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Tracing the evolution of bioluminescent light organs across the deep-sea shrimp family Sergestidae using a genomic skimming and phylogenetic approach

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ABSTRACT

Deep-sea shrimp of the family Sergestidae Dana, 1852 provide a unique system for studying the evolution of bioluminescence. Most species within the family possess autogenic bioluminescent photophores in one of three distinct forms: lensed photophores; non-lensed photophores; or internal organs of Pesta. This morphological diversity across the Sergestidae has resulted in recent major taxonomic revisions, dividing the two major genera (*Sergia* Stimpson, 1860 and *Sergestes* Milne Edwards, 1830) into 15. The present study capitalises on molecular data to construct an updated genus-level phylogeny of sergestid shrimp. DNA was successfully extracted from ~87 individuals belonging to 13 of the 15 newly proposed genera. A 'genome skimming' approach was implemented, allowing the capture of mitochondrial genomic data across 19 sergestid species. Additional individuals have been incorporated into the phylogeny through Sanger sequencing of both nuclear (*H3* and *NAK*) and mitochondrial (*16S* and *COI*) genes. The resulting molecular phylogeny is compared with previous morphological trees with specific attention to genus-level relationships. The *-sergestes* group was rendered non-monophyletic and the *-sergia* group was recovered as monophyletic. Ancestral state reconstructions of light organ type indicate that organs of Pesta is the ancestral state for the family. Non-lensed photophores evolved once across the *-sergia* group, but were later lost in the deepest living genus, *Sergia*. Lensed photophores also evolved once within the genera *Prehensilosergia* Vereshchaka, Olesen & Lunina, 2014, *Lucensosergia* Vereshchaka, Olesen & Lunina, 2014 and *Challengerosergia* Vereshchaka, Olesen & Lunina, 2014. These findings identify preliminary patterns across light organ type and species' depth distributions; however, future research that incorporates finer-scale depth data and more species is needed to confirm our findings.

Keywords: bioluminescence, Crustacea, deep sea, genome skimming, organs of Pesta, photophores, phylogenetics, Sergestidae, shrimps.

Introduction

Bioluminescence, or the biological production of light, plays a crucial ecological role throughout the world's oceans. This is evident in the high diversity and abundance of bioluminescent organisms within mesopelagic systems (Herring 1987; Haddock *et al.* 2010; Widder 2010). A recent study that quantified the prevalence of bioluminescence in pelagic marine systems across taxa found upwards of 54% in fish, 53% in cephalopods, 67% in crustaceans and over 90% in polychaetes, cnidarians, ctenophores and appendicularians (Martini and Haddock 2017). Initial estimates suggested that bioluminescence has evolved independently at least 40 times across the Metazoa (Haddock *et al.* 2010), and more recent findings have demonstrated at least 27 independent evolutionary events in ray-finned fish alone (Davis *et al.* 2016). This diversity of bioluminescent taxa has also led to a high diversity in bioluminescent form and function. The most understood functions of bioluminescence include counterillumination (Young *et al.* 1980;

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Fig. 1. The morphological diversity within Sergestidae: (*a*) *Robustosergia robusta* (Smith, 1882), (*b*) *Sergia tenuiremis* (Krøyer, 1855), (*c*) *Challengerosergia talismani* (Barnard, 1947), (*d*) *Challengerosergia hansjacobi* (Vereshchaka, 1994), (e) *Phorcosergia grandis* (Sund, 1920), (*f*) *Gardinerosergia splendens* (Sund, 1920), (*g*) *Parasergestes armatus* (Krøyer, 1855), (*h*) *Neosergestes edwardsii* (Krøyer, 1855), (*i*) *Deosergestes corniculum* (Krøyer, 1855), (*j*) *Deosergestes henseni* (Ortmann. 1893), (*k*) *Allosergestes pectinatus* (Sund, 1920) and (*l*) *Allosergestes sargassi* (Ortmann, 1893). Specimen photographs were provided by Dr Danté Fenolio.

Latz *et al.* 1988; McFall-Ngai 1990; Davis *et al.* 2020), hunting, antipredation (Porter and Porter 1979; Abrahams and Townsend 1993; Speiser *et al.* 2013; Delroisse *et al.* 2017) and intraspecific communication (Haddock *et al.* 2010; Rivers and Morin 2013). In animals, bioluminescence can be formed bacteriogenically (in which the light is produced by symbiotic bacteria), autogenically (in which the animal has the molecular machinery to produce the light itself by endogenous luciferases) or through the use of a kleptoprotein system (in which light-producing proteins are acquired from prey organisms for use by their predators) (Haddock *et al.* 2010; Widder 2010; Bessho-Uehara *et al.* 2020*a*, 2020*b*). Although bioluminescence has been studied widely across a diverse group of taxa, there are limited studies on the evolution of light organ types within major lineages.

The Sergestidae Dana, 1852 (Fig. 1), a family of deep-water shrimp that recently underwent a redefinition and major taxonomic revision (Vereshchaka 2017), provide a unique opportunity to study the evolution of light organs owing to the structural diversity present across the 15 genera and 71 species (Vereshchaka *et al.* 2014; Vereshchaka 2017). Whereas two genera lack bioluminescent organs entirely (*Sergia* Stimpson, 1860 and some species of

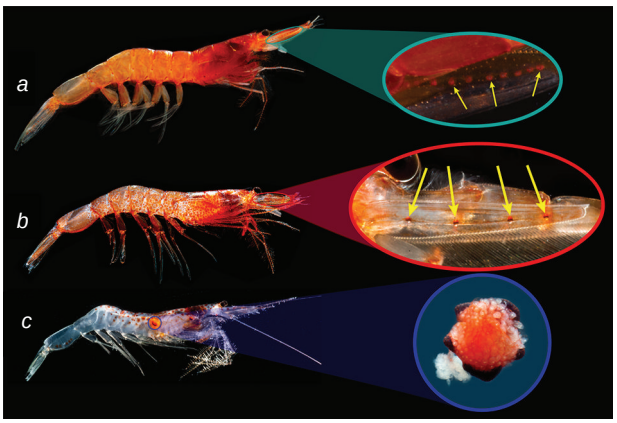

Fig. 2. The morphological diversity of light organs within the Sergestidae: (*a*) dermal non-lensed photophores exhibited in many *-sergia* group species; (*b*) dermal lensed photophores exhibited in many *-sergia* group species; and (*c*) a dissection of the posterior lobe of the hepatic photophore, organs of Pesta, found in every *-sergestes* group species. The exact arrangement and morphology of each of these light organ types varies by species and can be used to help differentiate the species of the family. Specimen and photophore photographs were provided by Dr Danté Fenolio.

