Expanding on *expansus*: A new species of *Scaphanocephalus* from North America and the Caribbean based on molecular and morphological data

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Abstract

Members of the genus *Scaphanocephalus* mature in accipitrids, particularly osprey, *Pandion haliaetus*, with metacercaria causing Black Spot Syndrome in reef fishes. In most of the world, only the type species, *Scaphanocephalus expansus* (Creplin, 1842) has been reported. Recent molecular studies in the Western Atlantic, Mediterranean and Persian Gulf reveal multiple species of *Scaphanocephalus*, but have relied on 28S rDNA, mainly from metacercariae, which limits both morphological identification and resolution of closely related species. Here we combine nuclear rDNA with mitochondrial sequences from adult worms collected in osprey across North America and the Caribbean to describe species and elucidate life cycles in *Scaphanocephalus*. A new species described herein can be distinguished from *S. expansus* based on overall body shape and size. Phylogenetic analysis of the whole mitochondrial genome of *Scaphanocephalus* indicates a close relationship with *Cryptocotyle*. We conclude that at least three species of *Scaphanocephalus* are present in the Americas and two others are in the Old World. Specimens in the Americas have similar or identical 28S to those in the Mediterranean and Persian Gulf, but amphi-Atlantic species are unlikely in light of divergence in cytochrome *c* oxidase I and the lack of amphi-Atlantic avian and fish hosts. Our results provide insight into the geographic distribution and taxonomy of a little-studied trematode recently linked to an emerging pathology in ecologically important reef fishes.

Keywords: Fluke; Black Spot Syndrome; coral reef fish; raptor; helminth

Introduction

Members of the genus *Scaphanocephalus* (Jägerskiöld, 1904) are opisthorchiid trematodes that mature almost exclusively in osprey, *Pandion haliaetus*. Metacercariae cause Black Spot Syndrome (BSS) in fishes (Kohl *et al.*, 2019; Malawauw *et al.*, 2024), which manifests as immunopathogenic pigmented spots on fish skin and fins (Kohl *et al.*, 2019; Dennis *et al.*, 2019; Cohen-Sánchez *et al.*, 2023a,b). Though little studied historically, *Scaphanocephalus* is receiving increased attention, likely because BSS appears to be increasing in prevalence (Elmer *et al.*, 2019, Cohen-Sánchez *et al.*, 2023a) in keystone herbivorous fishes of importance to coral reef health (Mumby *et al.*, 2007; Burkepile and Hay, 2008; Kohl *et al.*, 2019), and has been linked to economic impacts in fisheries (Shimose *et al.*, 2019).

Jägerskiöld (1904) named *Scaphanocephalus* for the wide forebody and narrow hindbody of adult worms that form a distinctive shape he likened to a spade. He erected the genus for a species Creplin (1842) originally described as *Monostomum expansum* from adults in osprey, having examined both Creplin's (1842) material as well as newly collected specimens from osprey in Egypt. Creplin (1842) did not mention a locality, but presumably the type specimens originated near Greifswald, Germany, where he lived and worked (Häckermann, 1876). *Scaphanocephalus expansus* has subsequently been reported from Europe, the Middle East, North America, the Gulf of Mexico, Caribbean, Japan, and Malaysia (reviewed by Kohl *et al.*, 2019). Two other species have been described: *Scaphanocephalus australis* (Johnston, 1916), described from the sea eagle, *Icthyophaga leucogaster*, to date endemic to Australia; and *Scaphanocephalus adamsi*, described from metacercariae found within split-level hogfish, *Bodianus mesothorax*, in the Philippines (Tubangui, 1933). Yamaguti (1942) considered *S. adamsi* a synonym of *S. expansus*, but Kifune and Kugi (1979) disagreed, based on adults of *S. adamsi* in *Buteo buteo burmanicus* from Kyushu, Japan, that presented morphological differences from *S. australis* and *S. expansus*.

Six recent studies have employed 28S rDNA to study diversity within *Scaphanocephalus* (Dennis *et al*., 2019; Kohl *et al*., 2019; Al Salem *et al*., 2021; Cohen-Sánchez *et al*., 2023a,b; González-García *et al*., 2023). Phylogenetic analysis reveals three 28S lineages of *Scaphanocephalus* in the Gulf of Mexico, Caribbean Sea, Persian Gulf and Mediterranean (González-García *et al.,* 2023). One 28S lineage occurs in both the New World and in the Persian Gulf (Al Salem *et al*., 2021) and another is in both the New World and the Mediterranean (Cohen-Sánchez *et al.,* 2023a,b; González-García *et al*., 2023). The third lineage, which to date has only been found in the Gulf of Mexico and Caribbean Sea, was identified as *S. expansus* after morphological study of metacercariae (Kohl *et al*., 2019) and adults (González-García *et al*., 2023). Collectively, these works leave several questions unresolved. Firstly, available 28S sequences indicate three species exist where only *S. expansus* has ever been reported (González-García *et al*., 2023), indicating undescribed diversity. Second, except for three sequences from González-García *et al.* (2023), molecular data are from metacercariae, which impedes identification because, as in all digeneans, species of *Scaphanocephalus* are conventionally distinguished based on the adult, which differs morphologically from the metacercaria. Third, although the morphological taxonomy clearly underestimates species diversity in this genus, the additional diversity indicated by 28S may also represent an underestimate, given that ribosomal markers may be insufficiently variable to resolve recently diverged species (Vilas *et al*., 2005). Finally, the current molecular view of species distributions in *Scaphanocephalus* is perplexing in that the species recognized in recent studies as *S. expansus* (Kohl *et al*., 2019; González-García *et al*., 2023) has only been recovered in Mexico and the Caribbean, and lacks a molecular link to the Old World, from which *S. expansus* was described by Creplin (1842).

