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Application of integrative taxonomy combining phylogenetic and geometric morphometric techniques in a snapping shrimp (*Alpheus* Fabricius, 1798) species complex (Decapoda: Caridea: Alpheidae)

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ABSTRACT

Snapping shrimps of the genus *Alpheus* Fabricius, 1798 exhibit remarkable diversity, with over 300 described species. The genus is well-known for its prevalence of species complexes, necessitating the need of new tools to facilitate species discrimination. Traditional taxonomic methods in *Alpheus* have primarily relied on the examination of morphological traits or comparative morphometric measurements, with an emphasis on variation in the major chela and rostro-orbital region. We applied an integrated approach that combines molecular genetics and geometric morphometrics to investigate the *A. gracilipes* Stimpson, 1860 species complex. We additionally applied geometric morphometric techniques to study the major chela and the rostrum across different species, and used three mitochondrial genes (12S, COI, and 16S) to reconstruct phylogenetic relationships of this complex. Our results demonstrate the first application of geometric morphometric techniques to *Alpheus* snapping shrimps, and highlight the significance of the major chela and rostrum as taxonomically informative traits. Furthermore, we use DNA barcodes and geometric morphometric techniques to the *A. gracilipes* species complex to reveal two previously unidentified cryptic species. We present the first phylogenetic reconstruction of this species complex, with new localities and expanded distribution ranges reported for many species.

KEYWORDS: Crustacea, mitochondrial genes, phylogeny, snapping shrimps, species delimitation

INTRODUCTION

Landmark-based geometric morphometrics has emerged as a valuable tool for both species identification and for exploring morphological variation between species (Sidlauskas *et al.*, 2011; Klingenberg & Marugán-Lobón, 2013; Karanovic *et al.*, 2018). This method integrates multivariate statistics and Cartesian coordinates to measure shape variation among species (Webster & Sheets, 2010; Zelditch *et al.*, 2012). A myriad of taxonomic and evolutionary investigations have demonstrated the efficacy of geometric morphometrics in distinguishing between closely related species (e.g., Mutanen & Pretorius, 2007; Ludoški *et al.*, 2008; Zuykova *et al.*, 2013; Mitrovski-Bogdanović *et al.*, 2014; Schwarzfeld & Sperling, 2014; Ruane, 2015; Meusel & Schwentner, 2017; Karanovic *et al.*, 2018; Grinang *et al.*, 2019; Moraes *et al.*, 2021), as well as distinguishing among different populations or sexes within a species (Bissaro *et al.*, 2013; Marchiori *et al.*, 2014; Ismail, 2018; Jabłońska *et al.*, 2021). Some of the most effective studies have adopted an integrative approach, combining morphological and molecular techniques (Zuykova *et al.*, 2013; Mitrovski-Bogdanović *et al.*, 2014; Castelin *et al.*, 2017; Meusel & Schwentner, 2017; Karanovic *et al.*, 2018; Jabłońska *et al.*, 2021). Geometric morphometrics appears to be more effective in diagnosing morphologically similar species

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when compared to subjective morphological assessments by expert taxonomists (Mutanen & Pretorius, 2007). Despite the success of geometric morphometrics in differentiating cryptic species, however, it has yet to be utilized in the snapping shrimp genus *Alpheus* Fabricius 1798.

Alpheus snapping shrimps are characterized by key morphological innovations such as asymmetrical chelae and rostro orbital hoods that completely cover the eyes. The major chela, largest of asymmetrical chelae, forms the characteristic "snapping" claw (see Anker *et al.*, 2006). The rostral orbital hoods are theorized to be potential adaptations that evolved in concert with their powerful snapping claws (Anker *et al.*, 2006). Notably, the current estimate of formally described species of *Alpheus* (336; World Register of Marine Species (WoRMS), 2023) is likely an underestimate as both molecular and morphological research suggests the presence of numerous cryptic species (Anker, 2001; Anker *et al.*, 2006; Hurt *et al.*, 2021). There are at least 40 cryptic-species complexes in *Alpheus* (Anker, 2001). Anker (2001) emphasized the necessity of incorporating molecular genetics and color patterns for accurate species identification. Consequently, numerous studies investigating *Alpheus* snapping shrimps have employed a range of techniques, including molecular methods, color patterns, and subtle morphological differences to reveal multiple cryptic species complexes (Knowlton & Keller, 1985; Anker, 2001, 2012; Nomura & Anker, 2005; Anker *et al.*, 2007, 2008; Mathews & Anker, 2009).

Variations in the shape of the major snapping chela and the rostro-orbital hoods have been used for much of *Alpheus* taxonomic history. These two characters form the basis of seven morphologically defined species groups (Coutière, 1899, 1905; Banner & Banner, 1982). Variation in these characters has also been useful at smaller taxonomic scales, such as distinguishing among closely related species in cryptic-species complexes (Nomura & Anker, 2005; Anker *et al.*, 2007). Researchers have assessed variation in these characters using various approaches. One method involves comparing measurements, such as the length-width ratio of the major chela (Nomura & Anker, 2005; Anker, 2012). Another uses the presence/absence of characters such as notches or sculpturing on the major chela (Anker *et al.*, 2009) to discriminate species. Finally, some researchers investigating *Alpheus* have also used a combination of multivariate statistics and linear measurements (i.e., traditional morphometric techniques) to diagnose species and to identify taxonomically informative traits (McClure & Wicksten, 1997). Certain species complexes, i.e., the *A. armatus* (Rathbun, 1901) complex, are nevertheless more problematic to resolve and often require the use of molecular genetics or discrimination using color patterns on live specimens for accurate species diagnosis (Knowlton & Keller, 1985; Hurt *et al.*, 2013). Accurately diagnosing and describing the full range of diversity in *Alpheus,* which includes a significant amount of material held in museum collections, necessitates exploration of new diagnostic methods that complement existing traditional taxonomic and morphological techniques to fully capture morphological differences among cryptic species.