Phorcosergia Vereshchaka, Olesen & Lunina, 2014), all others possess one of three distinct forms of autogenic light organs: (1) dermal lensed photophores; (2) dermal non-lensed photophores; or (3) hepatic photophores called organs of Pesta (OOP), which are modified hepatopancreas glands located internally under the carapace of the organism (Fig. 2). The ventral and lateral orientation of these distinct photophore types, and the wavelengths of light they produce, suggests a primary role in counterillumination (Latz and Case 1982; Latz 1995; Vereshchaka 2009), and recent studies have shown evidence for light organ photosensitivity (Bracken-Grissom *et al.* 2020; DeLeo and Bracken-Grissom 2020).

Within Sergestidae there are two main groups, previously classified as the two genera, *Sergestes* Milne Edwards, 1830 and *Sergia*, henceforth called the *-sergestes* group and *-sergia* group. Several revisions have since divided these into 15 genera (Judkins and Kensley 2008; Vereshchaka *et al.* 2014; Vereshchaka 2017), but the two major groups (*-sergia* and *-sergestes*) can be identified by the type of photophore they possess (either lensed, non-lensed or absent within the *-sergia* group of species, or with the OOP within the *-sergestes* group of species). For example, within the *-sergia* group, 16 species exhibit dermal non-lensed photophores (Fig. 2*a*), 14 species exhibit dermal lensed photophores (Fig. 2*b*) and 6 species lack bioluminescent organs completely (Vereshchaka 2000; Vereshchaka *et al.* 2014; A. L. Vereshchaka pers. comm.). Photophores can be found in species-specific patterns across the shrimp's body, usually on the antennal scales, the exopodite and endopodite of the uropod, pereiopods, mouthparts and the ventral and lateral

surfaces of the eyes, cephalothorax and abdomen (Vereshchaka 2000; Nowel *et al.* 2002). In species bearing the dermal lensed photophores, there exists a thick cuticular lens covering each photophore, having the appearance of small bubbles on the surface of the exoskeleton (Fig. 2*b*). The *-sergestes* group lacks dermal photophores entirely, but ubiquitously possesses the hepatic OOP. Found internally beneath the carapace of the animal, the OOP contains lobes of luminescent tissue (Fig. 2*c*). These lobes are orientated ventrally and are always found anteriorly and posteriorly on the organ, but there can sometimes be medial lobes depending on the species. These organs also exhibit species- or genus-specific patterns (i.e. bi-lobed, tri-lobed, fringed; Foxton 1972; Vereshchaka 2009; Vereshchaka *et al.* 2014) that can aid in identification.

Sergestid light organs are known to play a role in counterillumination, a form of deep-sea camouflage (Warner *et al.* 1979; Latz 1995). During counterillumination, an organism will emit light to match the wavelength and intensity of downwelling sunlight, which serves to 'hide' their silhouette from predators below (Clark 1963). Counterillumination is common in taxa that participate in diel vertical migration (DVM), the largest synchronised migration of biomass on the planet (Lampert 1989). DVM is a predator-avoidance behaviour in which animals migrate up to hundreds of metres into shallow waters at night to feed and retreat to deeper waters during the day for safety, constituting a major mechanism for the biological carbon pump (Hernández-León *et al.* 2019). Most sergestids exhibit DVM behaviour (Vereshchaka 2000, 2009); however, migration strength (the extent of vertical movement) can vary greatly. Some species migrate upwards of 1000 m across diverse light environments (i.e. from bathypelagic to epipelagic), whereas others migrate shorter distances (i.e. within the mesopelagic). Although sergestid depth distribution varies by species, previous studies have identified that the *-sergestes* species are more translucent and inhabit shallower depths in the water column than the *-sergia* group (Flock and Hopkins 1992; Vereshchaka 2000, 2009; Vereshchaka *et al.* 2014; A. L. Vereshchaka, pers. comm.). It is plausible that light organs have diversified across light environment, with larger organs (OOP) needed for counterillumination in shallower waters and no light organs needed in species that live in perpetual darkness. It is equally plausible that light organ type diversified as a result of drift or to maximise camouflage (accounting for depth and translucence) during DVM. To date, the drivers behind light organ diversity remain unclear; however, reconstructing the evolution of light organs across a phylogeny and comparing against a depth gradient may allow for a better understanding of photophore diversification.

Recent morphology-based taxonomic revisions of sergestid shrimp have led to the creation of entire new families and the expansion of the Sergestidae from two genera to 15 (Judkins and Kensley 2008; Vereshchaka 2009, 2017; Vereshchaka *et al.* 2014). However, no molecular-based phylogenies have been constructed to corroborate this reclassification. A molecular phylogeny will also allow for the investigation of light organ diversification. As such, the objective of this study is two-fold: to build the first molecular phylogeny of Sergestidae with an emphasis on genus-level relationships; and to investigate the evolution of bioluminescent light organs across an oceanic depth gradient.

Materials and methods

Taxon sampling

The majority of specimens used in this study were collected over the course of ten research expeditions into the Gulf of Mexico (GOM) and Florida Straits (FSs), with a combined total of 134 days at sea. Six of the ten expeditions occurred aboard the R/V *Point Sur* in the northern GOM as part of the Deep Pelagic Nekton Dynamics of the Gulf of Mexico (DEEPEND) consortium (http://www.deependconsortium.org). Two expeditions, funded by the National Science Foundation and henceforth abbreviated as 'NSF expeditions', occurred in the north-east Florida Straits between Miami and the island of Bimini aboard the R/V Walton Smith to study bioluminescence and vision in the deep sea. The remaining two expeditions occurred in the south-west Florida Straits, south and south-west of Key West, Florida as part of an Oceanography at Sea course for undergraduate students at Florida International University (FIU) aboard the R/V *W. T. Hogarth* and R/V *Weatherbird II* (henceforth abbreviated as 'OCB4005C expeditions' after the course number at FIU).

During the DEEPEND expeditions, sampling occurred twice-daily at each sampling site: once at noon and once at midnight, each sampling 0–1500 m depths. The expeditions occurred twice-annually in 2015 and 2016 (once in May (regional dry season) and once in August (regional wet season)) and once per year in 2017 and 2018. The DEEPEND expeditions employed a Multiple Opening/Closing Net and Environmental Sensing System rigged with six 3 mm mesh trawling nets. The rig allowed for the opening and closing of each net at discrete depth ranges, further allowing for collected samples to be binned by their depth range of collection at (0–200, 200–600, 600–1000, 1000–1200, 1200–1500 m, with the sixth net sampling the entire water column from 0 to 1500 m). For more details on DEEPEND sampling methods, see Cook *et al.* (2020).