The aim of this study was to describe species and provide a robust basis for phylogenetic conclusions within *Scaphanocephalus*. Adult worms collected from across North America and

the Caribbean were studied morphologically with sequencing targeted on both 28S (for comparability with past studies) and mitochondrial markers. Results were interpreted in a biogeographic context that considers the distribution of hosts and geographic barriers. Whole mitochondrial genome and nuclear ribosomal operon data were also obtained to clarify the phylogenetic placement of the genus *Scaphanocephalus* and to provide resources for future molecular phylogenetic work.

Materials and methods

Sample collection and processing

Specimens of *Scaphanocephalus* were newly collected from salvaged osprey carcasses (provided by wildlife rehabilitation centers) and from ocean surgeonfish (*Acanthurus tractus*) collected in North America and the Caribbean (**Table 1, Supplementary Table 1**). Adult worms were removed from osprey gastrointestinal tracts while metacercariae were mechanically excysted from fins and stored in 95% ethanol until molecular or morphological analysis. In specimens or subsamples of worms intended for molecular work, DNA was extracted using commercial kits following manufacturer instructions (e.g., NucleoSpin Tissue XS, Macherey Nagel, Allentown, PA, USA). Vouchers for morphological study were gradually rehydrated, stained with acetocarmine, gradually dehydrated in pure alcohol, cleared in clove oil, and mounted on slides using Permount. Voucher specimens were deposited in the Museum of Southwestern Biology (MSB:Para:49134–46). Line drawings of adults were made with a Nikon Alphaphot YS equipped with a camera lucida.

Extracted DNA from prior studies (Dennis *et al.*, 2019; Cohen Sánchez *et al.*, 2023b) was also re-analyzed in the present study. These extracts were from (1) a metacercaria (specimen XN4P4M) from a pearly razorfish (*Xyrichtys novacula*) caught offshore from the Balearic Islands in the Mediterranean Sea, from which Cohen Sánchez *et al.* (2023b) obtained 28S

sequence OK045682, and (2) two specimens from a doctorfish (*Acanthurus chirurgus*) in St. Kitts, from which Dennis *et al.* (2019) obtained 28S sequence MN160570.

Molecular analysis

Next generation sequencing

The DNA of an adult worm from a salvaged osprey from Florida was extracted and shotgun-sequenced on an Illumina HiSeq 4000 at Azenta (NJ, USA). Nuclear ribosomal DNA operon and whole mitochondrial genomes were assembled using Geneious Prime v2020.2.2 (Biomatters Ltd., Auckland NZ) from 150-bp paired-end reads built with Nextera adapters (llumina, San Diego, USA). The nuclear rDNA operons were iteratively assembled using custom settings with medium-low sensitivity read-mapping to a chimeric consensus of two 28S sequences of *Scaphanocephalus* (MN160569, MN160570) and the entire rDNA operon of *Diplostomum ardeae* (MT259036) until even and deep coverage of the assembly was obtained. The boundaries of rDNA subunit genes and the transcribed spacers were determined by aligning the assembly with other sequences (MH521249-52, Locke *et al.*, 2018). Mitochondrial genomes were assembled by mapping Illumina reads to a consensus of two mitochondrial genomes from the Heterophyidae: *Haplorchis taichu* (MG972809) and *Metagonimus yokogawai* (KC330755). The longest resulting fragment with good coverage was then extended in iterative assemblies until the molecule was circularized. Coding regions, tRNAs and rRNAs were annotated using MITOS (Bernt *et al.*, 2013) and through comparison with and KC330755 (unpublished), EU921260 and FJ381664 (Shekhovtsov *et al.*, 2010), KC330755 (unpublished), KT239342 (Na *et al.*, 2016), MG972809 (Le *et al.*, 2021), MH536507-13 (Locke *et al.*, 2018), and NC_063968 (unpublished).

As described in the results, two specimens (one from Montana, one from Florida) each yielded CO1 Sanger sequences that differed by a large and similar magnitude from all other sequences obtained. The specimens were suspected to belong to the same species, but their CO1 sequences could not be compared because different regions of this gene were amplified in each. After unsuccessful attempts to amplify and Sanger-sequence CO1 fragments allowing direct comparison, extracted DNA from the Florida specimen was subject to Minion sequencing with the goal of obtaining CO1 sequence comparable to the Montana specimen. The sequencing library was prepared using the Ligation Sequencing Kit v14 (SQK-LSK114, Oxford Nanopore Technologies) following the manufacturer's protocol. The library was sequenced on a whole Flongel Flow Cell (R10.4.1, Oxford Nanopore Technologies) with MinKnow v.21.05.12 software (Oxford Nanopore Technologies). Base-calling was performed in Guppy (v.5.0.12). NanoFilt v.2.8.0 (De Coster *et al.*, 2018) was used to process and filter all resulting reads with quality scores ≥Q10. MiniMap2 v.2.22 (Li, 2018) was used to remove non-trematode contaminant reads (host, human, bacteria).