One suitable group for exploring the utility of geometric morphometric techniques in snapping shrimps is the *Alpheus gracilipes* Stimpson, 1860 species complex (Nomura & Anker, 2005). This species complex has a broad distribution across the Indo-West Pacific (IWP) region and its species are common on reefs, in or under dead corals, and/or in submarine caves near shore (Miya, 1974; Bruce, 1999; Nomura & Anker, 2005). The seven species in this complex can be separated by subtle morphological variations, and to a somewhat greater extent, color patterns in live specimens (Nomura & Anker, 2005).

The type species, *A. gracilipes,* was originally described from Tahiti and was initially considered to have an extensive distribution across the IWP with a considerable amount of phenotypic variation (color and morphology) between different locations (Banner, 1953; Banner & Banner, 1967, 1981, 1982; Miya, 1974). Banner (1953), for example, described a specimen from the Hawaiian Islands with abdominal ocelli (eyespots), whereas Banner & Banner (1967) described two morphological forms of male *A. gracilipes* (one with "balaeniceps" minor chelae (e.g., chela densely adorned with setae resembling the upper jaw of a baleen whale (Barnard, 1950), and one without the setae), and noted variation in rostrum length amongst other characters. Bruce (1999) was the first to describe *A. soror* Bruce, 1999 as a distinct species using minor morphological differences and color patterns, remarking that *A. soror* had likely been misidentified as *A. gracilipes* by Coutière (1898), Banner (1953), and Miya (1974).

Nomura & Anker (2005) re-examined preserved museum specimens identified as *A. gracilipes* from southern Japan and other IWP localities using variation in morphological characters, including the major chelae and the rostro-orbital area. They also designated a neotype for *A. gracilipes* and described five new cryptic species: *A. fujitai* Nomura & Anker, 2005, *A. roseodigitalis* Nomura & Anker, 2005, *A. parvimaculatus* Nomura & Anker, 2005, *A. kuroshimensis* Nomura & Anker, 2005, and *A. angustilineatus* Nomura & Anker, 2005. The shape of the rostrum (e.g., the relative height of the rostrum vs. orbital hoods) and dimensions of the major chela (e.g., the length-width proportions; Nomura & Anker, 2005) are frequently used to distinguish the species of this complex. Intriguingly, Nomura and Anker (2005) suggested that *A. gracilipes* potentially represented multiple species and noted that while material from some localities (Japan, the Red Sea, Guam, New Caledonia, and the Hawaiian Islands) aligned well with the neotype, other records (from Australia, Indonesia, Thailand, and the Society Islands) likely did not refer to *A. gracilipes* sensu stricto. As such, the *A. gracilipes* species complex is well-suited for investigation using an integrated approach combining geometric morphometric techniques and molecular-based species delimitation methods.

We applied geometric morphometric techniques on two key characters crucial in the taxonomic treatments of species of *Alpheus,* the rostrum (Fig 1) and the major chela (Fig 2). We assessed the efficacy of landmark-based geometric morphometrics for distinguishing between highly morphologically similar species with the *A. gracilipes* species complex as our test group. We also applied molecular genetic techniques to three mitochondrial genes (COI, 12S, and 16S) to substantiate our geometric morphometric results and to provide the first phylogeny of this species complex. Finally, we applied a molecular species delimitation method (Assemble Species by Automatic Partitioning, ASAP) to the COI dataset to delimit species.

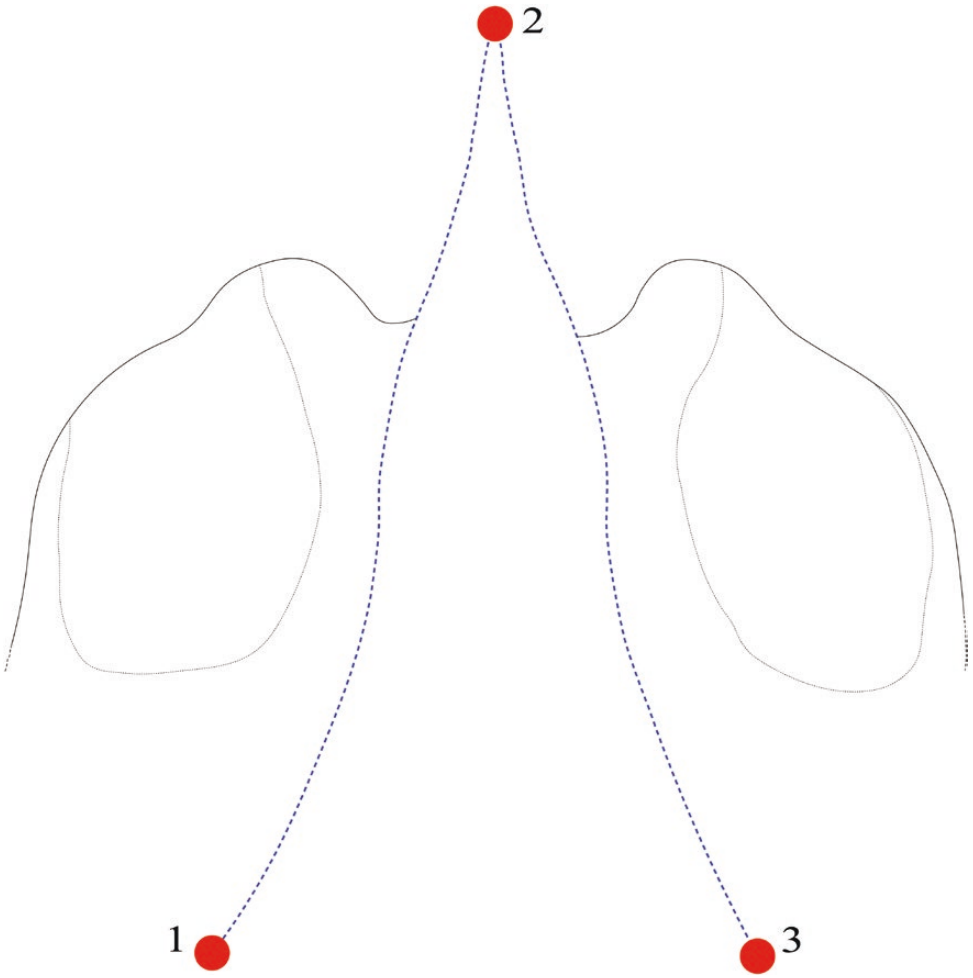

Figure 1. Landmarks and semi-landmarks on the rostrum of *Alpheus roseodigitalis* (UF 15756) identified for use in geometric morphometric analyses. Closed red dots denote landmarks ($N = 3$), dashed blue lines denote the curve along with semi-landmarks were placed.