The NSF expeditions occurred in July 2016 and 2017. Sampling was done continuously at each sampling site. Sampling depths were dependent on sea floor depth and time of day, with deeper trawls occurring during the day and shallower trawls conducted at night in anticipation of DVM events, but usually no deeper than 800–900 m at night. Specimens were collected using a 9-m^2 Tucker trawl fitted with a remote-closing, temperature-insulated cod-end

(see Frank and Widder 1999 for details). This equipment allowed for semi-discrete depth sampling and maintained *in situ* temperatures within the cod-end to increase the preservation of specimens.

During the OCB4005C expeditions, occurring in May 2017 and 2019, sampling methods followed similar procedures to those during the NSF expeditions, with continuous sampling at site depths dependent on sea floor topology and time of day. The 2017 expedition employed a 1-m^2 Tucker trawl with a standard cod-end. The 2019 expedition employed a 1-m^2 Tucker trawl capable of equipping up to three individually remote-opening and -closing nets. During the expedition, however, only two nets were equipped.

After trawl retrieval, specimens from each net were sorted into large trays and identified to the lowest taxonomic group as determined by morphology and dichotomous keys during each expedition. After identification, samples were catalogued and preserved in either 70 or 80% ethyl alcohol and immediately stored at −20°C onboard the vessel, or in an RNA-stabilising buffer (RNAlater brewed in the CRUSTOMICS laboratory at FIU) and left at room temperature for the duration of the cruise to allow the buffer to properly penetrate the specimens' tissues. Samples were transported from the vessel to the CRUSTOMICS laboratory on ice, where they were stored at −80°C until they were processed. Processing involved: (1) identification confirmations by Dr Heather Bracken-Grissom; (2) plucking muscle tissue from each specimen; (3) the assignment of individual voucher numbers to each specimen and corresponding tissue sample; (4) the cataloguing of each specimen with its associated voucher number and collection metadata into the Florida International Crustacean Collection (FICC) database; and finally (5) whole specimen preservation in 80% ethyl alcohol for storage in the FICC Museum for future molecular and morphological studies. Tissue plucking involved the gentle lifting of the third or fourth integument of the abdomen to expose the muscle tissue, which could be removed without disturbing the overall morphology of the specimen. The voucher number system helped to ensure each specimen remained reliably associated with its corresponding tissue sample and all downstream DNA extractions and analyses. Tissue samples were stored in 80% ethyl alcohol at −80°C.

For species not collected during these expeditions, tissue samples from species of the genera *Deosergestes* Judkins & Kensley, 2008, *Gardinerosergia* Vereshchaka, Olesen & Lunina, 2014, *Prehensilosergia* Vereshchaka, Olesen & Lunina, 2014 and *Lucensosergia* Vereshchaka, Olesen & Lunina, 2014, collected off Taiwan, Japan and Mozambique were donated from the National Taiwan Ocean University (NTOU) and Muséum national d'Histoire naturelle, Paris (MNHN). Two tissue samples of *Challengerosergia talismani* (Barnard, 1947) collected from Keyhole Point, Hawaii were donated from the University of Hawaii, and multiple specimens of *Sergia tenuiremis* (Krøyer, 1855) and *Phorcosergia grandis* (Sund, 1920) collected from the Canary Islands were donated from the University of Las Palmas de Gran Canaria. All three species are common in the GOM and were included alongside our own GOM samples. Tissue samples for two outgroup species, *Sicyonella liui* T.-Y. Chan, 2020 and *Acetes japonicus* Kishinouye, 1905, from within the same superfamily, Sergestoidea Dana, 1852, were also donated from the NTOU and MNHN, and were used to root our phylogenetic analyses.

Molecular analysis

DNA extractions

Genomic DNA (gDNA) was extracted from abdominal muscle tissue when available, or, in the case of several donated samples, from pleopod muscle tissue. For DNA extractions, the Qiagen DNeasy Blood and Tissue Kit (Catalogue number 69504) was used. In certain cases where tissue was limited or DNA degradation was suspected, DNA was instead extracted with a phenol chloroform protocol (modified from Sambrook and Russell 2006). DNA extraction quality was assessed using 1% gel electrophoresis. DNA extraction quantity was assessed using a dsDNA High Sensitivity Assay kit with a Qubit Fluorometer (ver. 2.0, Invitrogen, Life Technologies, CA, USA) following manufacturers protocols. DNA extractions were preserved at −20°C for downstream molecular work.

PCR and Sanger sequencing

Four partial genes were selected for phylogenetic analysis based on their reliability in resolving taxonomic relationships: (1) the nuclear protein-coding histone 3 (*H3*) (Colgan *et al.* 1998) of 328 base pairs (bp); (2) the nuclear protein-coding sodium–potassium ATPase α subunit (*NaK*) (Chu *et al.* 2009; Ma *et al.* 2009, Chakraborty *et al.* 2015) of 540 to 750 bp; (3) the mitochondrial protein-coding gene, cytochrome *c* oxidase subunit I (*COI*) of 721 to 798 bp (Folmer *et al.* 1994; Varela *et al.* 2021); and (4) the mitochondrial large ribosomal subunit (*16S*) of 363 to 527 bp (Crandall and Fitzpatrick 1996; Palumbi *et al.* 2002; Schubart *et al.* 2002; Yang *et al.* 2012). These genes were amplified by PCR using Promega's GoTaq Green Master Mix Protocol (Promega M7122). PCR amplifications were performed using a thermal cycler (Pro-Flex PCR system). Gene fragments were amplified using the following thermal profiles: initial denaturing for 2–5 min at 94°C; annealing for 35 cycles: 30–45 s at 95°C, 30 s at 40–69°C (depending on the taxon and primers used, see Table 1), 1 min at 72°C; and final extension 3 min at 72°C, with appropriate primers for each taxon and target gene region. Amplification success for all PCRs was verified using 2% gel electrophoresis. PCR products were sequenced through GENEWIZ Sanger Sequencing services (Genewiz, Boston, MA, USA) to produce whole-amplicon forward and reverse strand reads.

Table 1. Primer sequences used in this study including forward and reverse sequences for PCR amplification of cytochrome *c* oxidase subunit I (*COI*), mitochondrial large ribosomal subunit (*16S*), nuclear protein-coding sodium–potassium ATPase α subunit (*NaK*) and nuclear protein-coding histone 3 (*H3*) target regions, as well as the annealing temperature for each primer pair.