Sanger sequencing

Partial 28S was amplified using the primers LSU5 (Littlewood, 1994) and 1500R (Snyder and Tkach, 2001) with the following thermocycling conditions: 95°C for 30 s, followed by 30 cycles of 95°C for 30 s, 56°C for 45 s and 68°C for 60 s; and a final incubation at 68 ∘C for 5 min. The 5' or barcode region of the mitochondrial cytochrome *c* oxidase I (CO1) gene was amplified using the primers and protocols MplatF/R, MplatF/Dice11 and MplatF/Dice14 of Moszczynska *et al.* (2009) and Van Steenkiste *et al.* (2015), using the touchdown protocol described by Van Steenkiste *et al.* (2015). The 3' half of CO1 was amplified using primers JB3 (Morgan and Blair, 1998) and CO1-R (Miura *et al.*, 2005) using the cycling conditions of Miura *et al.* (2005).

Phylogenetic analysis

Sequences generated in the present study were aligned with data from the literature and discovered through BLAST searches (Altschul *et al.*, 1997) using MAFFT (Katoh and Standley 2013; **Supplementary Table 1**). Uncorrected p-distances were calculated using all alignment sites in MEGA X v.10.1.8 (Kumar *et al.*, 2018). Phylogenetic trees based on alignments stripped of gaps were based on substitution models selected using the Bayesian Information Criterion in MEGA X v.10.1.8 (Kumar *et al.*, 2018), or the nearest approximation available in Maximum Likelihood (ML) (RAxML, Stamatakis, 2014) and Bayesian Inference (BI, Huelsenbeck and Ronquist, 2001) implemented in Geneious. Phylogenetic accuracy in ML was estimated by bootstrapping the trees with 1000 replicates and in BI, with 2 runs of total chain length 1100000, subsampled every 200 generations, a burn-in length of 110000, yielding 4951 trees.

Results

Molecular data were obtained from 27 newly collected adult *Scaphanocephalus* specimens found within osprey from Montana, Virginia, Florida, and Curaçao, and two metacercariae from newly collected ocean surgeonfish (*Acanthurus tractus*) from Curaçao (**Table 1**). Mitochondrial data were also newly obtained from three specimens from which 28S was sequenced in prior studies, namely two metacercariae from St Kitts (specimens QSK29.1, QSK29.2, 28S sequence MN160570 from Dennis *et al.*, 2019) and one from the Spanish Mediterranean (specimen XN4P4M, 28S sequence K045682 from Cohen-Sánchez *et al.*, 2023b). As presented further below and summarized in **Table 2**, molecular and morphological data supported description of a new species. As many as four other putative species were not described, identified, or characterized morphologically due to a lack of vouchers of adult worms or of CO1 sequences: *Scaphanocephalus* sp. ne1, *Scaphanocephalus* sp. ne2,

Scaphanocephalus sp. pa1, *Scaphanocephalus* sp. pa2 (where 'ne' refers to Nearctic and 'pa' to Palearctic). Sequence-based identifications revealed mixed infections in osprey. One osprey from Florida was infected with six *S. robustus* n. sp. and one *Scaphanocephalus* sp. ne2, and a second osprey from Florida was infected with four *S. robustus* n. sp. and one *Scaphanocephalus* sp. ne1.

Description

Scaphanocephalus robustus n. sp.

Type host: *Pandion haliaetus*; other hosts: *Mugil curema*

Type Locality: Virginia, USA; other localities: Florida, USA; Curaçao; Celestún, Yucatán, Mexico

Voucher specimens: MSB:Para:49134–46

GenBank: 28S: PP436427-34, OR794154; CO1: PP456658–80

Description based on 15 hologenophores from *P. haliaetus* (**Table 3, Figure 1**): Dorsoventrally flattened, with wide lateral expansions in forebody; hindbody subcylindrical. Oral sucker small, subterminal. Short pre-pharynx followed by small pharynx and lengthy esophagus. Muscular genital complex (ventral sucker, gonotyl) in anterior third of body. Vitellaria in two lateral extracaecal fields, confluent posteriorly in most specimens, extending anteriorly to caecal bifurcation. Ovary multilobate, median, pretesticular; testes tandem, deeply lobed, in posterior third of body. Seminal receptacle submedian, elongate, extending laterally between ovary and anterior testis.

Remarks

Compared with descriptions of *S. expansus* originating in the Old World by Creplin (1842), Jägerskiöld (1904) and Dubois *et al.* (1960), *S. robustus* n. sp. is shorter in total length, with a relatively wide hindbody. In *S. robustus* n. sp., the width of the hindbody is mean 34% (range

26-46% ± 7%) of total length, while in descriptions of *S. expansus* from the Old World, this proportion is mean 25%, range 23-29% (Creplin, 1842), 20% (Jägerskiöld, 1904), and 28% (Foronda *et al.*, 2009). The relative width of the hindbody in adults of *S. adamsi* described by Kifune and Kugi (1979), from 44 to 46% of total length, was greater than in *S. robustus* n. sp. Foronda *et al.* (2009) described worms identified as *S. expansus* that were similar in length and proportion to *S. robustus* n. sp. but with smaller eggs.

Among New World species, *S. robustus* n. sp. can be distinguished from *Scaphanocephalus* sp. ne1, described by González-García *et al.* (2023) as *S. expansus*, by the longer eggs and, to a lesser extent, the wider anterior body of *S. robustus* n. sp. Morphometrically, *S. robustus* n. sp. most closely resembles *S. australis*, which Johnston (1916) described from *I. leucogaster* in Australia, but in *S. robustus* n. sp. eggs are slightly longer and narrower, and testes are deeply lobed, rather than solid bodies. *Scaphanocephalus robustus* n. sp.