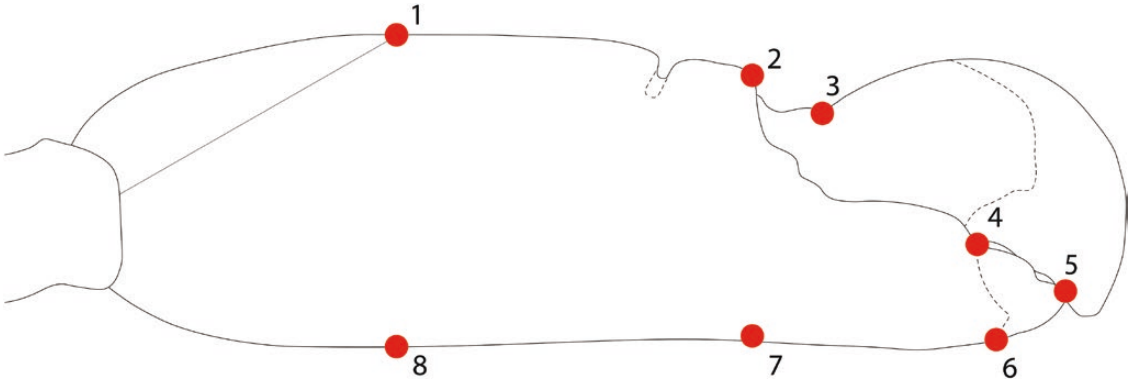

Figure 2. Landmarks on the major chela of *Alpheus roseodigitalis* identified for use in geometric morphometric analyses. Closed red dots denote landmarks ($N = 8$).

MATERIALS AND METHODS

Taxon sampling, genetics, and phylogeny construction

Specimens belonging to the *A. gracilipes* species complex (including *A. percyi* Coutière 1908, used as an outgroup) were acquired on loan with permission for sampling from the Florida Museum of Natural History (FLMNH), Gainesville, FL, USA and from the Muséum national d'Histoire naturelle (MNHN), Paris, France. All specimens were collected from the Indo-West Pacific region. Only adult shrimps with intact rostrums and major chelae were included in geometric morphometric analyses (Supplementary material Table S1).

We sequenced three mitochondrial genes frequently used in species-level phylogenetic reconstructions of shrimps (Harrison, 2004; Bracken-Grissom *et al.*, 2014; Hultgren *et al.*, 2014). These included the 16S large ribosomal subunit (~500 bp), the 12S small ribosomal subunit (~400 bp), and the 5′ barcoding end of the protein coding cytochrome oxidase I gene (COI: ~650 bp). Primer sequences used for amplification are listed in Table 1.

We extracted genomic DNA from pleopods, pereopod stumps, and/or eggs following manufacturer protocols for the Omega Bio-Tek E.Z.N.A.® Tissue DNA Kit (Omega Bio-Tek, Norcross, GA, USA). We then amplified each gene with polymerase chain reactions (PCR) performed in, 20 µl reactions consisting of 10 µl of Taq DNA polymerase, dNTPs, and reaction buffer (Promega 2X; Promega, Madison, WI, USA), 0.5 µl each of forward and reverse primer, 7 µl of PCR water, and 2 µl of extracted DNA. The following parameters were used for thermal cycling: 1 cycle of initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60.3 °C for 30 s, extension at 72 °C for 80 s, and a final extension at 72 °C for 10 min. Amplified DNA was visualized in an agarose gel. PCR products were incubated with shrimp alkaline phosphatase (rSAP) and exonuclease I (EXO) to remove residual primers and dNTPs. Bidirectional Sanger sequencing was performed using an ABI 3730XL automated sequencer platform (MCLAB, San Francisco, CA, USA).

Sequence data from the COI, 12S, and 16S genes was visualized and filtered for quality using Sequencher 5.4 (GeneCodes, Ann Arbor, MI, USA). Sequences were aligned using MUSCLE (multiple sequence comparison by log-expectation; Edgar, 2004), as implemented in the software AliView (v. 1.27; Larsson, 2014). The best-fit model of nucleotide substitution for COI (TIM3+I+G), 16S (TIM1+G), and 12S (TIM3+G) was determined using the Akaike Information Criterion (AIC) in JModelTest2 (Darriba *et al.*, 2012), run on XSEDE in the Cipres Science Gateway (Miller *et al.*, 2010). Bayesian Inference analysis was subsequently used to reconstruct the phylogenetic relationships for each individual locus and for the concatenated data set. For all analyses, the general model of evolution was coded for each individual locus (nst = 6, rates = gamma), but allowed MrBayes to estimate other model parameters individually for each locus (e.g., proportion of invariable sites, base frequencies, and nucleotide substitution rates). In the concatenated data set, we maximized species sampling across the genus by including two specimens that were missing data: *A. fujitai* (missing 12S data) and *A. gracilipes* C (missing 16S data). All other specimens in the concatenated dataset included data from all three genes. We also used sequences from *A. percyi,* a closely related species to the *A. gracilipes* complex (Hurt *et al.*, 2021), and *Synalpheus belizensis* Anker & Tóth, 2008, a sister taxon to the genus *Alpheus,* as outgroups in phylogenetic analyses. All trees were run on four chains with two runs, and a burn-in of 25% of the trees; the COI and 12S Bayesian gene trees were inferred based on 30,000,000 generations, the 16S gene tree was run for 50,000,000 generations, and the concatenated analysis with three loci was based on 50,000,000 generations. For all trees, the potential scale reduction factor (PSRF) values were ~1.00, and the standard deviation of split frequencies was < 0.001. Bayesian analyses were carried out using MrBayes on XSEDE in CIPRES Science Gateway, version 3.3 (Miller *et al.*, 2010). For COI gene trees, we also used several sequences of *A. gracilipes* and *A. soror* from GenBank (Supplementary material Table S1).

