live in freshwater streams (Yeo and Ng, 1996). Only a few species of *Alpheus* occur in temperate or cool-temperate waters, occurring as far north as eastern Russia and northern Japan, or as far south as New Zealand and southern Chile (Banner and Banner, 1982; Kim and Abele, 1988; Anker et al., 2016). A number of species occur in deep water, well below 100 m and down to at least 1000 m (Anker and Nizinski, 2011; Scioli and Anker, 2020). They also occupy a wide variety of microhabitats, ranging from coral reef crevices, burrows in mud or sand, spaces under rocks, coralline algae, mangrove roots, tide pools etc.; some species are well-known bioeroders of both living and dead corals (Banner and Banner, 1982; Werding, 1990; Williams et al., 2001; Anker et al., 2006; Anker and De Grave, 2016).

Although many species of *Alpheus* occupy a range of different abiotic habitats, some form intimate and well-studied symbioses with a great variety of marine taxa, including gobiid fishes (Karplus and Thompson, 2011), hermatypic corals (Glynn, 1976; McKeon et al., 2012; Rouzé et al., 2017), sponges (Banner and Banner, 1982), sea anemones (Hurt et al., 2013), echiuran worms (Anker et al., 2005, 2007a, 2015), and other decapod crustaceans, including smaller alpheid shrimps (Anker and Lazarus, 2015; Boltaña and Thiel, 2001). In particular, goby-shrimp symbioses represent one of the best model systems for understanding interspecific communication (Karplus and Thompson, 2011). Despite the fact that over 100 species of gobies from different genera and at least 30 species of *Alpheus* engage in these partnerships around the world, little is known about the taxonomy and phylogenetic relationships among goby-associated *Alpheus* spp., which lies in strong contrast to the well-resolved taxonomy and phylogeny of their partner gobies (Anker, 2000; Thompson et al., 2013). More broadly, it is unknown how many times symbiotic relationships with different taxonomic groups (e.g. gobiid fishes, corals, echiurans, and sponges) have evolved within *Alpheus*.

Examining the evolutionary factors that have resulted in the extraordinary morphological and ecological diversity of the genus *Alpheus*, and their close symbiotic relationships with a wide range of

Fig. 1. Ecological diversity of *Alpheus,* with emphasis on symbiotic associations: (A) *A. formosus,* free living under rocks and rubble, Florida; (B) *A. immaculatus,* associated with the sea anemone *Bartholomea annulata*, Florida (C); *A. lottini,* associated with the coral *Pocillopora damicornis*, Colombia; (D) *A.* aff. *djiboutensis,* associated with the goby *Cryptocentrus* cf. *strigilliceps*, Papua New Guinea; (E) *A. barbatus,* associated with the echiuran worm, *Ochetostoma erythrogrammon*, Japan. Photographic credits: Linda Ianniello (A, B), Juan Felipe Lazarus (C), Andrey Ryanskiy (D), Ryutaro Goto (E).

organisms, requires a phylogenetic framework establishing the relationships among *Alpheus* species. Coutière (1899, 1905) proposed seven informal species groups within *Alpheus*, i.e. the *A. macrocheles, A. sulcatus, A. obesomanus, A. crinitus, A. diadema, A. brevirostris*, and *A. edwardsii* species groups. These groups were defined largely on morphological characters of the major chela and the rostro-orbital region and have been amply used in alpheid taxonomy for most of the 20th century (De Man, 1911; Banner, 1953; Banner and Banner, 1982; Chace, 1988; Kim and Abele, 1988). However, the last and so far the most comprehensive molecular phylogenetic study of *Alpheus,* based on three genes sequenced from 53 species of primarily western Atlantic and eastern Pacific *Alpheus* spp*.,* indicated that some of Coutière's species groups were paraphyletic (Williams et al., 2001). Specifically, two of the most well-represented species groups, e.g. the *A. sulcatus* and *A. edwardsii* groups, did not form monophyletic clades. This and related studies also drew attention to the need for multiple markers (including nuclear markers) to delimit relationships within *Alpheus*, as some clades possessed COI pseudogenes (Williams and Knowlton, 2001). Much of the early phylogenetic work on *Alpheus* has focused on rates of molecular evolution and on examining sister species pairs separated by the Isthmus of Panama (Knowlton et al., 1993; Knowlton and Weigt, 1998; Hurt et al., 2009). The well-studied timing of the formation of the Isthmus of Panama has been used to calibrate the separation of 15 recognized transisthmian sister species of *Alpheus*, allowing absolute time estimates to be placed on the evolutionary history of this group (Hurt et al., 2009). Additional molecular phylogenetic work on *Alpheus* has primarily been used in the revision of individual species complexes (Anker et al., 2008a, a,b,b,c; Anker and Pachelle, 2013; Bracken-Grissom et al., 2014; Bracken-Grissom and Felder, 2014; Hurt et al., 2013; Mathews, 2006; Mathews and Anker, 2009); however, a comprehensive phylogeny spanning the morphological diversity and geographical distribution of this group is needed.

The most comprehensive cladistic analysis of the family Alpheidae, based on 122 morphological characters, indicated that the genus *Alpheus* may not be monophyletic and is in need of revision (Anker et al., 2006). Most importantly, three minor genera morphologically allied to *Alpheus*, viz. *Metalpheus, Pomagnathus*, and *Racilius,* were recovered nested within *Alpheus*. The single species of *Racilius* is well known as an obligate symbiont of *Galaxea* corals (Bruce, 1972), whereas species of *Pomagnathus* (monotypic) and *Metalpheus* (two or three species) are more frequently found in rock crevices, coralline algae, dead corals, and occasionally in living corals (Chace, 1937; Banner and Banner, 1982; Navas et al., 1999). *Pomagnathus* and *Metalpheus* share a suite of morphological characters, some related to their crevice-dwelling lifestyle, and without any doubts represent sister lineages (Anker et al., 2006). However, it is still unclear whether they are phylogenetically close to the exclusively symbiotic *Racilius*. More generally, the position of these three lineages within *Alpheus* remains somewhat problematic as they are morphologically more or less derived (Anker et al., 2006).

To focus on these and other unresolved questions within *Alpheus,* including the monophyly of Coutière's species groups, how these species groups are related, and the taxonomic status of *Metalpheus* and *Racilius,* we used a phylogenomic approach to reconstruct evolutionary relationships within *Alpheus*. The anchored hybrid enrichment (AHE: Lemmon et al., 2012) technique was used to generate more than 200 genome-wide markers for 61 species of *Alpheus* and two of the three related minor genera, and to construct phylogenetic trees using both the concatenated dataset and species-tree methods. We addressed several hypotheses using a majority-rule consensus tree. First, we mapped Coutiere's species groups onto the tree to examine the monophyly and taxonomic utility of these groups. We included representatives of *Metalpheus* and *Racilius* to test whether these taxa were nested within *Alpheus*, as suggested by Anker et al. (2006). Finally, we performed a preliminary examination of how many times specialized host symbioses evolved within *Alpheus*.