Although *S. robustus* n. sp. is morphologically distinguished from *S. expansus* based largely on metrical variation, caution is needed in making metrical comparisons among descriptions because authors have reported the range, both mean and range, mean and standard deviation, or unexplained figures, often without specifying the number of specimens in which a feature was measured. Notably, most measurements provided by Jägerskiöld (1904), who examined the type specimens of Creplin (1842), are inconsistent with or lack the variability of the original description. Foronda *et al.* (2009) reported the seminal receptacle to be wider than the hindbody, with a large standard deviation suggesting this was not a typographical error. González-García *et al.* (2023) apparently summed the standard deviations to the means to obtain a range of measurements in Foronda *et al.* (2009), thus underestimating morphometric variation in the latter study.

Phylogenetic results: Sanger sequencing

28S rDNA

The 19 new sequences of 28S generated in this study were aligned with 16 sequences collectively published by Kohl *et al.* (2019), Dennis *et al.* (2019), Al Salem *et al.* (2021), Cohen Sánchez *et al.* (2023b), and González-García *et al.* (2023). Phylogenetic analysis yielded three strongly supported clades (**Figure 2**). The largest clade consisted of *S. robustus* n. sp. (from North America, the Caribbean, and the Yucatán), with sequences from the Mediterranean nested within (including OK045682 from specimen XN4P4M, from which divergent CO1 was newly obtained in the present study, see below). Another 28S clade was formed by identical sequences from specimens from the Persian Gulf, and Montana and the Caribbean. A third clade, *Scaphanocephalus* sp. ne1, was formed by 28S from specimens collected in the Gulf of Mexico and Caribbean (Florida, St. Kitts, Curaçao, Bonaire, Campeche), including specimens identified as *S. expansus* by Kohl *et al.* (2019) and González-García *et al.* (2023).

Genetic distances among 28S sequences of the genus *Scaphanocephalus* were bimodally distributed, with a lack of p-distances greater than 0.57% and less than 1.01% (**Figure 3**). This gap in 28S p distances corresponded to intra and inter-clade variation in 28S (**Figure 2**), but not to comparisons within and between species, as recognized herein. For example, 28S distances between *S. robustus* n. sp. and *Scaphanocephalus* sp. pa1 averaged 0.27 (range 0-0.57)% and thus were similar in magnitude to intraspecific 28S distances in *Scaphanocephalus* (**Table 4**).

Cytochrome *c* oxidase I

Phylogenetic analysis of partial CO1 revealed four lineages with variable node support (**Figure 4**). The topology of the CO1 phylogeny was consistent with the 28S phylogeny (**Figure 2)** in that *Scaphanocephalus* sp. ne2 was the basal lineage, giving rise to other clades, including *Scaphanocephalus* sp. ne1, which emerged as reciprocally monophyletic in analyses of both markers. The CO1 phylogeny (**Figure 4**) differed from the 28S phylogeny (**Figure 2)**, in that

CO1 from the Mediterranean specimen of *Scaphanocephalus* sp. pa1 (XN4P4M) was separate from New World specimens of *S. robustus* n. sp., albeit with weak support. A concatenated alignment of 28S and CO1 generated to clarify the phylogenetic position and status of this Mediterranean specimen (XN4P4M) with respect to *S. robustus* n. sp. showed *Scaphanocephalus* sp. pa1 to be separate from the *S. robustus* n. sp. in both BI and ML (**Figure 5**).

Among the four species (*Scaphanocephalus* spp. ne1, ne2, pa1, and *S. robustus* n. sp.) from which CO1 sequences were available, p-distances were as high as 2.54% within and at least 5.0% between species (**Figure 3**, **Table 4**). Between the most closely related species (*S. robustus* n. sp., *Scaphanocephalus* sp. pa1), p distances averaged 5.5% (range 5.0-6.2%) and the 59th translated amino acid (glycine) from the single specimen of *Scaphanocephalus* sp. pa1 differed from the amino acid found in all specimens in the New World (alanine). Under the alternative interpretation that *Scaphanocephalus* sp. pa1 and *S. robustus* n. sp. represent a single, transatlantic species, CO1 variation within this species would average 1.27 (range 0-6.2)%.

Phylogenetic results: Next Generation Sequencing

Nuclear rDNA operon

Of 73,675,730 150-bp Illumina reads obtained from the DNA of an adult *Scaphanocephalus* from *P. haliaetus* in Fort Lauderdale, Florida, 52,581 were assembled to an rDNA operon contig 7,571 bp long, with mean coverage of 1,015 reads per site (range 477-1930) over subunit and internal transcribed spacer sites (GenBank accession: PP430581). The guanine + cytosine (G+C) content of the rDNA operon was slightly enriched (52.4%) and the 18S subunit was 1991 nt long, ITS1 798 nt, 5.8S 157 nt, ITS2 275, and 28S 4194 nt. Blast searches using the entire rDNA operon or portions thereof yielded top hits belonging to *Cryptocotyle lingua*, with

overall operon similarity of 7194/7618 identities, or 94.4% similarity with *C. lingua* (MW361240), including two large gaps (20 and 162 nt) in the ITS1 portion of the alignment.

Mitochondrial genome

The mitochondrial genome assembly of *Scaphanocephalus* sp. ne1 (GenBank accession: PP577105) was a circular molecule 14,188 bp in length, with mean of 2,279 (range 516 – 17,204) reads per site from the 5' end of cox3 to the 3' end of nad5 (i.e., excluding a difficult to assemble, non-coding, region repetitive that artefactually increases read depth). Annotations yielded 36 genes: 12 protein-coding genes (*cox1-3*, *nad1-6*, *nad4L*, *atp6*, *cob*), 22 transfer RNA genes, and 2 ribosomal RNA genes (**Table 5**) transcribed in the same direction (5'-3'). Mitochondrial coding genes were separated by short intergenic sequences except for *cox2* and *nad6*, which overlap by 17 bp. The start codon ATG was used in 7 protein-coding genes, followed by GTG codon in 4 genes, and TTG in 1, and all coding genes ended in the TAG stop codon except *nad2*, which terminated with TAA. The molecule was A+T enriched, like other opisthorchiid mt genomes (**Table 6**).