Target gene	Forward primer	Reverse primer	Annealing temperature
16S	16S_L2/L9	16S_1472	45°C
	5′-TGCCTGTTTATCAAAAACAT-3′	5′-AGATAGAAACCAACCTGG-3′	
	5′-CGCCTGTTTATCAAAAACAT-3′	(Crandall and Fitzpatrick 1996)	
	(Schubart *et al.* 2002; Palumbi *et al.* 2002)		
	16S_Serg_F2	16S_Serg_R1	68°C
	5′-GACCGTGCGAAGGTAGCATAATC-3′	5′-CCTGGCTYACGCCGGTCTGAACTC-3′	
	(This study)	(This study)	
COI	COI_LCO1490	COI_HCO2198	40°C
	5′-GGTCAACAAATCATAAAGATATTG-3′	5′-TAAACTTCAGGGTGACCAAAAAATCA-3′	
	(Folmer *et al.* 1994)	(Folmer *et al.* 1994)	
	COI_Crusty_F	COI_Crusty_R	45°C
	5′-YTCHWSDAAYCAYAARGAYATTGG-3′	5′-TANACYTCNGGRTGNCCRAARAAYCA-3′	
	(Varela *et al.* 2021)	(Varela *et al.* 2021)	
NaK	Nak_F	Nak_R	50°C
	5′-GTGTTCCTCATTGGTATCATTGT-3′	5′-CGGGYCTCCATGCTSAGCCARTG-3′	
	(Chakraborty *et al.* 2015)	(Chakraborty *et al.* 2015)	
H3	H3_AF	H3_AR	50°C
	5′-ATGGCTCGTACCAAGCAGACVGC-3′	5′-ATATCCTTRGGCATRATRGTGAC	
	(Colgan *et al.* 1998)	(Colgan *et al.* 1998)	

New *16S* primers were designed for this study (Serg_F, Serg_R) owing to difficulties in PCR amplification with current universal primers (Table 1). These primers were designed using a combination of sergestid *16S* sequences already acquired through Sanger sequencing, mitochondrial genome sequencing (see 'Genome skimming' below) and available sequences published on the GenBank nucleotide database (National Center for Biotechnology Information, see www.ncbi.nlm.nih.gov/genbank/). These sequences were aligned in Geneious Prime (ver. 2020.0.3, see http://www.geneious.com/) using the MAFFT alignment tool. Conserved 5′ and 3′ regions of the alignment rich in G/C nucleotides were selected for forward and reverse primers, respectively. Melting temperatures of the primers were calculated using Oligo Calculator (ver. 3.27, see http://biotools.nubic.northwestern.edu/OligoCalc.html; Kibbe 2007). Primers were manufactured through Integrated DNA Technologies (Owczarzy *et al.* 2008).

Genome skimming

A genomic skimming approach was used to acquire more genes for phylogenetic analysis of the Sergestidae and played an important role in Sanger sequencing primer design as previously mentioned. Genomic skimming is a method of sampling large portions of a specimen's entire genome. In short, it involves the fragmentation of gDNA, the sequencing of those fragments, and the reassembly and annotation of those sequences into useable genes or genome loci for various analyses (Dodsworth 2015). In this study, gDNA (55 µL, at ~200 ng of total mass) was sonicated on a Covaris ultrasonicator (LE220) at the University of Miami's Center for Genome Technology to create a peak fragment size of 200 bp (treatment time, 300 s; peak power, 450 W; duty factor, 30; cycles and bursts, 200). Following fragmentation, a tapestation was used to determine concentration, peak fragment size and molarity. DNA libraries were then made from size selected gDNA fragments (insert length of 200 bp) using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs, E7645/E7103). Libraries were assessed for quality on an Agilent Bioanalyzer before being pooled and sequenced on an Illumina HiSeq 3000/4000 to acquire 150 bp paired-end reads (GENEWIZ NextGen Sequencing service; South Plainfield, NJ, USA).

Mitochondrial genomes (mtDNA) were assembled from the raw DNAseq reads on FIU's high-performance cluster using NOVOplasty: Organelle Assembler (Dierckxsens *et al.* 2017) using the following settings: insert size, 200; insert

size auto, yes; read length, 150; type, mito; genome range, 12 000–20 000; k-mer, 39; insert range, 1.6; insert range strict, 1.2; single or paired, PE. *16S* and *COI* seed sequences were selected for the assembly from GenBank's nucleotide database, based on relatedness to each specimen. See Supplementary Table S1 for seed sequences used in this study. Assembled mtDNA was annotated with MITOS: Web Server (Bernt *et al.* 2013) using default settings and invertebrate genetic code to return protein-coding, rRNA, and tRNA gene sequences. Using this method, 12 whole mitochondrial genes were selected for use in this study based on their phylogenetic information, as determined by single-gene tree screening. These 12 genes included the large and small ribosomal subunits (*16S* and *12S*) and 10 protein coding genes: two adenosine triphosphate (ATP)-synthase enzyme subunits, *ATP6* and *ATP8*; four subunits of the ubiquinol–cytochrome *c* reductase complex, *COB*, *COI*, *COII* and *COIII*; and four subunits of the nicotinamide adenine dinucleotide + hydrogen (NADH) dehygrogenase complex, *NAD1*, *NAD2*, *NAD4* and *NAD5*.

Phylogenetic analysis

Forward and reverse strands from Sanger sequencing were assembled with the program Geneious Prime, using the *de novo* assembly function at the 'highest sensitivity/slow' setting and trimmed manually. MtDNA and Sanger sequences were screened for indels and stop codons to identify and exclude pseudogenes. They were then compared with reported sequences in GenBank using the Basic Local Alignment Search Tool (BLAST) to identify and remove any contaminated sequences. Finally, they were further screened for otherwise anomalous sequences by aligning each gene set using the MAFFT alignment tool in Geneious Prime followed by single-gene tree estimations. IQ-TREE (ver. 2.0.4, see http://www.iqtree.org/; Nguyen *et al.* 2015) was used with the edge-unlinked branch lengths to determine models of evolution and to build maximum likelihood (ML) single-gene trees for each gene alignment using an ultrafast bootstrapping approximation (UFBoot, ver. 2, in IQ-TREE; Hoang *et al.* 2018) with 1000 replicates. The resulting single-gene trees are available as supplementary materials (Supplementary Fig. S1–S13).

The single-gene alignments were then concatenated using Geneious Prime into a single dataset and a ML or Bayesian (BAY) phylogenetic tree reconstruction was done using IQ-TREE or MrBayes (ver. 3.2, see https://nbisweden.github.io/MrBayes/index.html; Ronquist *et al.* 2012) respectively. ModelFinder (see http://www.iqtree.org/ModelFinder/; Kalyaanamoorthy *et al.* 2017) was used to determine partitioning and models of evolution. Branch support for the ML analysis was assessed in triplicate using IQ-TREE's UFBoot, Shimodaira–Hasegawa-like approximation ratio likelihood test (Guindon *et al.* 2010) and approximate Bayesian analysis (aBayes) (Anisimova *et al.* 2011) with 10 000 replicates each. Branch support values are presented on the final phylogeny. The BAY analysis was conducted using MrBayes with two independent runs using three heated and one cold Metropolis-coupled Markov chain Monte Carlo chains for 10 000 000 generations. Chains were sampled every 1000 generations, and convergence of the analysis was validated by the standard deviation of split frequencies achieving <0.01. Posterior probability support values were added to the final ML topology.