2. Material and methods

2.1. Tissue samples

A total of 68 species of *Alpheus*, as well as one species of each *Metalpheus* and *Racilius*, were selected for phylogenetic analyses, in order to maximize representation from the seven morphologically defined species groups and two putatively closely related genera. DNA extractions were obtained from subsamples of specimens deposited in the Florida Museum of Natural History, University of Florida, Gainesville, FL (FLMNH UF), the Oxford University Museum of Natural History, Oxford, United Kingdom (OUMNH.ZC.), and in some cases frozen tissue or ethanol-preserved specimens currently held at Seattle University (SU, Hultgren) and Tennessee Tech (TT, Hurt) and available upon request. Museum catalog numbers for all specimens used for DNA analyses are listed in Appendix A. Extraction of genomic DNA was performed using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. Extracted DNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific) and the quality of DNA was confirmed by electrophoresis on a 2% agarose gel. Since some of these species belonged to putative cryptic species complexes (Table 1, Appendix A), we also sequenced most specimens for gene regions used in previous studies of cryptic species in *Alpheus,* including the COI 5′ barcoding region (Anker et al., 2008a, 2008b, 2008c; Hurt et al., 2009, 2013), and/or the16S rRNA and COI 3′ region (Williams et al., 2001; Mathews and Anker, 2009; Bracken-Grissom et al., 2014). We used universal COI barcoding primers for the 5′ region (Geller et al., 2013), and otherwise used standard primers and PCR conditions described in previous papers on alpheid shrimps (Mathews and Anker, 2009; Bracken-Grissom et al., 2014; Hultgren et al., 2014). For some small museum specimens, there was not enough tissue for separate barcoding reactions thereforebarcodes are not provided. Genbank accession numbers for new sequences and additional verification of specimens used in this study (e.g., citations in the taxonomic literature) are presented in Appendix A.

2.2. Anchored hybrid enrichment

Genome-wide sequence data was generated using the anchored hybrid enrichment (AHE) method (Lemmon et al., 2012). Anchored hybrid enrichment library preparation and sequencing were performed at the Center for Anchored Phylogenomics at Florida State University (www.anchoredphylogeny.com). In this method, genomic DNA was first fragmented by sonication to 300–800 bp in length on a Covaris E220 Focused ultrasonicator. Fragmented DNA was then indexed using a Beckman-Coulter Biomek FXp liquid-handling robot. Indexed libraries were pooled and hybrid enrichment was performed on an Agilent Custom SureSelect kit. Custom probes used for hybridization were designed to hybridize to targeted fragments across the infraorder Caridea (Wolfe et al., 2018). Enriched libraries were then sequenced on an Illumina HiSeq2000 at the Translational Science Laboratory in the College of Medicine at Florida State University. All sequences are deposited in the NCBI (Sequence Read Archive, BioProjectID PRJNA689880) depository.

2.3. Sequence assembly methods and filtering

Sequence reads passing the Cassava high-chastity filter were first demultiplexed using the 8-bp indexes, with no mismatches tolerated. Overlapping, paired reads were merged and adapters were trimmed following (Rokyta et al., 2012). A quasi *de novo* assembly was then performed for each individual as described in Prum et al. (2015) and Hamilton et al. (2016), but with the following being used as references: *Lysmata wurdemanni, Neocaridina denticulata, Halocaridina rubra, Pandalus latirostris* and *Periclimenes rathbunae*. Consensus sequences were constructed from assembly clusters formed from more than 20 reads.

Table 1

Species of *Alpheus* (*A.*), two allied genera *Metalpheus* (*M.*) and *Racilius* (*R.*), and *Synalpheus* (*S.*) [outgroup] used in anchored hybrid enrichment phylogenetic reconstructions. Clade numbers correspond to the phylogenetic clade assignments in Fig. 2. Species groups refer to morphological classifications summarized in Banner & Banner (1982) and Kim and Abele (1988), except for the *A. leviusculus* and *A. paracrinitus* groups, more recently proposed by Anker et al. (2009) and Anker (2020) Asterisks indicate species with known symbiotic associations. Abbreviations used for genera distribution of taxa: CA – Central Atlantic; EA – East Atlantic; EP – East Pacific; IWP – Indo-West Pacific; IO – Indian Ocean; WA – West Atlantic; WP – West Pacific. Species complexes (known or presumed) are highlighted with (SC).