In phylogenetic analysis of mitochondrial genomes, *Scaphanocephalus* and *Cryptocotyle* emerged as sisters while the families Opisthorchidae and Heterophyidae were not monophyletic (**Figure 6**).

Minion sequencing was used to resolve the status of two adult specimens from two salvaged osprey that yielded Sanger sequences from different parts of the CO1 gene (Supplementary Figure 3). The p distances of the barcode CO1 Sanger sequence (3' region, PP456682) from a specimen of *Scaphanocephalus* sp. ne2 from Montana averaged 16.5% (range 16.3-16.6%) to CO1 from all other specimens, while the 3' CO1 Sanger sequence (PP456683) of a worm from Florida averaged 17.2% (range 16.3-17.8%). However, these two Sanger sequences did not

overlap and could not be compared, and repeated attempts to amplify and sequence CO1 fragments allowing direct comparison were unsuccessful. Minion sequencing of the Florida specimen yielded 27,411 reads (average length of 2,618 nt) and 7457 contigs averaging 2751.1 (range 115-6963, SD ± 1200.7) nt in length, some assembled from hundreds of reads. Most (95%) were filtered out as contamination after mapping to bacterial, human or avian scaffolds. The remaining 356 contigs were mean 593.3, range 165-2370, SD ± 306.0 in length. Of the latter, a 198-nt contig mapped to the CO1 sequence obtained from Montana with 90.2% similarity. All variation between the Minion contig of the Florida specimen and the Montana specimen occurred in the first (5') 47 and last (3') 29 nt; the central 122-nt (PP456684) were identical in the Minion contig and the PCR-amplified, Sanger-sequenced CO1 of *Scaphanocephalus* sp. ne2 (PP456682) from Montana. Quality scores of the Minion contig were lower in these 5' and 3' marginal regions (mean quality 15.0, range 3-43, SD ± 8.8), and trimmed by default parameters in Geneious. In contrast, the central 122-bp of the Minion contig identical to the Sanger sequence of *Scaphanocephalus* sp. ne2 had mean quality 26.7, range 6-90, SD ± 13.1. We interpret these results as indicating the Florida specimen also belongs to *Scaphanocephalus* sp. ne2.

Discussion

Here we report a large-scale, molecular phylogenetic study of the trematode genus *Scaphanocephalus*. We describe a new species and find evidence for four others (two in North America and two in Europe), ultimately concluding that none of these four can be reliably identified as *S. expansus* based on currently available data. We provide mitogenomic confirmation of the close relationship between this genus and *Cryptocotyle*, previously suspected based on morphology and phylogenetic analysis of a smaller number of nuclear characters. The genomic resources provided here will be useful in ongoing and future work

that is needed in view of the association of *Scaphanocephalus* with BSS, an emerging pathogenesis in reef fishes.

The existence of multiple, genetically distinct species within the geographic distribution encompassed by descriptions of *S. expansus* casts morphometric variation in descriptions of this species in a new light. For example, measurements provided by Jägerskiöld (1904, in Egypt) are mostly smaller than those of Creplin (1842, likely in Germany). In France, Dubois *et al.* (1960) reported worms smaller than in both these prior accounts, yet oral suckers and internal organs were larger. Next, Foronda *et al.* (2009) reported even shorter body lengths in worms from the Canary Islands than Dubois *et al.* (1960), along with eggs highly variable in size. Recently González-García *et al.* (2023) report worms from Mexico smaller than those from Creplin (1842), Jägerskiöld (1904) and Dubois *et al.* (1960). All of these authors attributed such differences to morphological variability within *S. expansus*, but the inclusion of multiple species in these descriptions of geographically widespread specimens seems likely to have contributed to this morphometric variation.

Even without the species diversity revealed by recent molecular studies, the cosmopolitan range of *S. expansus* implied by records in the literature (reviewed by Kohl *et al.*, 2019) is questionable considering host distributions. While osprey definitive hosts conduct impressive latitudinal migrations, the American and European osprey constitute genetically distinct subspecies with flyways that do not overlap (Ferguson-Lees and Christie, 2001; Monti *et al.*, 2015; Monti, 2021). Thus, transatlantic distribution of worms by osprey is unlikely. Mackrill (2023) emphasizes the unprecedentedness of a recent transatlantic journey from Scotland to Barbados by one osprey, speculating that the bird rested on oceangoing boats. Transatlantic transport of *Scaphanocephalus* in other migratory bird species is also unlikely, due to the high specificity for osprey. Another way that *S. expansus* could theoretically maintain population connectivity across the Atlantic is via transport of metacercariae in migrating fish hosts, but

this also seems improbable. Metacercariae of *Scaphanocephalus* are recorded mainly in reef-associated fishes poorly adapted for long-distance migration across pelagic habitats. Transatlantic distributions are reported in just 116/2605 (4%) of reef fish species (Floeter *et al.*, 2008; see also Evans *et al.*, 2020). Recent molecular studies (Cohen-Sánchez *et al.*, 2023; González-García *et al.*, 2023) encountered metacercariae of *Scaphanocephalus* in fish species (*X. novacula*, *M. curema*) reported on both sides of the Atlantic in resources such as FishBase (Froese and Pauly, 2023). However, both these fishes consist of geographically disjunct, cryptic species (Nirchio *et al.*, 2019; Durand and Borsa, 2015). Overall, given the distribution and mobility of known hosts, the parasites described from osprey in Germany by Creplin (1842) are unlikely to be conspecific with specimens in North America and the western Atlantic; indeed, any individual species of *Scaphanocephalus* distributed on both sides of the Atlantic seems doubtful. This view is in keeping with the general tendency of molecular data to reveal multiple, geographically isolated species of digeneans within samples of allegedly cosmopolitan species (Locke *et al.*, 2021).