Species assignment and species delimitation analyses

The COI gene tree (Fig. 3) and the consensus tree (Fig. 4) both recovered three distinct paraphyletic clades within specimens identified as *A. gracilipes*, suggesting that there may be additional cryptic species within specimens identified as *A. gracilipes* (*A. gracilipes* A, B, and C). We therefore used Assemble Species by Automatic Partitioning (ASAP) (Puillandre *et al.*, 2021; https://bioinfo.mnhn.fr/abi/public/asap/) and used Kimura (K80) as a substitution model. This species delimitation method uses pairwise genetic distances instead of phylogenetic reconstruction to implement a hierarchical clustering algorithm to sort single-gene sequences (such as COI) into proposed species partitions (Puillandre *et al.*, 2021).

Species identifications were tentative for some museum specimens ("cf" or "aff" herein), or specimens were identified only to the general species complex (e.g., *A. gracilipes* species complex). We therefore used Kimura 2-parameter distances for the COI data and examined branching relationships within all three gene trees to clarify relationships and species assignments. For example, two specimens (UF 15674 and MNHN-IU-2013-1475) initially identified as *A. parvimaculatus* using morphology, consistently formed a separate clade in the COI gene tree (Fig. 3). They also grouped with other *A. gracilipes* B individuals in the

Table 1. List of 16S, 12S, and COI primers used in this study. References: 1, Palumbi *et al.* (1991); 2, Folmer *et al.* (1994); 3, primers created by CH and AC for this study.

Gene	Primer	Primer Pair	Sequence 5′ → 3′	Reference
16S	16Sar	16Sbr	CGC CTG TTT ATC AAA AAC AT	1
16S	16Sbr	16Sar	CCG GTY TGA ACT CAG ATC AYG T	1
COI	LCOI1490	COIL2	GGT CAA ATC ATA AAG ATA TTG G	2
COI	alp202	COIL2	TAG CCT TCA AAG TTT CCA ATA GGG	3
COI	COIL2	LCOI1490	ACT TCA GGG TGA CCG AAG AAT CAG AA	3
COI	COIL2	alp202	ACT TCA GGG TGA CCG AAG AAT CAG AA	3
12S	12SAC1	12SAC2	GTG ATC CTC CCA TTG TAA GTG G	3
12S	12SAC2	12SAC1	GCC AGC CGC GGT TAT AC	3