Species	Clade	Species Group	Distribution	Ecology
A. barbatus (SC)	VII	*A. brevirostris*	IWP	Associated with echiurans*
A. brevirostris	VIII	*A. brevirostris*	IWP	In burrows on muddy-sandy bottoms
A. djiboutensis (SC)	VIII	*A. brevirostris*	IWP	In burrows in sand/rubble, typically associated with gobies*
A. floridanus	VIII	*A. brevirostris*	WA	In burrows in sand, occasionally associated with gobies*
A. glaber	–	*A. brevirostris*	EA	In burrows on muddy bottoms
A. kagoshimanus	VIII	*A. brevirostris*	WP	In burrows on muddy bottom
A. miersi	VIII	*A. brevirostris*	IWP	Coral rubble
A. naos	VII	*A. brevirostris*	EP	Associate of echiurans*
A. novaezealandiae	VIII	*A. brevirostris*	IWP	Under rocks
A. platyunguiculatus	VIII	*A. brevirostris*	IWP	In burrows in sand/rubble
A. rapacida 1 (SC)	VIII	*A. brevirostris*	IWP	In burrows in sand/mud, often associated with gobies*
A. rapacida 2 (SC)	VIII	*A. brevirostris*	IWP	In burrows in sand/rubble
A. samudra	VIII	*A. brevirostris*	IO	In burrows on muddy bottoms
A. savuensis	VIII	*A. brevirostris*	IWP	In burrows in sand/rubble
A. bucephalus	VI	*A. crinitus*	IWP	In algal tubes among dead corals
A. cristulifrons	VI	*A. crinitus*	WA	Coral rubble
A. frontalis (SC)	VI	*A. crinitus*	IWP	In algal tubes under rocks or in coral crevices
A. pachychirus	VI	*A. crinitus*	IWP	In algal tubes under rocks or in coral crevices
A. spongiarum (SC?)	VI	*A. crinitus*	IWP	Associated with sponges*
A. diadema	V	*A. diadema*	IIWP	Coral rubble
A. gracilipes	V	*A. diadema*	IWP	Coral rubble
A. paracrinitus	–	*A. diadema*	WA	Under rocks and in coral rubble
A. percyi	V	*A. diadema*	IWP	Coral rubble
A. amarillo	VII	*A. edwardsii*	WA	Under rocks
A. armillatus	VII	*A. edwardsii*	WA	Under rocks
A. bahamensis	VII	*A. edwardsii*	WA	Semi-endolithic in coral rubble
A. bouvieri	–	*A. edwardsii* → *A. leviusculus*	WA + EA	In coral rubble and coral rock crevices
A. cyanoteles	–	*A. edwardsii*	WP	Freshwater streams
A. echiurophilus	IV	*A. edwardsii*	IWP	Associated with echiurans*
A. intrinsecus	II	*A. edwardsii*	WA + EA	Under rocks on sand/mud
A. japonicus	IV	*A. edwardsii*	WP	In burrows on muddy bottoms
A. lacertosus	VII	*A. edwardsii*	EP	Under rocks
A. malabaricus (SC)	IV	*A. edwardsii*	IWP	In burrows on muddy-sandy bottoms
A. nuttingi	VII	*A. edwardsii*	WA	Under rocks and coral rubble
A. parvirostris	IV	*A. edwardsii* → *A. leviusculus*	IWP	Coral rubble
A. schmitti	VII	*A. edwardsii*	WA	Rock crevices
A. strenuus	IV	*A. edwardsii*	IWP	Under rocks
A. umbo	VII	*A. edwardsii*	EP	Endolithic in rock crevices
A. vladivostokiensis	IV	*A. edwardsii*	WP	Under rocks on muddy bottoms
A. amblyonyx	I	*A. macrocheles*	WA	Coral rubble
A. cedrici	I	*A. macrocheles*	CA	Under rocks
A. crockeri (SC?)	I	*A. macrocheles*	EA + IWP	Under rocks and coral rubble
A. deuteropus	I	*A. macrocheles*	IWP	Endolithic in living corals
A. inca	I	*A. macrocheles*	EP	Under intertidal rocks
A. perplexus	I	*A. obesomanus/A. macrocheles*	IWP	Endolithic in dead corals
A. malleodigitus (SC)	IV	*A. obesomanus*	IWP	Endolithic in dead corals
A. simus	III	*A. obesomanus*	WA	Endolithic in dead corals
A. armatus	II	*A. sulcatus*	WA	Associated with sea anemones*
A. blachei	III	*A. sulcatus*	EA	Under rocks
A. formosus	III	*A. sulcatus*	WA	Under rocks and coral rubble
A. gracilis (SC?)	III	*A. sulcatus*	IWP	Coral rubble
A. immaculatus	II	*A. sulcatus*	WA	Associated with sea anemone*
A. lottini (SC)	III	*A. sulcatus*	IWP + EP	Associated with pocilloporid corals*
A. packardii (SC)	V	*A. sulcatus*	WA	Under rocks and in coral rubble
A. panamensis	III	*A. sulcatus*	EP	Under rocks
A. polystictus	II	*A. sulcatus*	WA	Associated with sea anemone*
A. roquensis	II	*A. sulcatus*	WA	Associated with sea anemones*
A. splendidus (SC?)	III	*A. sulcatus*	IWP	Under rocks
A. thomasi	III	*A. sulcatus*	WA	Under rocks and in coral rubble
A. websteri	III	*A. sulcatus*	WA	Under rocks and coral rubble
A. wonkimi	III	*A. sulcatus*	EP	Semi-endolithic in rock crevices
M. rostratipes	–	N/A	EP	In rock and dead coral crevices
R. compressus	III	N/A	WA + IWP	Associated with euphylliid corals*
S. herricki		Outgroup	IWP	Associated with sponges*
S. occidentalis		Outgroup	WA	Associated with sponges*

Ambiguities in the consensus sequences were called if variation across reads at a site could not be attributed to sequencing error (0.01% error rate assumed). Consensus sequences were then collected into orthologous groups using pairwise sequence distances and a neighbor-joining approach, as described in Hamilton et al. (2016). Sequences within each group were aligned in Mafft using the following flags:

–genafpair, –maxiterate 1000, –quiet (v7.023b; Katoh and Standley, 2013). Each alignment was then inspected for misaligned regions sequences, which were masked if, within a 20-bp window, more than 15 bases differed from the common base at that site. The mingoodsites parameter was set to 15 because it provided the best balance between masking the large majority of the misaligned regions, but not over-masking to the point where real sequence variation across species was lost. After masking, sites containing fewer than 50% unambiguous characters were removed from the alignment (see Hamilton et al. (2016) for methodological details).

We investigated the amount and distribution of missing data using AliStat v1.7 (Wong et al., 2019), which provides a completeness score for the alignment (C_a = Number of unambiguous characters/number of sequences * length of the alignment) and produces a heat map of the distribution of missing data across the concatenated alignment. The concatenated alignment is deposited in TREEBASE (submission ID 27245).

2.4. Phylogenetic methods

Bayesian and maximum likelihood criteria were used for phylogenetic reconstructions from concatenated datasets. Bayesian phylogenetic analyses were implemented in Exabayes v1.4 (Aberer et al., 2014) on the concatenated dataset partitioned by loci. Searches were initiated with a random-order addition parsimony tree under the GTR + GAMMA model of nucleotide substitution. We performed four independent runs, each with four coupled Markov Chain Monte Carlo (MCMC) chains for a minimum of 1,000,000 generations and sampling every 500 generations, set to terminate when the average standard deviation of the split frequencies was below 0.01. The first 25% of sampled topologies were discarded as burn-in. The "consense" function was used to construct a majority-rule consensus tree. Sampled trees from the four MCMC chains were combined and the maximum clade credibility tree was identified using TreeAnnotator v. 1.8.4 on the CIPRES portal (Rambaut and Drummond, 2015). Maximum Likelihood (ML) reconstructions were performed with RAxML-HPC v. 8 (CIPRES portal) using the GTRCAT model of nucleotides substitution. The concatenated dataset was partitioned by locus. Nodal support was assessed using nonparametric bootstrapping (BS) with 1000 replicates.

Three different methods were used to estimate species-tree phylogenies including MP-EST, STAR, and ASTRAL. MP-EST and STAR species tree reconstructions were performed on the Species Tree Analysis Web (STRAW) server (Shaw et al., 2013). MP-EST estimates species trees from rooted gene-trees by maximizing a pseudo-likelihood function of triplets of taxa to estimate the topology and branch lengths of the overall species tree; the MP-EST method accepts rooted gene trees as input files (Liu et al., 2010). Maximum likelihood gene trees were generated for each of the 211 loci under the GTRGAMMA model using RAxML (Stamatakis, 2006). Node support was estimated from 100 bootstrap replicates per locus (21,100 total gene-trees). The STAR method for species tree reconstruction uses the same rooted gene trees as in the MP-EST method as input files. First, ranks of coalescent times are calculated for all pairs of species in each gene tree. A neighbor-joining tree is then constructed from a distance matrix where pairwise distances are equal to two times the average ranks of coalescences across loci. Node support was estimated from 100 bootstrap replicates per locus. Finally, we used the Accurate Species TRee ALgorithm (ASTRAL) as performed by ASTRAL II (Mirarab and Warnow, 2015). This method finds the species tree with the largest number of quartet trees within a set of input gene trees. Individual, unrooted RAxML gene trees were used as input files. Support for each node was calculated using the local posterior probabilities method (Sayyari and Mirarab, 2016).