Character evolution

Ancestral State Reconstruction (ASR) was run with MESQUITE (ver. 3.6, see www.mesquiteproject.org) using a pruned version of the final tree to trace light organ structure across the Sergestidae. Light organ structure can be divided into four major categories and each species in our tree was assigned one of four character states (Supplementary Table S2) including: no light organ (0); OOP (1); non-lensed photophores (2); or lensed photophores (3). The analysis was executed using both maximum parsimony (MP) and ML methods owing to debate between the efficacy of each (Cunningham *et al.* 1998; Royer-Carenzi *et al.* 2013). Although MP reconstructs character evolution using the fewest possible character changes over time, ML reconstructions make use of possible rates of character evolution, propagation of character states across terminal taxa and branch lengths to account for all possible character states at each node (Pagel 1997; Cunningham *et al.* 1998). MP has been criticised for underestimating rates of evolutionary change because it lacks consideration for branch lengths (Fitch and Bruschi 1987; Fitch and Beintema 1990; Huelsenbeck and Lander 2003; Crisp and Cook 2005). Regardless, both of our reconstructions were largely in agreement with one another.

Alongside the ASR tree, we mapped daytime (light blue) and nighttime (dark blue) depth distributions for each species to investigate if these distributions correlated with major groups. Those ranges were compiled and summarised from past literature (Vereshchaka 2000, 2009) for all species in the *-sergia* and *-sergestes* group based on data collected during three global research expeditions in the early- to mid-1900s. In the latter report, several species were noted as being reported at depths possibly unreliable due to equipment malfunctions, so other studies summarised and cited within Vereshchaka's report were considered alongside his own (Foxton 1972; Donaldson 1975; Walters 1976; Flock and Hopkins 1992; Cartes *et al.* 1994) (Supplementary Table S3).

Results

Phylogenetic analysis

In total, 87 individuals across 24 species were used to construct this phylogeny, spanning 13 of the 15 genera of

Sergestidae and two genera within the recently described families, Acetidae and Sicyonellidae (Vereshchaka 2017). From the Sergestidae, representatives of the genera *Cornutosergestes* Vereshchaka, Olesen & Lunina, 2014 and *Scintillosergia* Vereshchaka, Olesen & Lunina, 2014 (among other species across genera) were unavailable owing to the lack of specimens or molecular grade material. In total, 263 partial gene regions were sequenced using Sanger sequencing methods, including 55 *16S* sequences, 73 *COI* sequences, 85 *H3* sequences and 50 *NaK* sequences. The genomic skimming approach resulted in 22 complete mitochondrial genomes across 18 species and one mostly complete mitochondrial genome of *Prehensilosergia prehensilis* (Spence Bate, 1881) within the Sergestidae. From these mitochondrial genomes, two rRNA genes (*16S* and *12S*) and 10 protein coding genes (*ATP6*, *ATP8*, *COB*, *COX1*, *COX2*, *COX3*, *NAD1*, *NAD2*, *NAD4* and *NAD5*) were included in the final dataset. Other regions were discarded because they contained no phylogenetic signal (resulting in large polytomies) as determined by single-gene constructions. The final concatenated dataset (Supplementary Table S4) includes 437 novel sequences and 13 294 bp. All gene sequences used in this dataset are available in GenBank.

Single-gene tree analyses were run for each gene alignment to examine congruence in topology (Supplementary Fig. S1–S13). Across the concatenated phylogenetic analyses (ML and BAY), the topology was identical and similar support was recovered. The ML topology was used for the final tree and support values were included on branches where statistical support was recovered (Fig. 3).

The final phylogeny included 13 genera, 21 species and 85 individuals across the Sergestidae (Fig. 3). The *-sergestes* group, which comprises six genera, was recovered as non-monophyletic and the *-sergia* group, comprising seven genera, was recovered as monophyletic. Within the *-sergestes* group, the genus *Deosergestes* Judkins & Kensley, 2008 was recovered as non-monophyletic (albeit very low support) with *D. coalitus* (Burkenroad, 1940) and *D. henseni* (Ortmann, 1893) falling out separately from a clade containing *D. corniculum* (Krøyer 1855) and *D. paraseminudus* (Crosnier & Forest, 1973). The genus *Allosergestes* Judkins & Kensley, 2008 is recovered with very high support (100/100/1/1) and cryptic diversity is found within the species *A. pectinatus* (Sund, 1920) from the Florida Straits. A monophyletic clade that contains the genera *Sergestes*, *Neosergestes* Judkins & Kensley, 2008 and *Parasergestes* Judkins & Kensley, 2008, is strongly supported (100/100/1/1) with *Neosergestes* forming a sister relationship with *Parasergestes*. *Eusergestes arcticus* (Krøyer, 1855) is the sister species to a monophyletic *-sergia* group. Within the *-sergia* group, *Gardinerosergia* is the earliest branching genus and forms a monophyletic group including *G. splendens* (Sund, 1920) and *G. inequalis* (Burkenroad, 1940) (100/100/1/1). Surprisingly, *Robustosergia* Vereshchaka, Olesen & Lunina, 2014, including *R. regalis* (Gordon, 1939) and *R. robusta* (Smith, 1882), is recovered as non-monophyletic. A strongly supported clade that includes *Prehensilosergia, Lucensosergia* and *Challengerosergia* Vereshchaka, Olesen & Lunina, 2014 (97/99/1/1) falls sister to a clade composed of *Sergia* and *Phorcosergia* (100/100/1/1). Population structure can be found within *P. grandis* with a break between the eastern and western Atlantic. All single and concatenated alignments and their resulting tree files can be found at https://doi.org/10.5061/dryad.vdncjsxvk.

Evolution of light organs

Light organ structure was traced over the final tree and divided into four characters states: lacking light organs (0); exhibiting OOP (1); exhibiting non-lensed photophores (2); or exhibiting lensed photophores (3). ML and parsimony analyses were executed, but because we did not find any notable differences in the results, here we only show the ML results (Fig. 4). The reconstructions from both analyses support that the most recent common ancestor of all sergestids was OOP-bearing. All members of the *-sergestes* group retained an OOP until the divergence of the *-sergia* group. The non-lensed photophore evolved once during the emergence of the *-sergia* group and was recovered as the ancestral state for the clade. Non-lensed photophores were lost completely within the species *Sergia tenuiremis* (lacking light organs completely) and lensed photophores evolved once within a group that includes *Prehensilosergia*, *Lucensosergia* and *Challengerosergia*.