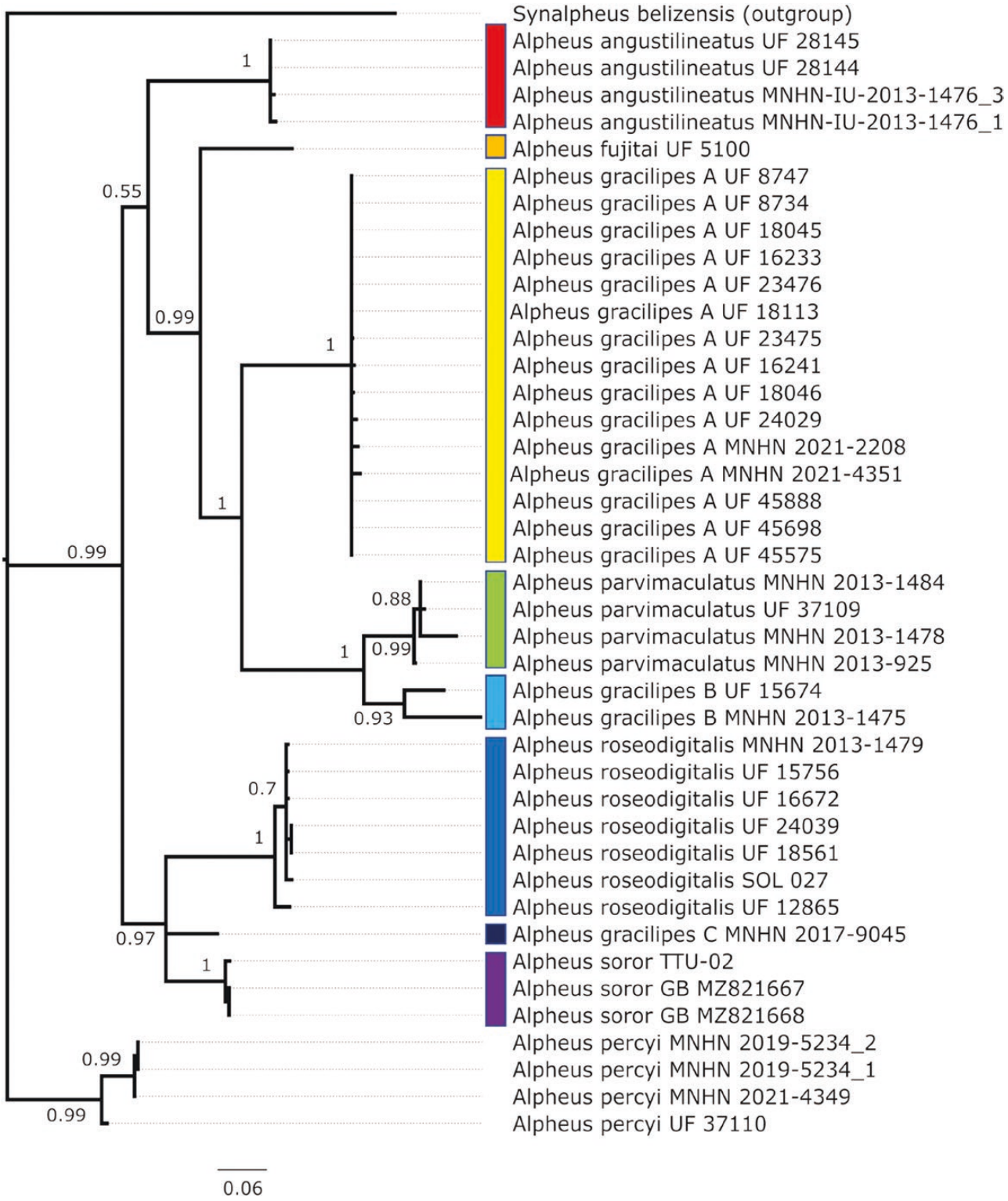

Figure 3. COI gene tree for the *Alpheus gracilipes* species complex, with species delimitation analysis mapped on. Numbers above branches indicate Bayesian posterior probability values; color blocks indicate species according to ASAP species delimitation.

12S/16S gene trees (Supplemental material Figs. S2, S3), leading to their subsequent reassignment as *A. gracilipes* B. Two additional specimens were re-identified using these methods: UF 8747, originally identified as *A.* cf. *kuroshimensis,* was re-identified as *A. gracilipes* A, and UF 37110, originally identified as *A. gracilipes*, was re-identified as *A. percyi.*

Landmark-based geometric morphometrics

We applied geometric morphometric techniques to study two taxonomically informative traits frequently used in the taxonomy of *Alpheus* (Coutière 1899, 1905; Anker *et al.*, 2006, 2008). Images of the rostrum (N = 23) and the major chela (N = 20) from *A. gracilipes* A (N = 6), *A. gracilipes* B (N = 1),

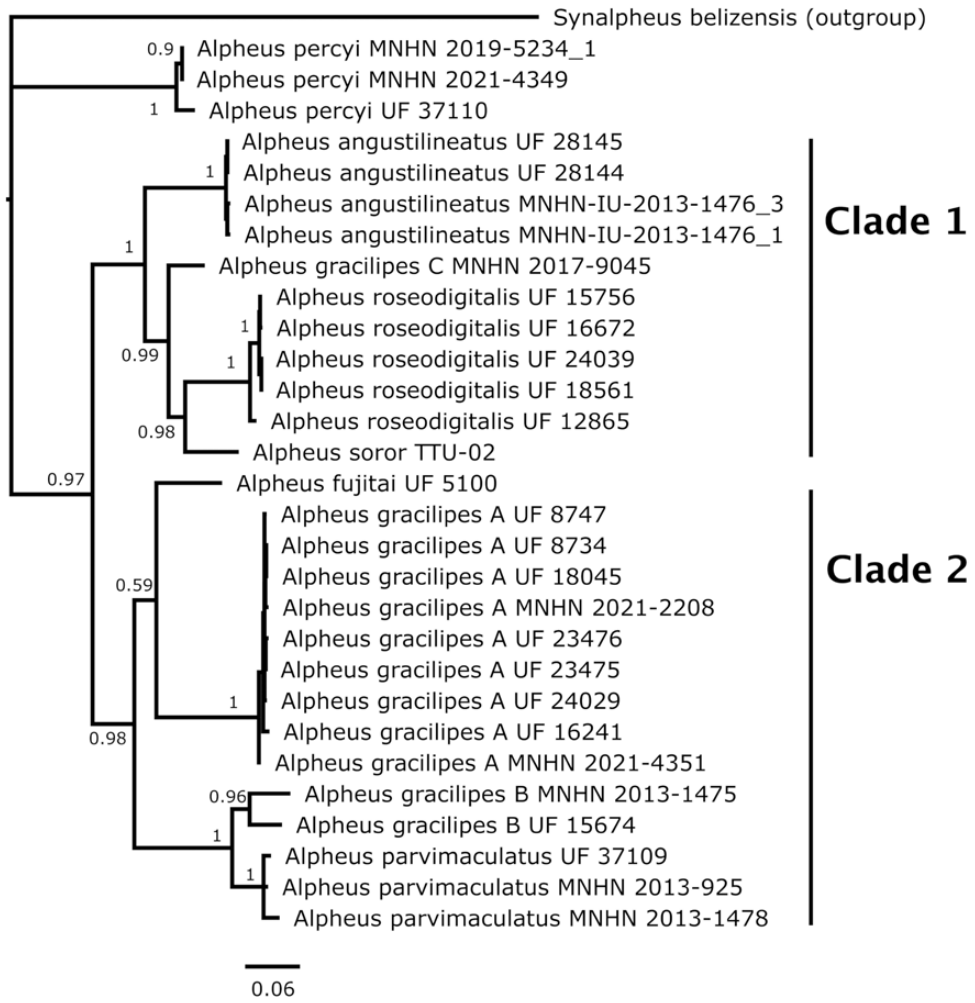

Figure 4. Concatenated Bayesian phylogenetic tree for the *Alpheus gracilipes* species complex, based on a combined analysis of COI, 16S, and 12S. Branch lengths indicate genetic distance; numbers above or below each branch represent Bayesian posterior probabilities.

A. roseodigitalis ($N = 6$), *A. percyi* ($N = 3$), *A. parvimaculatus* ($N = 3$), and *A. angustilineatus* ($N = 4$) were digitized using a stereomicroscope (Motic DM143 FBGG-C; Motic Optical, Hong Kong). All photographs were taken by the same person (AC) to minimize errors and maximize consistency.