Topological comparisons between all trees were performed using the Robinson-Foulds (RF) metric (Robinson and Foulds, 1981) as computed by the package Phangorn (Schliep, 2010) in the R statistical platform. The RF metric counts the number of branch partitions that appear in one tree but not the other, summed over both trees. The maximum score is equal to two times the number of possible partitions. The resulting RF scores were reported as a percentage of maximum RF scores ($2(n − 2)$).

2.5. Molecular dating

Divergence times were inferred using the auto-correlated relaxed clock model as performed by PhyloBayes v4.1c (Lartillot et al., 2013). A birth death prior was used for estimating divergence times and hyper-parmaters for the birth death process, p1 and p2, were treated as free parameters. Our Bayesian (GTR + gamma) tree was used as an input topology. *Synalpheus occidentalis* and *S. herricki* were used as outgroups to root the tree. We used three well-justified calibration intervals for dating nodes. A divergence time of 18–30 Ma was applied to the crown node for the entire *Alpheus* group, based on the presence of several claw morphotypes dated to the Miocene, ca. 18 Ma (Hyžný et al., 2017; Lima et al., 2020). Fossil samples used for dating were recovered from a broad geographical range including the United States, Europe, Japan, and Africa. These claw morphotypes exhibited several distinguishing features that linked them to extant representatives of the genus *Alpheus,* including the presence of rows of setal pores along the fingertip crests, the presence of basal pits that in extant *Alpheus* serve as sensory structures. Divergence times for transisthmian sister-species pairs *A. schmitti/A. umbo* and *A. panamensis/A. formosus* pairs were based on the final closure of the Isthmus of Panama (3 Ma; Knowlton and Weigt, 1998; Lessios 2008; but see Bacon et al., 2015). We set an informative prior for the age root of the tree as recommended by the authors (Lartillot et al., 2009) at 30 Ma, according to the oldest fossil record for the family Alpheidae (Hyžný et al., 2017).

3. Results

3.1. Sequence assembly

Sequence data was generated for 240 loci greater than 250 bp in length using the AHE protocol. A minimum of 100 loci were sequenced for 65 out of the 73 species (90.4%) with an average of 165 loci greater than 250 bp in length. The average length of the sequenced loci was 403 bp. The final alignment was comprised of 65 terminal species and 72,003 bp sites after filtering and trimming. Eight taxa had low sequence coverage (less than 50 loci greater than 250 bp in length) and were not included in the final alignment. Alignments for 29 loci were removed because they did not contain sequences for at least 50% of the taxa, reducing the number of loci in the final alignment to 211. The completeness score for the entire dataset was 0.770. Gaps in the dataset were uniformly distributed with regards to species groups with the exception of outgroup taxa (*Synalpheus* spp.), which had a lower average completeness score of 0.403 (Appendix B).

3.2. Phylogenetic reconstructions

Topological congruence was highest for comparisons between the Bayesian and ML analyses of the concatenated dataset with an RF score of 1.5%. Both trees were well supported statistically. The two methods used to summarize sampled Bayesian trees, the majority rule consensus tree and the maximum clade credibility tree (Appendix C), shared nearly identical topologies. There was less topological agreement among species trees methods and between the species trees and concatenated gene trees (Appendices E–G). The RF scores for these comparisons averaged 18% (SD = 3.6%). In addition, species trees were less well supported and less resolved than the concatenated phylogenies. In particular, the MP-EST tree included multiple polytomies throughout the topology. We present the Bayesian analysis of the concatenated dataset along with ML bootstrap support at each node (Fig. 2). The maximum likelihood topology is included as Appendix D.

All five tree reconstruction methods resolved the same eight major clades. The topologies of concatenated tree methods were nearly identical; however, there were conflicts regarding the arrangements of the eight major clades among species-tree reconstructions. The discrepancies and lack of resolution among species-tree reconstructions may reflect differences in the topology of individual gene trees at some nodes; the well-supported topologies in concatenated reconstructions may be driven by a strong signal from a subset of genes (Shen et al. 2017). Nevertheless, the assignment of individual species to major clades is supported by both species-tree and concatenation methods, indicating that these general patterns are consistent across genes.

Clade I included only species of the morphologically well-defined *A. macrocheles* species group: *A. perplexus*, *A. deuteropus*, *A. cedrici*, *A. amblyonyx*, *A. inca*, and *A. crockeri*. Monophyly of this species group is fully supported by all five reconstruction methods. Clade I was also recovered as the oldest of the eight clades, with the divergence time estimated at 21 ± 2.2 Ma (Fig. 3). *Metalpheus rostratipes* was recovered as a sister to Clade I in all reconstructions, and this relationship was highly supported.

Clade II included members of the western Atlantic *A. armatus* species complex, which belongs to the *A. sulcatus* group. *Alpheus armatus*, *A. roquensis*, *A. immaculatus*, and *A. polystictus* are all known obligate symbionts of sea anemones, seemingly with highly species-specific associations (Knowlton and Keller, 1983, 1985; Almeida and Anker, 2011). The estimated divergence time for the four sequenced species was 3.8 ± 0.9 Ma. All phylogenetic reconstruction methods recovered the *amphi*-Atlantic *A. intrinsecus*, from the *A. edwardsii* group, as a sister of Clade II.

Clade III was recovered as sister to Clade II + *A. intrinsecus* in both ML and Bayesian analyses. Clade III includes mostly members of the *A. sulcatus* group (Coutière, 1899; Banner and Banner, 1982), but also

Racilius compressus, an obligate symbiont of oculinid corals with a strongly compressed body (Bruce, 1972; Anker and De Grave, 2016) and the coral-boring *A. simus* from the *A. obesomanus* group (Holthuis, 1980; Banner and Banner, 1982).

Clades IV–VIII were recovered within a larger, statistically well-supported clade. Clade IV primarily included taxa from the heterogeneous *A. edwardsii* group, such as the free-living, rock-sheltering or burrowing *A. japonicus*, *A. vladivostokiensis*, *A. strenuus*, and *A. malabaricus*. It also included members of the recently proposed *A. leviusculus* and *A. paracrinitus* groups (sensu Anker et al., 2009; Anker, 2020), i.e. *A. parvirostris* and *A. paracrinitus,* and *A. obesomanus* group (*A. malleodigitus*), making it one of the most heterogeneous clades of *Alpheus*. Clade V contained three members of the *A. diadema* species group: *A. diadema*, *A. percyi,* and *A. gracilipes*. Clade VI included five species from the ecologically highly interesting *A. crinitus* group, including the sponge dwelling *A. spongiarum* and the algal weaver *A. frontalis*. The *A. normanni* complex, which was placed in the *A. sulcatus* group (e.g. Kim and Abele, 1988), and here represented by *A. packardii*, was recovered as a sister of Clade VI. Finally, Clade VII included seven species currently assigned to the morphologically highly diversified *A. edwardsii* group: *A. lacertosus*, *A. amarillo*, *A. armillatus*, *A. nuttingi*, *A. bahamensis*, *A. schmitti* and *A. umbo*, the latter two representing transisthmian sister species (Knowlton and Weigt, 1998).