These two considerations – unclear morphological foundations in *S. expansus*, and disjunct host assemblages across the Atlantic – influence our interpretation of phylogenetic results in *Scaphanocephalus*. Essentially, while 28S data indicate three species, we argue that five species are more likely present among currently sequenced specimens, for two reasons. First, 28S may be identical in species of digeneans distinguishable though life history, morphology, and other markers, and, secondly, two of the 28S lineages in *Scaphanocephalus* have implausible transoceanic distributions. Examples of digenean species that are poorly distinguishable with 28S and other commonly used nuclear ribosomal markers include members of *Hysteromorpha* (Locke *et al.*, 2018), *Pseudoheterolebes* (Martin *et al.*, 2018), *Bivesicula* (Cribb *et al.*, 2022), *Posthodiplostomum* (Achatz *et al.*, 2021), and *Transversotrema* (Cutmore *et al.*, 2023). In neither *S. robustus* n. sp. nor *Scaphanocephalus* sp. ne2 do the 28S

data provide sufficiently strong support for conspecificity of specimens separated by oceanic barriers. In the case of *S. robustus* n. sp., American isolates can be distinguished from Palearctic species of *Scaphanocephalus* both morphologically and by 5-7% divergence in CO1, which is higher than usually observed within species of digeneans (e.g., Vilas *et al.*, 2005).

Among the three lineages of 28S rDNA recovered in 33 specimens, *Scaphanocephalus* sp. ne1 has been recovered in the Gulf of Mexico, Caribbean, and North America, but not the Old World, where Creplin (1842) described *S. expansus*. González-García *et al.* (2023) identified adult specimens of this species as *S. expansus*, based on morphological similarity to prior descriptions. However, we doubt these specimens correspond to *S. expansus* because, as mentioned above, trans-Oceanic distributions are unlikely in species of *Scaphanocephalus*; no molecular evidence supports such a distribution in *Scaphanocephalus* sp. ne1; and the likely inclusion of multiple species in the morphological concept of *S. expansus* s.l. weakens identification on morphological grounds.

The mitochondrial genome sequence provided here provides strong and independent support of both early and more recent systematic hypotheses, the latter based only on nuclear rDNA subunit phylogenies. In creating *Scaphanocephalus*, Jägerskiöld (1904) noted its close relationship to *Cryptocotyle* (Osborn 1903). Kohl *et al.* (2019) were the first to support this close relationship between *Scaphanocephalus* and *Cryptocotyle* using molecular data, namely 28S. At the family level, the paraphyly newly observed here in the evolution of mitochondrial genomes, with the Opisthorchidae nested within Heterophyidae, was first reported by Olson *et al.* (2003) and has emerged repeatedly in increasingly taxon-dense analyses of rDNA subunits by Thaenkham *et al.* (2011), Kuzmina *et al.* (2018), Pérez-Ponce de León and Hernández-Mena (2019), Tatonova and Besprozvannykh (2019), and Sokolov *et al.* (2022).

The new molecular data provided herein will be useful for resolving the status of both the unidentified lineages already encountered, and of *S. adamsi*, considered a synonym of *S.*

expansus by Yamaguti (1942). The rDNA operon, mitochondrial genome and CO1 sequences will facilitate surveys and phylogenetic work based on commonly used markers heretofore unused in *Scaphanocephalus* (e.g., 18S, ITS, NAD1). For example, these data can help identify the first intermediate host of *Scaphanocephalus*. The use of littorinoid and truncatelloid snails by members of *Cryptocotyle* (Stunkard, 1930; Rothschild, 1938; Wootton, 1957; Tatonova and Besprozvannykh, 2019) suggests coastal-dwelling members of these clades may serve as hosts for *Scaphanocephalus*. Thus far, all known life cycles of opisthorchioid trematodes, which include the heterophyids, opisthorchids, and cryptogonimids, involve snails in one of three superfamilies: Cerithioidea, Truncatelloidea, and Littorinoidea (with *Cryptocotyle* as the sole representative using this last superfamily) (Cribb *et al*., 2001).

Identification of a gastropod intermediate host of *Scaphanocephalus* may shed light on regional variation in BSS epidemiology, which is a research priority due to potential effects on algal grazers that contribute to coral reef health (Mumby *et al*., 2007; Burkpile and Hay, 2008; Kohl *et al*., 2019). BSS appears to have increased in prevalence in the Caribbean in recent decades (Elmer *et al*., 2019), and Cohen-Sánchez *et al*. (2023) also note a recent increase in reports in the Mediterranean. In most of the world, the cause of BSS has been treated as a single, cosmopolitan species, *S. expansus*, but we argue that the global distribution of definitive (Monti *et al*., 2015) and intermediate hosts (Costello *et al*., 2017) suggests *S. expansus*, described initially from Germany, is probably limited to Eurasia and Africa. This predicted continental separation of species in *Scaphanocephalus* is supported by newly obtained CO1 distinguishing a new American species, *S. robustus*, from a European species with matching 28S. To clarify the status of other species of *Scaphanocephalus*, mitochondrial sequences are needed from morphologically characterized adults of *Scaphanocephalus* in Europe. Such data would resolve the conflict between our conclusions and those Al Salem *et al*. (2021), who maintained that identical 28S in the Persian Gulf and Caribbean indicated a cosmopolitan

species; and of González-García *et al.* (2023), who concluded the distribution of *Scaphanocephalus* 'expansus' to be cosmopolitan, based on adult morphology.