Discussion

The Sergestidae has recently undergone substantial taxonomic revisions based on morphological analyses (Judkins and Kensley 2008; Vereshchaka 2009, 2017; Vereshchaka *et al.* 2014). To examine genus-level relationships and compare against previous morphological trees, we constructed the first molecular phylogeny for this family, using both genomic skimming and phylogenetic methods. Using this molecular phylogeny, bioluminescent light organs were traced across the tree to investigate the evolution of the different morphologies (OOP, non-lensed photophore, lensed photophore, absence of photophore). Further, by compiling day and night depth distribution data for all species of sergestids, we compared light organ type with depth distributions to identify preliminary patterns of organ diversification and light environment.

Evolutionary relationships within the Sergestidae

Our tree included 85 sergestids, 21 of the 71 species, and 13 of the 15 recognised genera within Sergestidae, and represents the first molecular phylogeny for the group (Fig. 3). Our first goal was to investigate the evolutionary relatedness

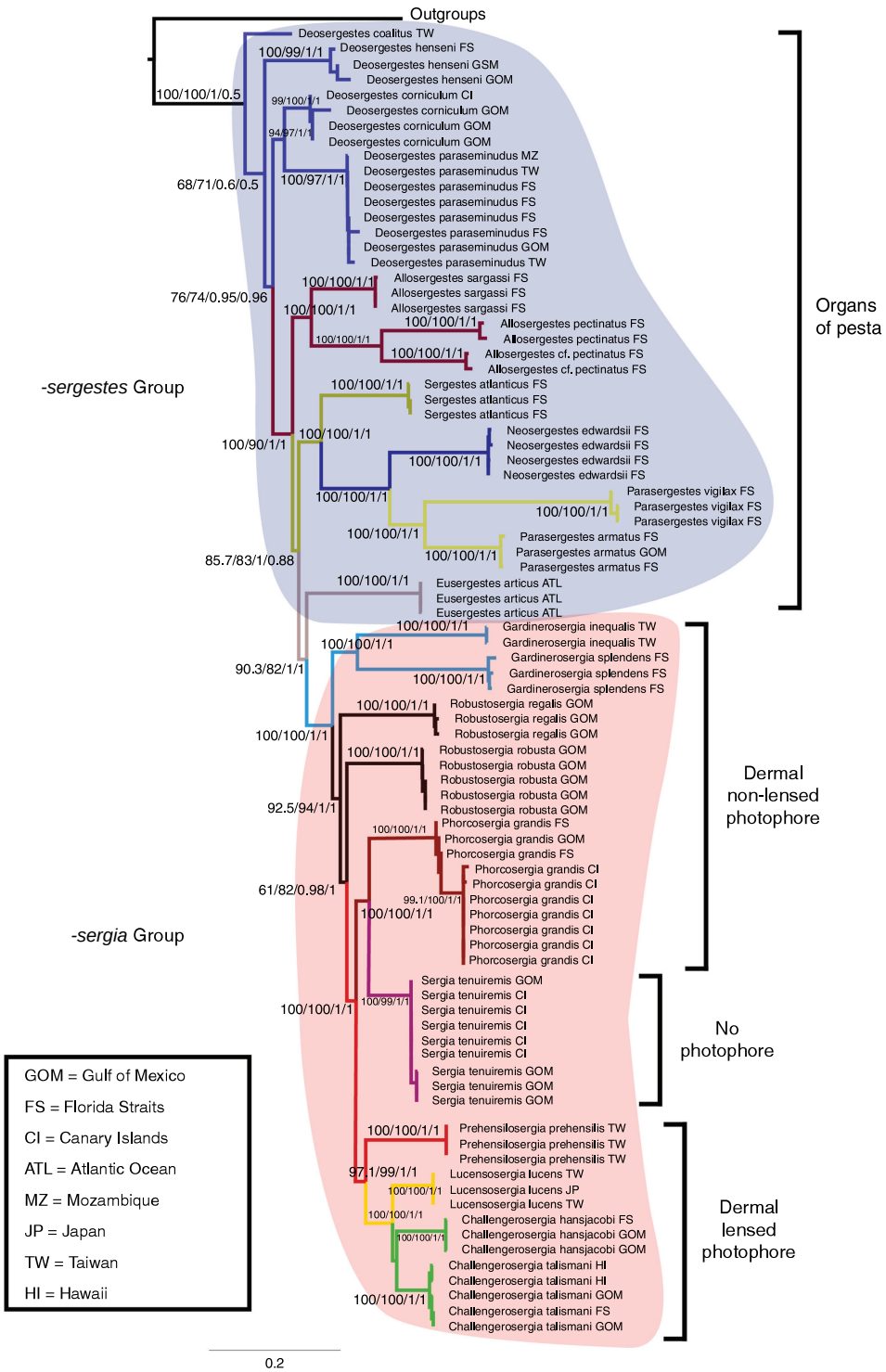

Fig. 3. A maximum likelihood phylogeny including 24 species and 87 individuals across Sergestidae. The two major groups have been identified in the tree (*-sergestes*, *-sergia*) and the branches of the phylogeny colour-coded by genus. Each individual has been tagged with collection locality to help identify possible population structure. Shimodaira–Hasegawa-like approximation ratio likelihood test (SH-aLRT), ultrafast bootstrap (UFBoot), approximate Bayesian analysis (aBayes) and posterior probability (Bayesian) support values are presented along branches in this order. Values >95, >80, >0.95, >0.95, are considered strongly supported.

across genera. In comparison with the most recent morphological analyses of the family (Vereshchaka *et al.* 2014; Vereshchaka 2017), we found a lot of congruence with new taxonomic revisions but with some differences in deep splits. The two major groups within the family, *-sergestes* and *-sergia*, (differentiated primarily by the type of bioluminescent organs, OOP vs dermal photophore, respectively), do not form reciprocally monophyletic groups in accordance with the most recent morphological phylogeny (Vereshchaka 2017). We found the *-sergestes* group to be non-monophyletic and *-sergia* group to be monophyletic, with the *-sergestes* group branching early in the evolution of the family, similar to a recent hypothesis of evolutionary relatedness (Vereshchaka 2017).