The *A. barbatus* complex, here represented by *A. naos* and *A. barbatus*, and previously considered as part of the *A. brevirostris* group (e.g. Banner and Banner, 1982; Anker et al., 2007a), was found to be the sister clade to Clade VII. Most of the remaining members of the *A. brevirostris* group included in our analyses formed a well-supported clade, Clade VIII, which corresponded to *A. brevirostris* group *s. str.*. These taxa are *A. brevirostris*, *A. aff. rapacida 1*, *A. aff. rapacida 2, A. samudra, A. djiboutensis, A. miersi, A. savuensis, A. platyunguiculatus* (two genetically
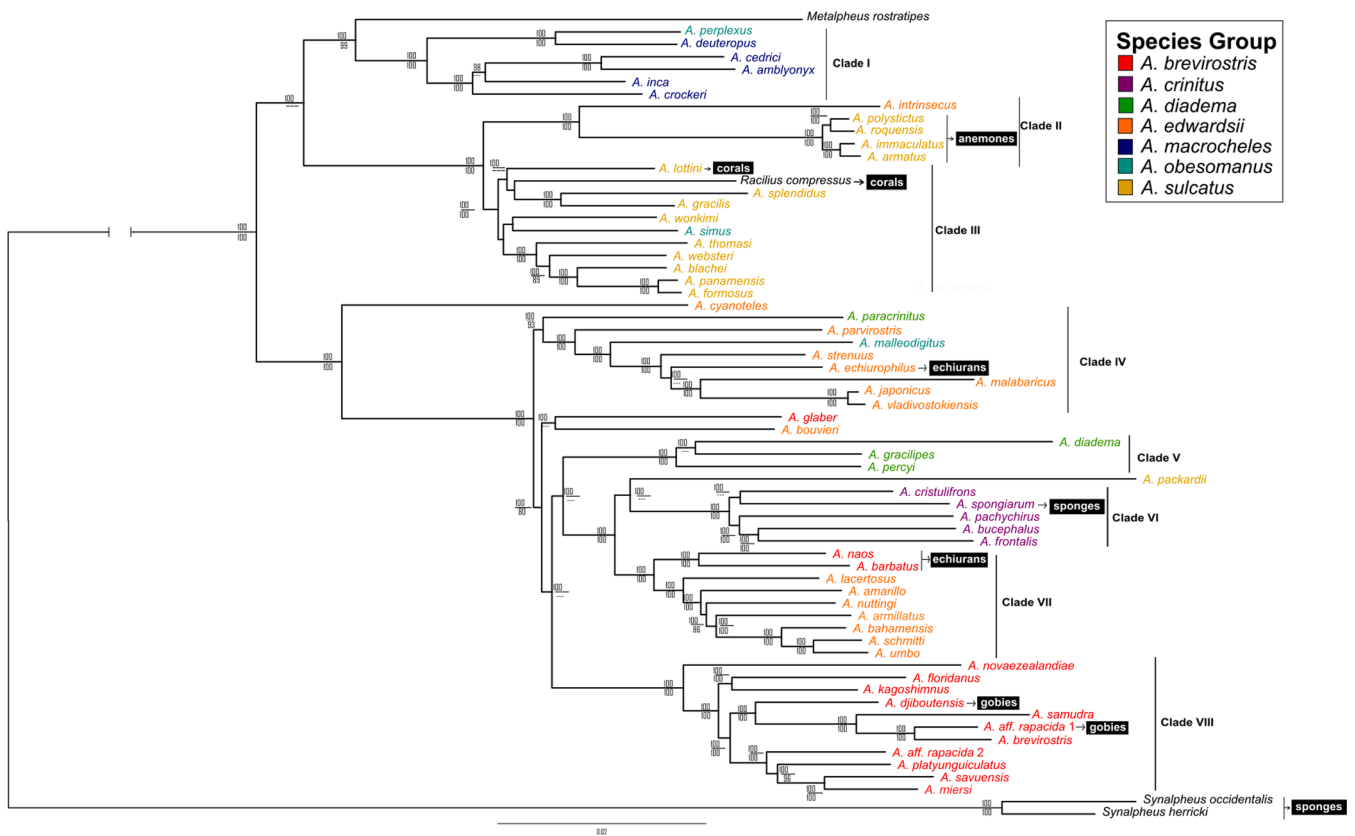