Two-dimensional (2D) landmarks were digitized using a thin-plate-spline (tps) software series (Rohlf, 2015) on the major chelae and rostrums of highly morphologically similar species from the *A. gracilipes* species complex. We used tpsUtil (Rohlf, 2015) to create two .tps files with digitized images of each morphological character and used tpsDig2 (Rohlf, 2015) to digitize landmarks (Figs. 1, 2). We placed a combination of type I (homologous points), II (maximum point in a curvature), and III (mathematically derived) landmarks on the rostrums and the major chelae. The shape of the rostrum (Fig. 1) was captured using three type I landmarks and two curves with 23 semi-landmarks (special type III landmark), and the major chela (Fig. 2) with a combination of five type I, II, and III landmarks. We finally edited each .tps file to convert semi-landmarks to landmarks to prepare the shape data for further analyses.

A generalized Procrustes analysis (GPA) was used to standardize shape data by scaling, translating, and rotating superimposed shape outlines (Adams & Otárola-Castillo, 2013). We used procrustes ANOVA (pANOVA) to compare the mean shapes for the major chelae and rostrums among different species. A principal component analysis (PCA) was used to visualize shape variation in the form of a scatter plot, and canonical variate analysis (CVA) was used to maximize the distance between individuals of different groups (i.e., species) while minimizing the distance between individuals of the same groups. All geometric morphometric analyses, including GPA, pANOVA, PCA, and CVA, were performed in MorphoJ (Klingenberg, 2011). Graphs were created using the morpho (Schlager, 2017) and ggplot2 (Wickham, 2016) packages in RStudio (R Studio Team, 2020).

RESULTS

Species delimitation using the COI gene

The COI gene tree and the ASAP delimitation analysis revealed that five species within the *A. gracilipes* complex (*A. angustilineatus, A. parvimaculatus, A. roseodigitalis, A. fujitai,* and *A soror*) formed distinct clades with high Bayesian posterior probabilities (bpp > 0.99; Fig. 3). Specimens identified

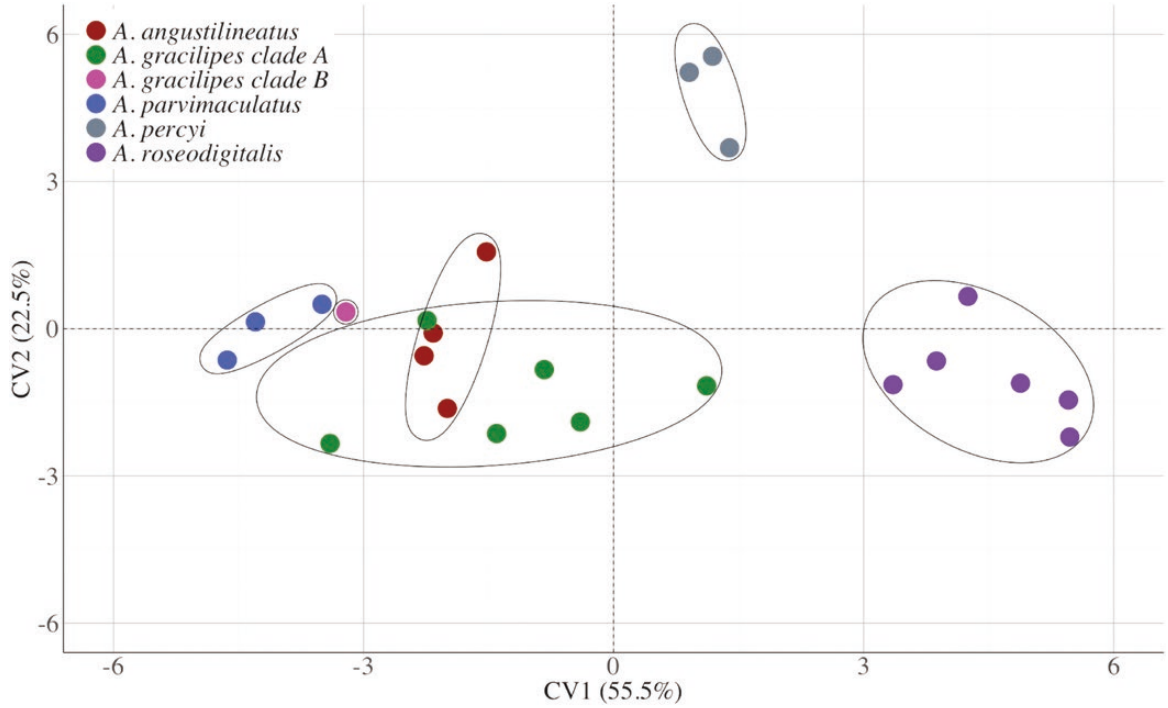

Figure 5. Canonical variate analysis (CVA) plot of rostrum shape data of species in the *Alpheus gracilipes* species complex

Table 2. Results of Procrustes ANOVA of rostrum shape and major chela shape for species of the *A. gracilipes* species complex. SS, sum-of-squares; MS,mean square; *df*, degrees of freedom; F, F statistics; *P*, associated probability level

Body structure	Effect	SS	MS	*df*	F	*P*
Rostrum	Individual	0.09080718	0.00019321	470	3.96	< 0.0001
	Residual	0.07802607	4.8827E-05	1598		
Major Chela	Individual	0.05376424	0.00134411	40	8.54	< 0.0001
	Residual	0.01762188	0.00015734	112		

as *A. gracilipes* were found to be paraphyletic and grouped in three primary clades (Figs. 3, 4): The ASAP analysis recognized *A. gracilipes* A, *A. gracilipes* B, and *A. gracilipes* C as distinct species (Fig. 3).