Supplementary material.

The supplementary material for this article can be found at [DOI].

Data availability.

Sequence data are available at GenBank accessions: OR794154-40 (partial 28S); PP456658-84 (partial CO1); PP456658-84; PP577105 (mitochondrial genome); PP430581 (rDNA operon). Specimen vouchers are deposited in the Museum of Southwestern Biology (MSB:Para:49134–46).

Author's contribution.

SAL and PTJJ conceived and designed the study. All authors contributed to data gathering – obtaining samples or sequences. SAL generated a draft manuscript that all authors revised and approved.

Competing interests.

The authors declare there are no conflicts of interest.

Ethical standards.

Vertebrate hosts used in this research were salvaged osprey (permit MBPER0028773)

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Figure 1. Line drawing of type specimen of *Scaphanocephalus robustus* n. sp. from *Pandion hal* from Virginia, USA. Hologenophore for sequence PP456670 (cytochrome *c* oxidase I) and PP436435 (28S), deposited in the Museum of Southwestern Biology (MSB:Para:49138). Scale bar is 1 mm.
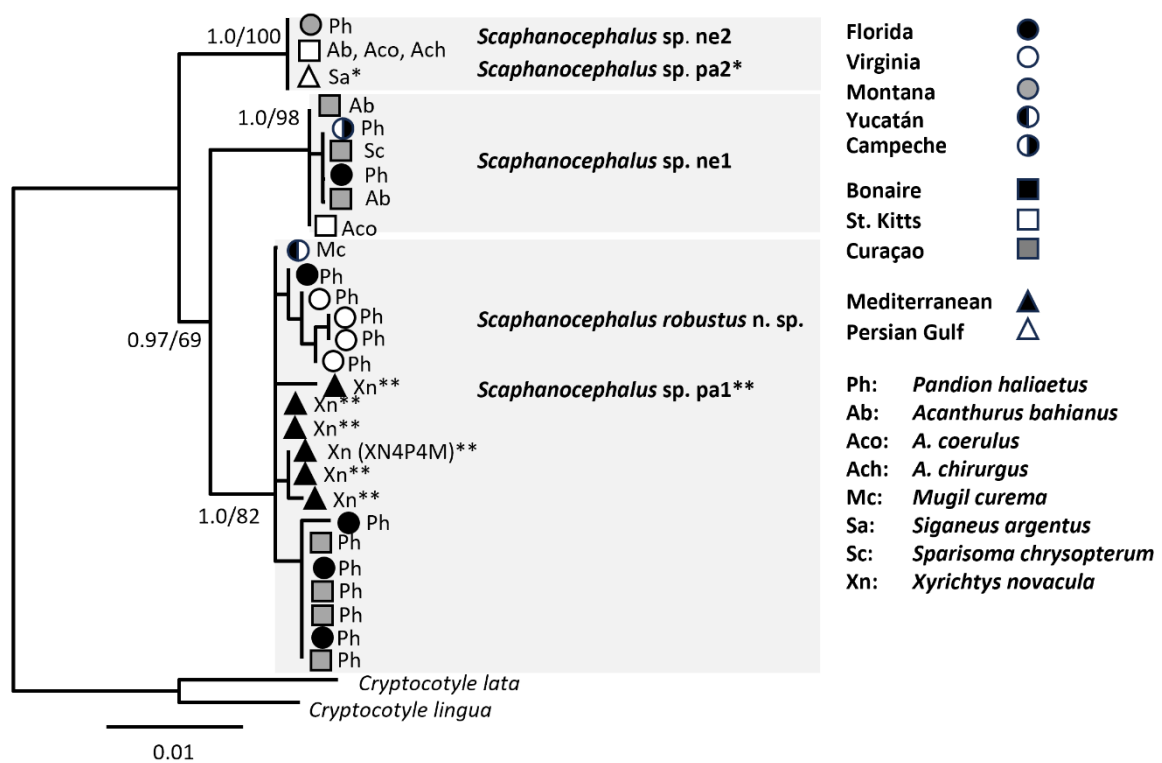