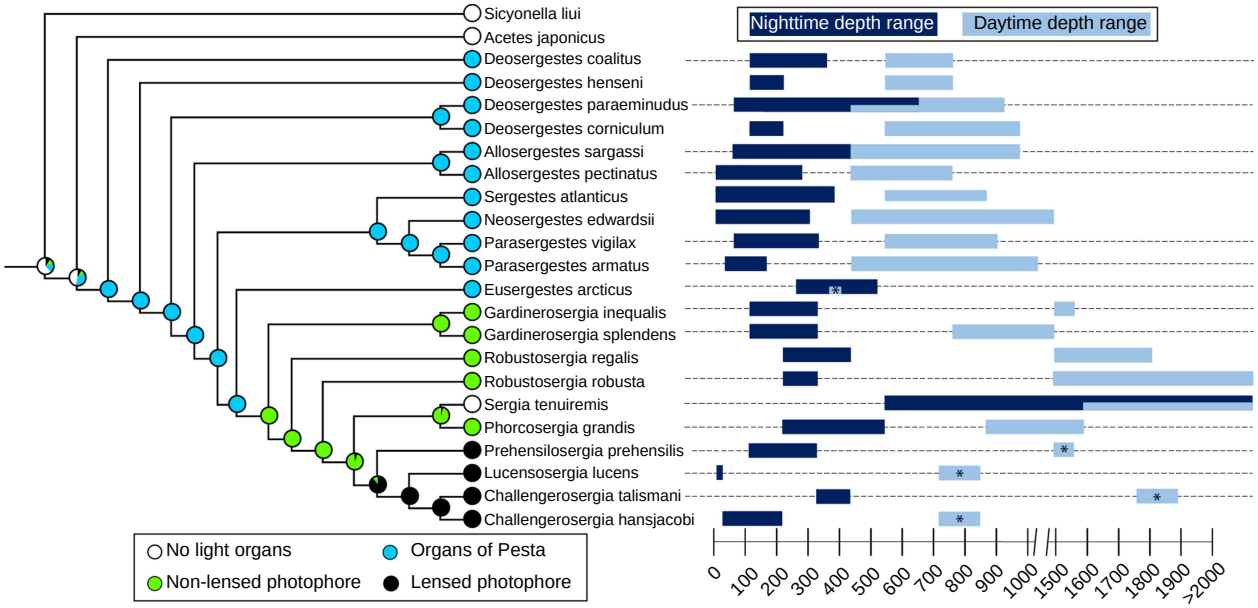

Fig. 4. An ancestral state reconstruction (ASR) of the Sergestidae family with light organ type traced over a pruned version of the maximum likelihood topology. Blue nodes represent hepatic organs of Pesta (OOP)-bearing species, green nodes represent dermal non-lensed photophore-bearing species, black nodes represent dermal lensed photophore-bearing species and white nodes represent species that completely lack light organs. Ancestral nodes states are predicted with the proportion of the node coloured to represent the likelihood of each state. To the right of the ASR is a bar graph representing the daytime and nighttime depth distributions (in metres) for each species, as summarised in Vereshchaka (2000, 2009). Light blue bars represent daytime depths and dark blue bars represent nighttime depths. Species denoted by an asterisk (*) are known to be benthopelagic during the daytime, whereas all others are more commonly mesopelagic.

Within the *-sergestes* group, we found nearly ubiquitous agreement with previous findings concerning the new genus reclassifications (Vereshchaka *et al.* 2014; Vereshchaka 2017). It must be acknowledged that several species are missing in our current tree, but in the cases where multiple species of a single genus are included, a monophyletic clade was recovered with the exception of *Deosergestes*. Within *Deosergestes*, non-monophyly is likely due to the low support at the deep branches, and with more informative genes, a monophyletic *Deosergestes* is likely. At the inter-genus level, we found both similarities and differences with previous morphological phylogenies (Vereshchaka *et al.* 2014; Vereshchaka 2017). One discordance is the non-monophyletic relationship of *Deosergestes* and *Allosergestes* in our tree compared with previous findings recovering a monophyletic clade containing both genera (Vereshchaka *et al.* 2014; Vereshchaka 2017). Agreement across morphological and molecular trees is seen in the strongly supported relationship of *Neosergestes* and *Parasergestes* (100/100/1). In our molecular tree, *Sergestes* is also recovered as a significantly supported sister lineage to this clade. This relationship was not recovered in a previous morphological phylogeny (Vereshchaka *et al.* 2014); however, it is not in disagreement with more recent findings (Vereshchaka 2017). Our interpretations are limited owing to the lack of *Cornutosergestes* reprentatives; if species of this genus were included, it is very possible this genus would show an affinity to *Sergestes*, as proposed in an earlier tree (Vereshchaka *et al.* 2014). The polytomy recovered in previous morphological trees (Vereshchaka *et al.* 2014; Vereshchaka 2017) does not rule out a monophyletic *Neosergestes*, *Parasergestes*, *Cornutosergestes* and *Sergestes,* but lack of informative characters could not resolve the branches. *Eusergestes* falls as a distinct lineage to the other genera, similar to previous findings (Vereshchaka *et al.* 2014; Vereshchaka 2017), but as the sister genus to the *-sergia* group.

Within the *-sergia* group, we found nearly ubiquitous support for the new genus-level reclassification, with the only exception being the *Robustosergia* (Fig. 3). However, short branch lengths and moderate support for some of our deep branches could question this finding. At the inter-genus level, we found one significant similarity to a previous morphological phylogeny (Vereshchaka *et al.* 2014): the genera exhibiting lensed photophores (*Lucensosergia, Prehensilosergia* and *Challengerosergia*) fall out together in a strongly supported monophyletic clade (97/99/1/1). One major difference in our molecular tree and previous trees is the placement of *Sergia*, a genus that lacks photophores. Previous findings place this genus as the earliest branching lineage within the *-sergia* group, but we found high support (100/99/1/1) for it as sister to *Phorcosergia*. Other conflicts

with previous findings include the placement of *Robustosergia* and *Gardinerosergia*. In previous studies, *Robustosergia* consistently forms a monophyly with *Phorcosergia* (Vereshchaka *et al*. 2014; Vereshchaka 2017), and *Gardinerosergia* forms either a monophyly (Vereshchaka *et al*. 2014) or a close relationship by polytomy (Vereshchaka 2017) with the lensed photophore clade including *Prehensilosergia, Lucensosergia* and *Challengerosergia*. Our findings did not recover either of these relationships.

Another significant finding of our analysis is the possible presence of cryptic speciation and population structure within currently classified species (Fig. 3). The first example can be seen in the species *Allosergestes pectinatus*. Here, we recovered two distinct clades within the Florida Straits with branch lengths equivalent to other species divergences within sergestids. This division is in accordance with a recent a study that used the barcoding genes *16S* and *COI* (Varela *et al*. 2021); however, our study corroborates these findings based on an expanded gene set. As reported by Varela *et al*. (2021), morphological characters support this division and current studies are formally describing this new species. Population structure can also be seen within *Phorcosergia grandis*, with clear indication of genetic divergence across the western and eastern Atlantic populations. It is also possible that a similar pattern can be seen in *Sergia tenuiremis*, but higher resolution population genomics methods must be used to reveal if any patterns exist. Pacific–Atlantic population structure may also exist within *Challengerosergia talismani,* but again, a higher resolution method should be implemented.