Fig. 2. Phylogenetic hypothesis for the genus *Alpheus* based on Bayesian GTR + gamma analysis of the concatenated dataset. Support for individual nodes is indicated as posterior probabilities (above line). Maximum likelihood bootstrap support is indicated below the line. Clade assignments are shown to the right of the tree. Colors indicate assignment to traditional morphological species groups originally defined by Coutière (1899). Black boxes show known symbiotic associations.
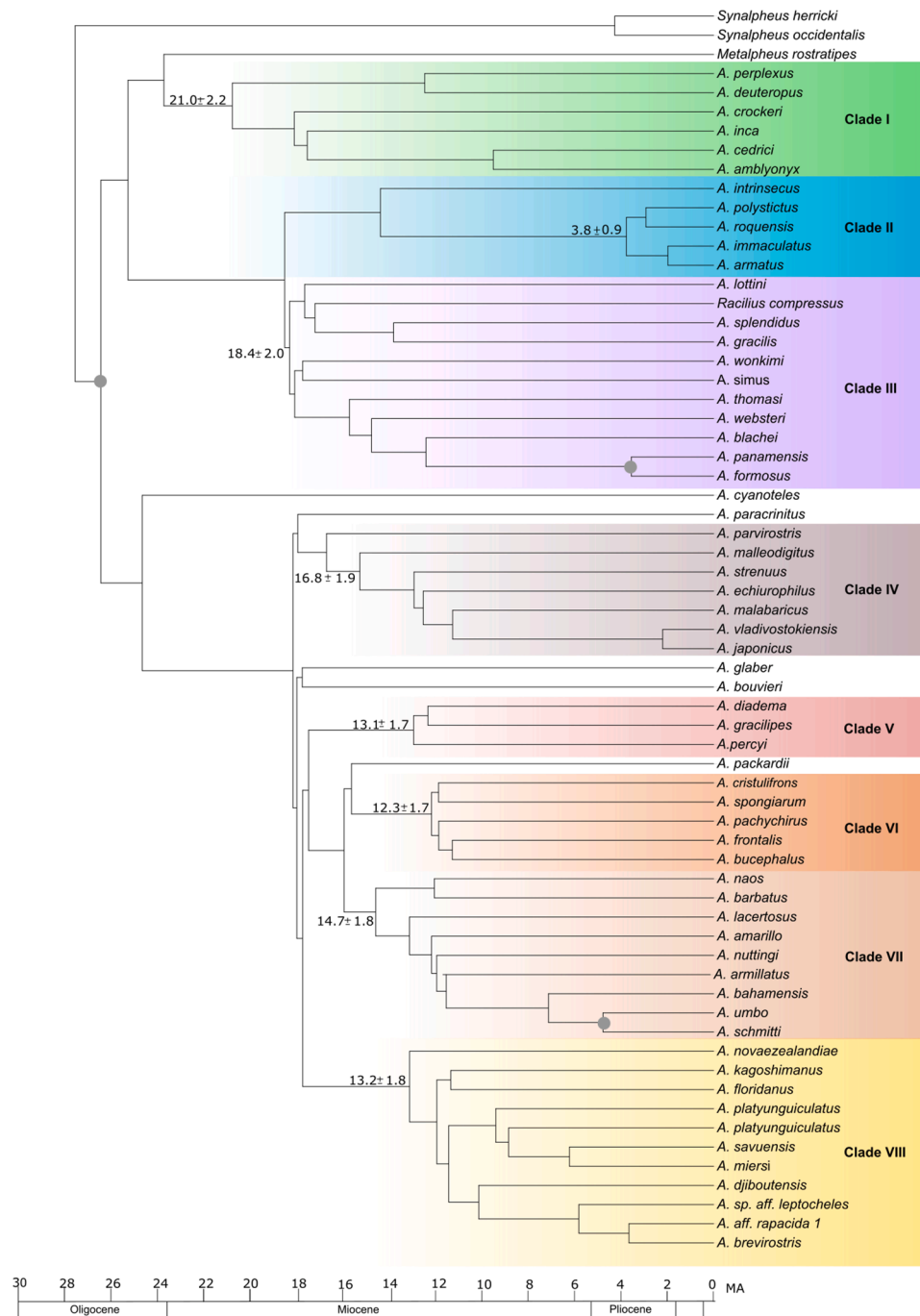
Fig. 3. Divergence time estimates for *Alpheus* based on the Bayesian (GTR + gamma) phylogenetic topology (Fig. 2). Node ages were estimated in PhyloBayes. Numbers at nodes indicate age estimates for the major clades ± standard error. Gray circles represent nodes with external calibrations. Colors are used to indicate assignment of OTUs to clades.

distinct taxa), *A. floridanus*, *A. kagoshimanus* and *A. novaezealandiae.*

Three taxa could not be assigned to any of the eight major clades of *Alpheus* defined above. Two morphologically and ecologically very different taxa, *A. bouvieri* and *A. glaber*, clustered together, forming the sister clade to Clades V-VIII*,* whilst the freshwater *A. cyanoteles*, with all characteristics of the *A. edwardsii* group, was recovered as sister lineage to Clades IV–VIII.

Relationships among the eight major clades of *Alpheus* were identical for Bayesian and ML reconstructions, however minor differences in the placement of Clade V (=part of the *A. diadema* group) were observed in the ASTRAL tree. All methods recovered a deep split between Clades I–III and Clades IV–VIII that occurred 26.7 ± 2.82 Ma, based on the

Phylobayes analysis. Within this first division, Clade II (=*A. armatus* complex + *A. intrinsecus*) and Clade III (=part of the *A. sulcatus* group) were recovered as well-supported sister groups. In all three analysis, the clade composed of Clade IV and *A. paracrinitus* was basal to Clades V–VIII, whereas Clades VI (*A. crinitus* group) and VII (part of the *A. edwardsii* group + *A. barbatus* complex) were recovered as sister clades.

4. Discussion

The genus *Alpheus* has been a valuable model system for studies of morphological adaptation (Mellon and Stephens, 1978; Anker et al.,

2006; Kaji et al., 2018), molecular evolution (Knowlton and Weigt, 1998; Hurt et al., 2009), symbioses (Glynn, 1976; Karplus and Thompson, 2011; McKeon et al., 2012) and speciation in the marine environment (Knowlton, 1986; Hurt et al., 2013). However, evolutionary studies within *Alpheus* have been limited in scope due to the scarcity of information regarding the interspecific relationships within this ecologically important and hyperdiverse lineage. The phylogenomic approach applied here to reconstruct evolutionary relationships between selected taxa of *Alpheus* (and two closely allied genera) is the first attempt to produce a hypothesis for this fascinating marine invertebrate clade. The selected taxa span a great deal of morphological variation, ecological adaptations and global distribution within *Alpheus*. Our results reveal or corroborate the paraphyly or polyphyly of several informal species groups defined on morphological grounds, and allow us to make first hypotheses on the evolution of particular cheliped morphologies and life styles, including symbioses and endolithic habits.

4.1. Monophyly of morphologically defined species groups

Taxonomy of *Alpheus* has traditionally relied on morphological variation in the shape of the frontal margin of the carapace combined with the features of the asymmetrical chelipeds, especially the major (snapping) claw. These features have formed the basis for delimiting the seven informal species groups established and used by most earlier workers since Coutière (1899). Although these groups have provided a useful framework for taxonomic studies within *Alpheus*, some groups have long been suspected to be non-monophyletic (Williams et al., 2001; Anker et al., 2009; Anker, 2020).

In the phylogeny presented here, monophyletic status is confirmed for only two morphologically well-defined species groups: the *A. macrocheles* group and the *A. crinitus* group (Clades I and VI, respectively). The *A. macrocheles* group includes mainly free-living species, but also includes sponge-associated taxa (Anker et al., 2008d), as well as endolithic snapping shrimps, e.g. *A. deuteropus* and *A. perplexus* (Banner, 1956; Banner and Banner, 1982). The latter species was tentatively placed in the *A. obesomanus* group, despite the presence of some characters alluding to its affinities with the *A. macrocheles* group (Banner, 1956; Banner and Banner, 1966). However, the remnants of longitudinal crests on the major claw (typical for the *A. macrocheles* group), together with the present molecular results, confirm that *A. perplexus* is indeed a derived member of the *A. macrocheles* group.

The *A. crinitus* species group (Clade VI) was recovered as monophyletic and is composed of at least 20 species (some representing species complexes) distributed mainly in the Indo-West Pacific, with only a few representatives in the eastern Pacific and Atlantic (including *A. cristulifrons*). The majority of the members of the *A. crinitus* group (e.g. *A. spongiarum*) are sponge associates, occupying a specific ecological niche similar to that of *Synalpheus* spp. However, several species in the *A. crinitus* group live in coral rubble, dead coral heads, or at the base of living corals, where they fabricate sausage-shaped, ramified domiciles made from various species of filamentous algae (*A. frontalis*, *A. pachychirus*, *A. bucephalus*). Other species of the *A. crinitus* group live deep in rock crevices, nodules of coralline algae, etc. and do not fabricate algal tubes. A more comprehensive phylogenetic investigation of the *A. crinitus* group is needed to address several evolutionary questions, such as colonization of sponges and corresponding morphological convergence and competition with sponge-dwelling *Synalpheus*, as well as evolution of algal felting.