Bayesian molecular phylogeny

Bayesian Inference analysis of the concatenated data set revealed the presence of two major clades (Fig. 4), which generally aligned well with the phylogenetic relationships in the individual gene trees (Fig. 3, Supplementary material Figs. S2, S3). In the concatenated tree, clade 1 (bpp = 1.0) consisted of a well-supported clade of *A. angustilineatus* (bpp = 1.0), which was sister to a clade containing specimens of *A. roseodigitalis* (bpp = 1.0), *A. soror,* and *A. gracilipes* C. Clade 2 consisted of *A. gracilipes* A (bpp = 1.0) with *A. fujitai* as an outgroup. This clade was the sister to a clade that contained two specimens of *A. gracilipes* B (bpp = 0.96) and a clade with *A. parvimaculatus* (bpp = 1.0). Its branching pattern was similar in 16S gene tree (Supplementary material Fig. S2**).** Overall, the branching patterns between species clades were also similar in the 12S gene tree, except that the single specimen of *A. gracilipes* C formed a polytomy with a clade of *A. angustilineatus* specimens and a clade of *A. roseodigitalis* and *A. soror* (Supplementary material Fig. S3).

Geometric morphometrics

Results of the CVA for the rostrum (Fig. 5) demonstrated that its shape differed among the species we used. The first two canonical variates accounted for 73% of total variation, with CV1and CV2 accounting for 49.8% and 23.2% of total shape variation, respectively. Canonical variate 1 discriminated *A. percyi* and *A. roseodigitalis* from the other species whereas CV2 separated *A. percyi* from the rest. Notably, both *A. gracilipes* A and *A. angustilineatus* exhibited an overlap in their distribution while *A. gracilipes* B clustered more closely with *A. parvimaculatus,* indicating a narrow and elongated rostrum. Lower CV1 scores in the CVA morphospace corresponded to a narrow and elongated rostrum, whereas higher scores corresponded to a broad and short rostrum. Likewise, lower CV2 values corresponded to a narrow base while higher values corresponded to a broad base (Fig. 5). The pANOVA revealed significant variation in the shape of the rostrum among the different species used in this study ($P < 0.0001$; Table 2)

The results of the CVA (Fig. 6) for the major chelae demonstrated significant morphological variation among the species

used. The first two canonical variates accounted for 82.0% and 9.5% of total variation, respectively, for a cumulative total of 91.5%. The CVA morphospace showed that *A. roseodigitalis, A. gracilipes* B, and *A. parvimaculatus* grouped more distinctly from the remaining species. Lower CV1 scores corresponded with a broader major chela whereas higher CV1 scores corresponded to a narrower major chela. Similarly, lower CV2 scores corresponded to a shorter chela while higher CV2 scores corresponded to a shorter chela. Notably, *A. gracilipes* B demonstrated overlap with both *A. percyi* and *A. angustilineatus* while these two species did not overlap with each other. The results of the pANOVA revealed significant variation in the shape of the major chela among the different species used in this study ($P < 0.0001$; Table 2).

DISCUSSION

We used an integrative approach combining geometric morphometric and molecular genetic techniques to study the *A. gracilipes* species complex. We worked primarily on specimens from two major natural history museums (FLMNH & MNHN). We used three mitochondrial genes to recreate the first molecular phylogenetic tree for this complex and perform species delimitation analyses. The phylogenetic reconstructions revealed that specimens identified as *A. gracilipes* grouped into at least three separate paraphyletic clades (*A. gracilipes* A, B, and C), indicating the potential presence of two additional cryptic species, *A. gracilipes* A and *A. gracilipes* B. One or more of these specimens may correspond to the "aberrant" records of *A. gracilipes* described in Nomura & Anker (2005), which the authors noted likely do not correspond to *A. gracilipes* sensu stricto. Additional sequencing and morphological examination of specimens identified as *A. gracilipes* or *A. gracilipes* complex is necessary to further elucidate the nature of these potentially new species.

We also used geometric morphometrics to provide morphological evidence supporting molecular diagnoses of cryptic species of snapping shrimps identified as *A. gracilipes*. Specifically, these techniques discriminated between *A. gracilipes* A and *A. gracilipes* B, which formed two separate clades in each gene tree (Fig 3, Supplementary material Figs. S2, S3) and in the combined tree (Fig. 4). These two putative cryptic species, however, did not overlap in either the rostrum morphospace (Fig. 5) or the major chela morphospace (Fig. 6). This result suggests that noticeable morphological differences accompany the substantial genetic differences found between *A. gracilipes* A and B. Finally, the geometric morphometric analyses of *A.* percyi, a closely related sister species which was the outgroup to all other species in the *gracilipes* complex, yielded mixed results and showed that while *A. percyi* formed its own distinct cluster in the rostrum morphospace (Fig 5), it overlapped with *A. gracilipes* A and *A. angustilineatus* in the major chelae morphospace (Fig. 6). Our findings demonstrate the value of using integrative taxonomic techniques to contribute to the understanding of cryptic species within *Alpheus*.

We report new distribution ranges for the *A. gracilipes* species complex. *Alpheus angustilineatus,* initially described from southern Japan, New Caledonia, and Fiji (Nomura & Anker, 2005), as well as in Lombok, Indonesia (Anker *et al.*, 2015), is reported in Guam and Papua New Guinea (Supplementary material Table S1). Similarly, *A. fujitai*, originally described from the Ryukyu Islands, Japan, is recorded in Palau and Singapore (GenBank MN690016; Supplementary material Table S1) as well. *Alpheus roseodigitalis*, originally described from