Evolution of light organs over a depth gradient

Another primary objective was to trace the evolution of bioluminescent light organ type across our molecular phylogeny (Fig. 4). Our results indicate the most recent common ancestor of sergestid shrimp had an OOP, which persisted throughout the evolution of the *-sergestes* group. Morphology of the organs of Pesta differs significantly across the *-sergestes* group and with significant variation in the posterior, lateral and anterior arrangements (Foxton 1972; Schweikert *et al*. 2020). Previous research speculated light organ pattern may be used in conspecific recognition, but a recent study testing the visual ability and acuity of sergestid shrimps found they were likely incapable of differentiating the various patterns (Schweikert *et al*. 2020). Schweikert *et al*. (2020) hypothesised that, although the light organs might still be used for communication at close distances, pattern variability is likely a result of genetic drift.

To further investigate light organ type in relation to depth (light environment) and migratory behaviour, day and night vertical distributions were compiled for all species and charted alongside ancestral reconstruction findings (Foxton 1972; Donaldson 1975; Walters 1976; Flock and Hopkins 1992; Cartes *et al*. 1994; Vereshchaka 2000, 2009) (Fig. 4, Supplementary Table S3). We see similar nighttime depth distributions across all sergestids, but an overall shallower daytime depth distribution of the *-sergestes* group when compared with the *-sergia* group (Foxton 1972; Donaldson 1975; Walters 1976; Flock and Hopkins 1992; Cartes *et al*. 1994; Vereshchaka 2000, 2009). The evolution of larger light organs, such as the OOPs in the *-sergestes* group, may be correlated with their role in counterillumination during the daytime depths, needing more light in shallower systems. Members of the *-sergestes* also tend to be translucent (when their chromatophores are retracted) and the larger OOPs may have also evolved to help camouflage the darker internal organs within the carapace during light emission. Unique among the *-sergestes* group is *Eusergestes arcticus*, which inhabits benthopelagic zones during the daytime (Cartes *et al*. 1994; Vereshchaka 2009), remaining in close proximity to the sea floor along continental shelves and margins. This differs from other *-sergestes* species, which generally inhabit the open waters of the mesopelagic. Despite this contrasting lifestyle, *E. arcticus* exhibits no obvious variation in light organ structure compared with other *-sergestes*. However, the phylogenetic placement as the closest related *-sergestes* to the deeper-living, dermal photophore-bearing *-sergia* group is interesting, and could indicate an evolutionary transition from shallower daytime depths to deeper waters.

The transition from hepatic OOPs to dermal photophores can be seen with the emergence of the *-sergia* species, in which most species possess either lensed or non-lensed photophores. Photophores are displayed in species-specific patterns across the bodies of the shrimps and are often used in taxonomic descriptions to aid in identification. As with OOPs, photophores are also thought to function in counterillumination. Recent evidence also indicates that, not only are photophores capable of emitting light, but they might also have the ability to detect light (Bracken-Grissom *et al*. 2020; DeLeo and Bracken-Grissom 2020), suggesting an expanded role in counterillumination. The most recent common ancestor of *-sergia* species had non-lensed photophores, which is retained in most extant species with the exception of the genus *Sergia*. Our results revealed that *Sergia tenuiremis*, which lacks light organs completely, evolved from a non-lensed ancestor. Although we were unable to include any representatives in this study, the genus *Phorcosergia* also includes species that have not been observed with light organs: *P. wolffi* (Vereshchaka, 1994) and *P. plumea* (Illig, 1927) (Vereshchaka, pers. comm.). Owing to their lack of inclusion in this study, it is best to refrain from discussing them with confidence; however, if they fall within *Phorcosergia*, it is possible photophores were lost twice across the sergestid tree of life. As with the genus *Sergia,* all photophore-absent species are amongst the deepest dwelling in the family, with the members of the genus *Phorcosergia* often observed at depths >1000 m during the

day and members of the genus *Sergia* often exceeding 2000-m depth during the day. A reasonable hypothesis is that light organs were lost as some species transitioned into deeper waters where counterillumination would be less important, but more detailed examination is needed.

Our results also suggest that lensed photophores evolved once across the family, within a clade containing *Lucensosergia*, *Prehensilosergia* and *Challengerosergia*. All species in this clade are lensed photophore-bearing, and all uniquely exhibit benthopelagic habitat ranges as mentioned in previous accounts (Vereshchaka 2000; Vereshchaka *et al.* 2014). It is possible this benthopelagic lifestyle is correlated with the evolution of the lensed photophore, and future studies investigating the structural and emittance properties of lenses may help elucidate their purpose and function. Speculatively, the lenses might function to focus emitted light from the photophore ventrally, preventing its lateral diffusion into the water column. Lanternfish possess sophisticated photophores with lenses, which recently were determined to function in this way (Paitio *et al.* 2020). For benthopelagic animals, such as these -sergia group species, which are often against the pitch black backdrop of the steep continental slope or seamounts they inhabit, preventing lateral light diffusion could be a necessary adaptation to avoid detection from predators from a side-on perspective, while still providing protection against predators below. Overall, our findings suggest that light organ type may be correlated with either depth distribution or habitat type, or a combination of the two; however, future research that incorporates finer-scale depth data and more species is needed to confirm our findings.

Conclusions and future directions

Here, we provide the first molecular phylogeny for the Sergestidae with the goal of investigating genus-level relationships for comparison with previous morphological phylogenies. With a few exceptions (*Deosergestes* and *Robustosergia*), our findings corroborate the recent morphology-based reclassification of the genera within the family Sergestidae (Vereshchaka 2017), though interpretations are limited due to lack of species representation. Evidence of cryptic speciation (*Allosergestes pectinatus*) and possible population structure (*Phorcosergia grandis*, *Challengerosergia talismani* and *Sergia tenuiremis*) within the family were recovered, which warrant further investigation. Ancestral state reconstruction indicates the OOP light organ type represents the ancestral state of Sergestidae and persists across the *-sergestes* group. Lensed photophores evolved once across the family within *Prehensilosergia, Lucensosergia* and *Challengerosergia*, and photophores were lost once in the deepest living genus, *Sergia*. We found that OOPs are associated with shallower daytime depth distributions within the *-sergestes* group, whereas dermal photophores (lensed and non-lensed) are associated with generally deeper daytime depth distributions of the *-sergia* group. Future studies that evaluate the evolution of lensed photophores in the benthopelagic habitat as opposed to non-lensed photophores of mesopelagic species could be interesting avenues of exploration. The exact functions of lensed photophores are still unknown, and future studies that integrate structural, physiological and spectral properties of these structures may illuminate their role in the benthopelagic environment.

Supplementary material

Supplementary material is available online.

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