All other species groups of *Alpheus*, as defined by Banner and Banner (1982) and Kim and Abele (1988), i.e. the *A. sulcatus, A. diadema, A. obesomanus, A. edwardsii* and *A. brevirostris* groups, are not monophyletic as currently defined.

The *A. sulcatus* group was previously shown to be non-monophyletic based on the position of *A. sulcatus* as sister to all other clades of *Alpheus* (Williams et al., 2001). Our analysis also shows that the *A. armatus* complex, considered as part of the *A. sulcatus* group (e.g. Zimmer 1913)

forms its own clade together with *A. intrinsecus*, Clade II. Recovery of the *amphi*-Atlantic *A. intrinsecus* as sister to the *A. armatus* complex is at least partly consistent with morphology, for instance, with the general configuration of the rostro-orbital region (Crosnier and Forest, 1966; Knowlton and Keller, 1983). Relationships between the species of the *A. armatus* complex in our AHE tree were identical to those recovered by Hurt et al. (2013), adding support for the utility of the AHE method for phylogenetic reconstructions. However, the estimated divergence time for this clade is notably more recent than previous coalescent-based divergence time estimates of 10–20 Ma (Hurt et al., 2013).

Most of the remaining members of the *A. sulcatus* species group were recovered within Clade III. The majority of the species in Clade III are free-living, typically dwelling under rocks and coral rubble or in coral rock crevices (*A. formosus, A. panamensis, A. splendidus, A. gracilis, A. blachei, A. websteri, A. thomasi*). Clade III also includes two snapping shrimps that bore into rocks and corals, the large and powerful *A. wonkimi* (Anker and Pachelle, 2013) and the highly specialized endolithic *A. simus*, formerly placed in the *A. obesomanus* group (represented by *A. malleodigitus*), because of its reduced rostrum and the major claw with a peculiar hammer-shaped dactylus (Holthuis, 1980). The only symbiotic member of this clade in our tree is *A. lottini*, which in fact is a complex of several cryptic species all associated with pocilloporid corals (Rouzé et al., 2017). Even after combining Clades II and III, the *A. sulcatus* group is still non-monophyletic as it includes representatives of the *A. obesomanus* and *A. edwardsii* groups (*A. simus* and *A. intrinsecus*, respectively), the genus *Racilius* (*R. compressus*).

The small but morphologically difficult to define *A. diadema* group was recovered as non-monophyletic with respect to *A. paracrinitus*, although three species with the typical features of the *A. diadema* group (Banner and Banner, 1982) clustered together in the Clade V. The endolithic *A. obesomanus* group was also non-monophyletic, being divided between Clades III (*A. simus*) and IV (*A. malleodigitus*).

The *A. edwardsii* group is morphologically and ecologically most diversified group within the genus *Alpheus*. Not surprisingly, this group was recovered as non-monophyletic, with most representatives forming large parts of Clades IV and VII. These results are consistent with the findings of Williams et al. (2001), showing the *A. edwardsii* group to be non-monophyletic with respect to the *A. bouvieri/A. hebes* clade, which was since reassigned to the *A. leviusculus* group (Anker et al., 2009, 2015). In addition, two species formerly assigned to the *A. edwardsii* group, *A. parvirostris* (later tentatively transferred to *A. leviusculus* group, see Anker et al., 2009) and *A. cyanoteles*, were recovered outside of the major clades, in rather questionable positions.

The *A. brevirostris* group is morphologically and ecologically relatively homogenous, especially when compared with the *A. edwardsii* group (Coutière, 1899; Banner and Banner, 1982). Most species of the *A. brevirostris* group are burrowers in soft sediments and some deepwater species have extremely elongated claws, such as *A. kagoshimanus* (Hayashi and Nagata, 2000). Several members of the *A. brevirostris* group (e.g. *A. rapacida, A. djiboutensis* and *A. floridanus*) are obligate or facultative associates of gobies (Karplus and Thompson, 2011). Exclusion of *A. naos* and *A. barbatus* from Clade VIII, which includes most of the other sequenced members of the *A. brevirostris* groups, indicates that this group is non-monophyletic as currently defined.

Since *Metalpheus* and *Racilius* were found embedded within *Alpheus* (the former as sister to Clade I and the latter nested within Clade III), the latter genus is paraphyletic in its present definition.

4.2. Comparisons with prior phylogenetic work

The most comprehensive previous phylogenetic analysis of *Alpheus* was based on three genes and included 53 species, primarily from the Western Atlantic and Eastern Pacific (Williams et al., 2001). Given a lack of overlap in taxa, direct comparisons between this work and ours are limited. Nevertheless, we can compare the general relationships between major clades identified in the two studies. Williams et al. (2001;

Fig. 6, consensus tree) partitioned *Alpheus* into three major clades, therein called Clade I, II and III, hereafter W-I, W-II and W-III. Clade W-I roughly corresponds to Clades IV–VIII; these five clades form a well-supported monophyletic group in the present phylogeny. Clade W-II formed a sister group to Clade W-III and included members of the *A. macrocheles* group and most members of the *A. sulcatus* group. This relationship is generally supported by our results, with Clade I (=*A. macrocheles* group) recovered as sister group to Clades II + III (mainly *A. armatus* complex and a large part of the *A. sulcatus* group). Most importantly, both Williams et al. (2001) and our study suggest that the *A. sulcatus* and *A. edwardsii* groups are polyphyletic.

Anker et al. (2006) recovered *Metalpheus* (together with probable sister genus *Pomagnathus*) and *Racilius* within *Alpheus* and argued that these genera may in fact represent highly derived members of *Alpheus*. These three genera were originally separated from *Alpheus* based on a few characters, such as "imperfect" orbital hoods and reduction of epipods on pereiopods. However, the general shape of the claws and other morphological features, coupled with our molecular results (at least for *Metalpheus* and *Racilius*), strongly suggest that they are indeed nested within *Alpheus* resulting in its paraphyly. Therefore, it is likely that *Metalpheus*, *Pomagnathus* and *Racilius* will be synonymized with *Alpheus* in the near future. However, such an action will require a formal invalidation of three widely used generic names and a significant emendation of the generic diagnosis of *Alpheus*, which are beyond the scope of the present study.

4.3. Host and habitat use

Our phylogeny suggests that symbiotic associations with other marine animals have evolved multiple times. Of the 65 species in our phylogeny, 14 exhibit symbiotic life styles (i.e. regularly recorded on the surface or inside body cavities of their animal hosts) (Horká et al., 2016), or share a burrow with partner animals, such as gobies (Karplus and Thompson, 2011). Symbiotic taxa (Fig. 1, Table 1) occur in at least seven non-related lineages (Fig. 2). Symbiotic lifestyles and habitat specialization have likely placed strong selective constraints on the evolution of body form, resulting in convergent evolution of some morphological features. For example, symbioses with echiuran worms evolved at least twice within *Alpheus*, once in the *A. barbatus* complex (Clade VII) and once in *A. echiurophilus* (Clade IV). Although not closely related, these snapping shrimps have a similarly shaped minor claw and also display similar, red-white color patterns (Anker et al., 2007a, 2015). The strong lateral compression of the body is seen in *A. lottini* (*s. lat.*), associated with pocilloporid corals, and especially in *Racilius compressus*, a highly derived symbiont of euphylliidcorals of the genus *Galaxea*.