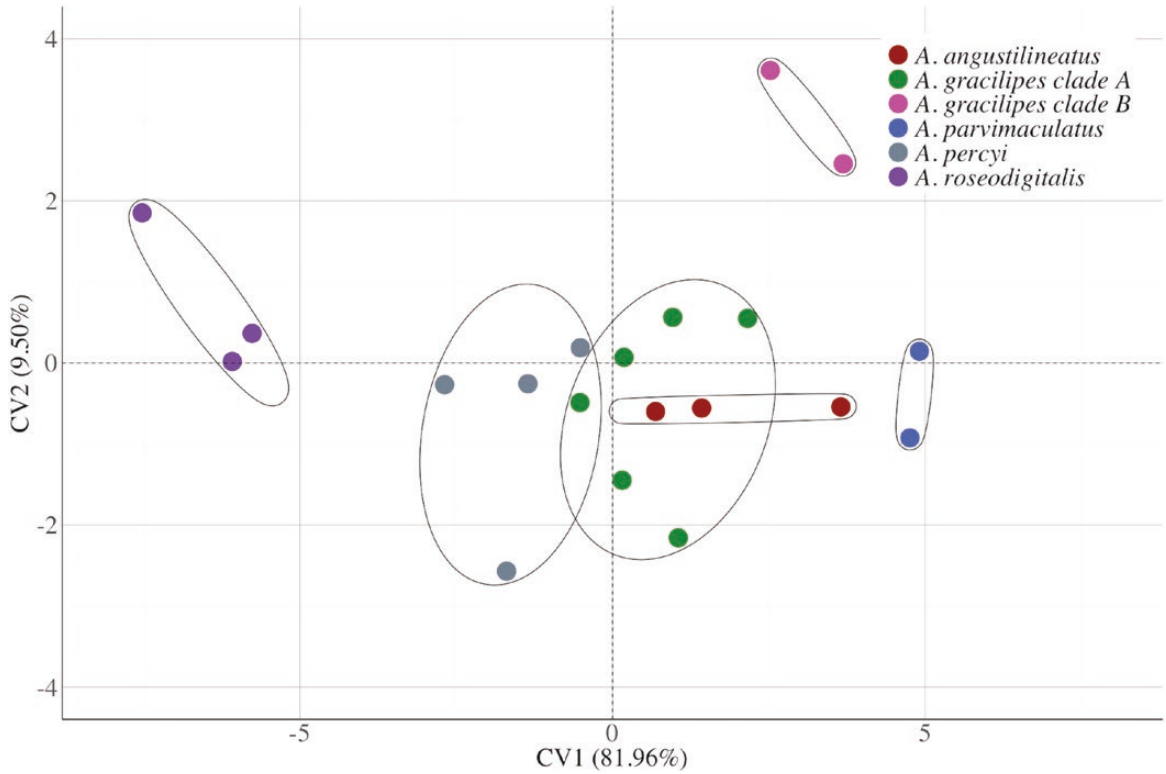

Figure 6. Canonical variate analysis (CVA) plot of major chela shape data of species in the *Alpheus gracilipes* species complex

southern Japan, Taiwan, Madagascar, Chagos Archipelago, Seychelles, Mauritius, and Maldives, is now also reported to occur in the Society Islands (French Polynesia), northern Australia (Queensland), and Réunion (Supplementary material Table S1). *Alpheus soror,* initially described from Sri Lanka (Bruce, 1999), is now also recorded in the Gulf of Mannar, India. *Alpheus gracilipes* A was recovered from the Society Islands, Hawaiian Islands, and New Caledonia, (and likely is distributed widely across the Pacific (Nomura & Anker, 2005)). *Alpheus gracilipes* B was recovered from Papua New Guinea and from the Society Islands and the single specimen of *A. gracilipes* C was from Bali, Indonesia. *Alpheus parvimaculatus,* originally described from southern Japan, northern Australia, and Indonesia (Nomura & Anker, 2005) is also recorded from Papua New Guinea and the Red Sea off Saudi Arabia (Supplementary material Table S1). The only specimen tentatively identified as *A.* cf. *kuroshimensis* (MNHN-IU-2013-925), grouped with *A. parvimaculatus,* suggesting additional examination of museum specimens identified as *A. kuroshimensis* for accurate species diagnoses.

Numerous studies in crustacean taxa have highlighted the efficacy of geometric morphometrics in discriminating cryptic species of prawns and shrimps (i.e., Bissaro *et al.*, 2013; Castelin *et al.*, 2017; Jabłońska *et al.*, 2021; Moraes *et al.*, 2021) as well as in other crustaceans (i.e., Marchiori *et al.*, 2014; Ismail, 2018; Grinang *et al.*, 2019). To our knowledge, however, our investigation represents the first application of geometric morphometrics in *Alpheus.* Our morphometric analyses were constrained to a subset of the *A. gracilipes* species complex due to complete absence or availability of fewer than two specimens for several species: *A. fujitai, A. gracilipes* C, and *A. soror*. The only sequence we had for *A. soror* was acquired from a privately owned aquarium. Additionally, we were unable to include the neotype for *A. gracilipes* (MNHN) in our analyses as type material was not available for loan. Therefore, we were limited in our investigation of the correlation between morphospace and phylogenetic similarity. Nevertheless, we did include two closely related sister species, *A. parvimaculatus* and *A. gracilipes B* (average COI genetic distance = 11.5%), in our geometric morphometric analyses. Although these two species grouped closely together in the rostrum morphospace (Fig 5), they grouped more distantly in the major chela morphospace (Fig. 6).

Our results provide a reproducible framework for employing an integrative taxonomic approach combining molecular genetics and geometric morphometrics to investigate *Alpheus*. Future studies should utilize a larger overall sample size, a more robust sample size per species, and possibly a larger range of morphological characters to facilitate discrimination of which characters best separate different species of *Alpheus*. Although most of the shrimps of this species complex do not exhibit sexual dimorphism, other species in this genus do (Costa-Souza *et al.*, 2019). As such, future investigations of *Alpheus* employing geometric morphometrics should consider sexual dimorphism as a factor by including sex as a variable in the morphometric analyses.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Crustacean Biology* online.

S1 Table. Snapping shrimp specimens used for phylogenetic reconstruction and geometric morphometric analyses.

S2 Figure. Bayesian gene tree based on 16S sequences.

S3 Figure. Bayesian gene tree based on 12S sequences.

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