Evolutionary patterns of symbiosis in *Alpheus* parallel some patterns observed in other speciose caridean groups, such as Palaemonidae and Hippolytidae (Baeza 2010, Horká, 2017). Phylogenetic reconstructions of Palaemonidae have demonstrated multiple transitions to symbiosis with a taxonomically diverse range of hosts, including numerous cnidarian and sponge hosts (Horká et al., 2016). Symbiotic palaemonid shrimps share some of the morphological features observed in symbiotic alpheids. Two species of palaemonid shrimp, *Anapontonia denticauda* and *Ischnopontonia lophos* are obligate associates of *Galaxea* alongside *R. compressus*. Notably, all three species share morphologically derived body plans with similar tail fan structures and laterally compressed bodies (Marin and Britavev, 2014), suggesting similar morphological adaptations to shared selective pressure. The phylogenetic placement of several species was unexpected based on both morphological characters and ecological traits and may be a consequence of convergent evolution in response to similar habitat use. The endolithic *A. malleodigitus* and *A. simus* are ecologically and morphologically similar species currently placed in the *A. obesomanus* group (Kropp, 1987), but were recovered in different clades in our AHE phylogeny. Another endolithic species, *A. perplexus*, was initially assigned, albeit with some doubts, to the *A. obesomanus* group (Banner, 1956), but was here recovered as a

member of the *A. macrocheles* group (Clade I). The evolution of a similar suite of morphological characters as adaptations to endolithic life style, including reduction of the rostrum and the hammer-shaped dactylus of the major claw, in three different clades constitute a strong evidence for convergent evolution.

4.4. Taxon sampling and unresolved questions

Additional taxon sampling is still needed to address key taxonomic questions and overall relationships within *Alpheus*. Two of the phylogenetically most problematic species groups, *A. sulcatus* and *A. edwardsii* groups, are under-represented in our AHE analysis. For instance, *A. sulcatus* (not included in the present phylogeny) was previously found to be unrelated to any of the other species of the *A. sulcatus* group, and may represent a sister lineage to all other clades (Williams et al., 2001). However, it must be noted that *A. sulcatus* is yet another species complex, and is presently being revised (Anker, in prep.). Several other species from the *A. sulcatus* group, such as the deep-water *A. compressus*, *A. soelae* and *A. canaliculatus*, the crevice-dwelling *A. rugimanus*, *A. villosus*, *A. acutofemoratus*, and the semi-endolithic *A. architectus* (possibly more closely related to *A. wonkimi* and *A. malleator*), will need to be included to have a better representation of this group.

Additional taxa from the *A. edwardsii* species group need to be included in future analyses to test whether they are phylogenetically closer to some taxa in Clade IV (=mainly Indo-West Pacific members of the *A. edwardsii* group) or are part of Clade VII (=eastern Pacific and Atlantic members of the *A. edwardsii* group). Some of the species form species complexes, including *A. edwardsii*, *A. pacificus*, *A. lobidens*, *A. pareuchirus*, and *A. euphrosyne,* that remain to be resolved taxonomically (Anker, 2001; Anker and De Grave, 2016; A. Anker, unpubl. data). If the geographical separation of the *A. edwardsii* group is confirmed by additional sequencing (Indo-West Pacific vs. eastern Pacific/Atlantic), it would mean that the *edwardsii*-type major claw evolved independently within *Alpheus*, i.e. in Clades IV and VII (not counting *A. cyanoteles*). The same may also be true for the *A. leviusculus* group, as defined by Anker et al. (2009), since in the present analysis, *A. parvirostris* and *A. bouvieri* did not form a monophyletic group.

Additional sampling could also resolve the status and position of taxa that could not be assigned to one of the major clades. For example, *A. cyanoteles* was recovered as sister to a clade that combined Clades IV–VIII, a result that is unexpected morphologically and warrants further investigation with additional species. Two morphologically and ecologically very different taxa, *A. bouvieri* and *A. glaber,* were recovered as sister species, but their sister status may be the result of long-branch attraction due to incomplete taxon sampling. Another example is *A. paracrinitus*, which was recovered as sister to all other taxa of the morphologically and ecologically heterogeneous Clade IV. This species is part of a pantropical species complex (Anker, 2001) with at least 10 species, forming a morphologically well-defined group. Its position within Clade IV will need to be confirmed by inclusion of additional taxa from this complex, as well as from taxa with intermediate characters between *A. paracrinitus* and the *A. diadema* group, such as *A. lanceloti* and *A. philoctetes*.

5. Conclusions

The well-supported phylogeny of the genus *Alpheus* presented here will provide a foundation for exploration of factors that drive species diversification. This is also the first worldwide molecular phylogeny of *Alpheus*, specifically targeting the morphological and ecological diversity within this model group of marine crustaceans. *Alpheus* exhibits an astonishing diversity in body form, color patterns, habitat use, and other life history strategies and offers unparalleled opportunities for testing evolutionary hypotheses, when coupled with a robust phylogenetic hypothesis. One promising area is the application of morphometric tools for testing hypotheses regarding convergent evolution and

adaptation. The commonness of closely related species in sympatry within *Alpheus* raises pertinent questions about the role of host specificity and habitat specialization on reproductive isolation. Coevolution between snapping shrimps and their hosts or partners is another promising research field. Finally, the presence of multiple sister species pairs across the Isthmus of Panama facilitates the calibration of evolutionary processes to absolute time. Transisthmian sister species have been used most extensively to study molecular evolution (Knowlton and Weigt, 1998; Hurt et al., 2009), but can also be used to calibrate the rate of divergence of morphological traits (Marko and Jackson, 2001; Marko and Moran, 2002). Additional taxon sampling, especially within underrepresented morphological groups (especially the polyphyletic *A. sulcatus* and *A. edwardsii* groups), ecological guilds (e.g., deep-water and symbiotic taxa) or biogeographic regions (South Africa, temperate Australia, western Indian Ocean, Japan, eastern Atlantic) is needed to examine factors that have contributed to the extraordinary diversity of this group.

CRediT authorship contribution statement

Carla Hurt: Conceptualization, Methodology. **Kristin Hultgren:** Conceptualization, Methodology. **Arthur Anker:** Conceptualization. **Alan R. Lemmon:** Methodology. **Emily Moriarty Lemmon:** Methodology. **Heather Bracken-Grissom:** Methodology.

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Appendix A. Supplementary material

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