Figure 2. Phylogenetic analysis of 30 partial 28S sequences of *Scaphanocephalus* in the present (16 sequences) and prior studies (12 sequences). The Maximum Likelihood topology is shown and nodes are annotated with posterior probability (Bayesian Inference) / bootstrap support (1000 replicates in Maximum Likelihood). The trimmed alignment was 1048 nt in length. The ML tree was generated using nucleotide substitution model GTR+G; the BI tree using HKY+G. The same tree, expanded with individual sequences labelled with GenBank accessions, is available in **Supplementary Figure 1**).
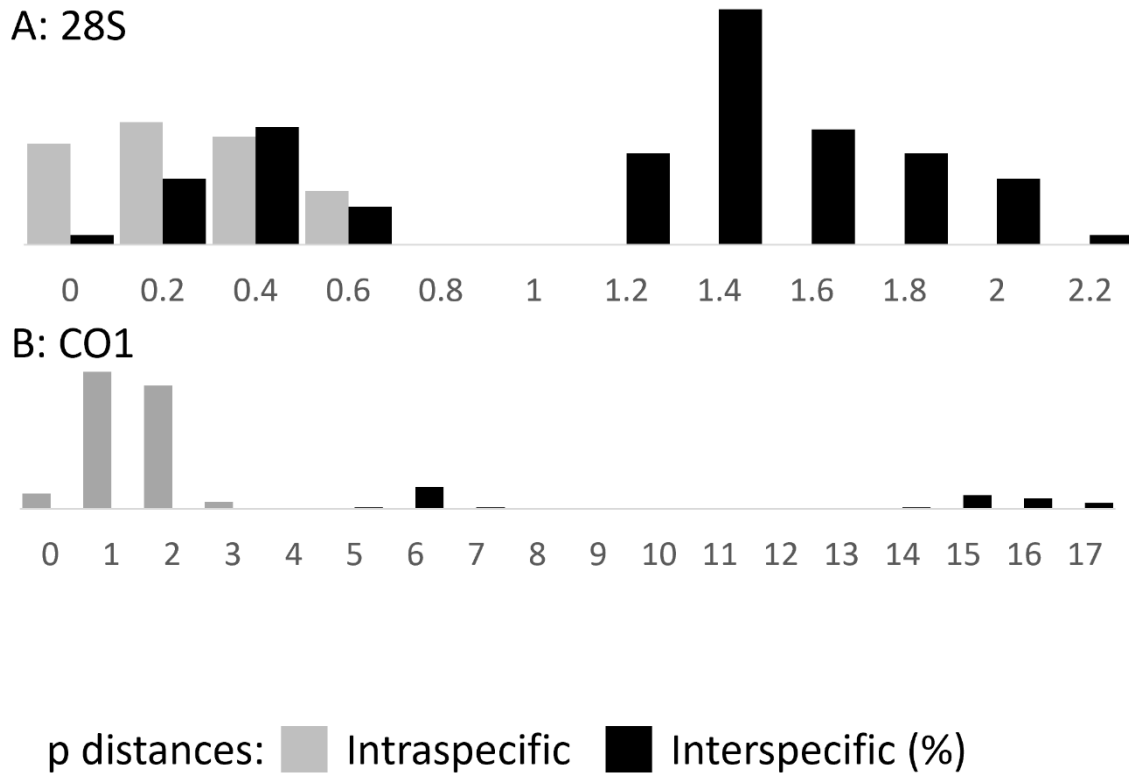
Figure 3. Histogram of uncorrected p distances in *Scaphanocephalus* among A) 33 partial 28S sequences from the present and prior studies and B) 28 partial cytochrome *c* oxidase 1 sequences from the present study.
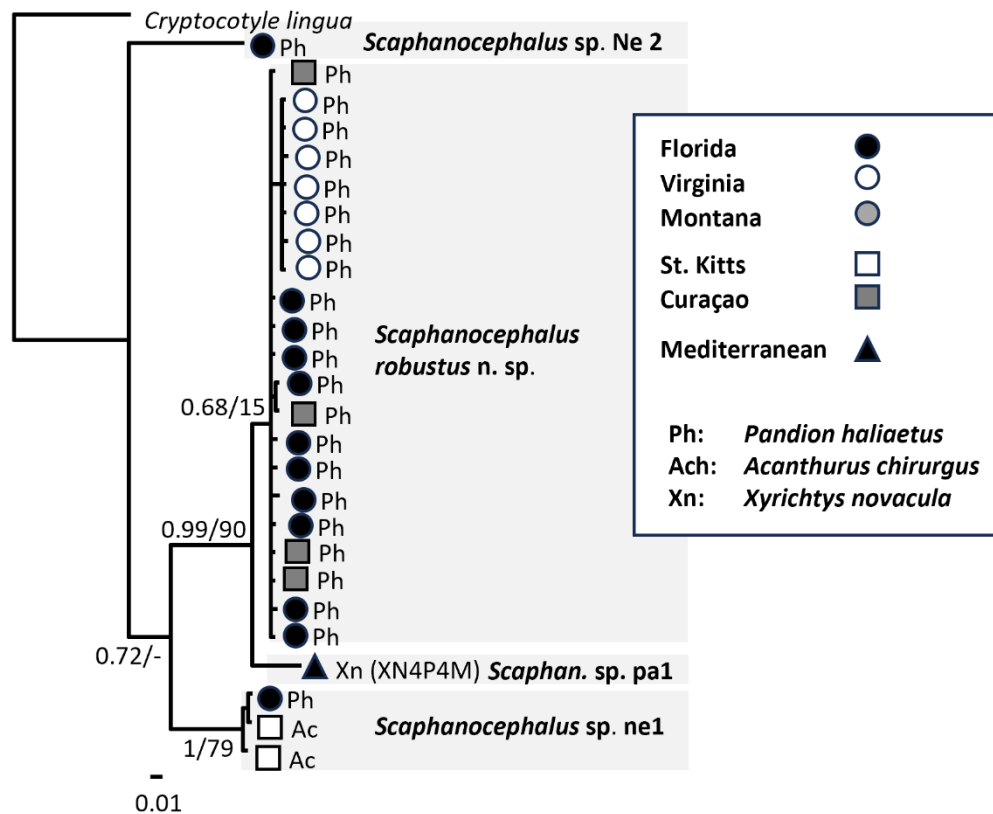
Figure 4. Phylogenetic analysis of 26 partial CO1 sequences of *Scaphanocephalus* generated in the present study. The Bayesian inference topology is shown with nodes annotated with posterior probability (Bayesian Inference, BI)/bootstrap support (1000 replicates in ML) based on a 415-nt alignment. The ML tree was generated using substitution model GTR+I; the BI tree using HKY+I.
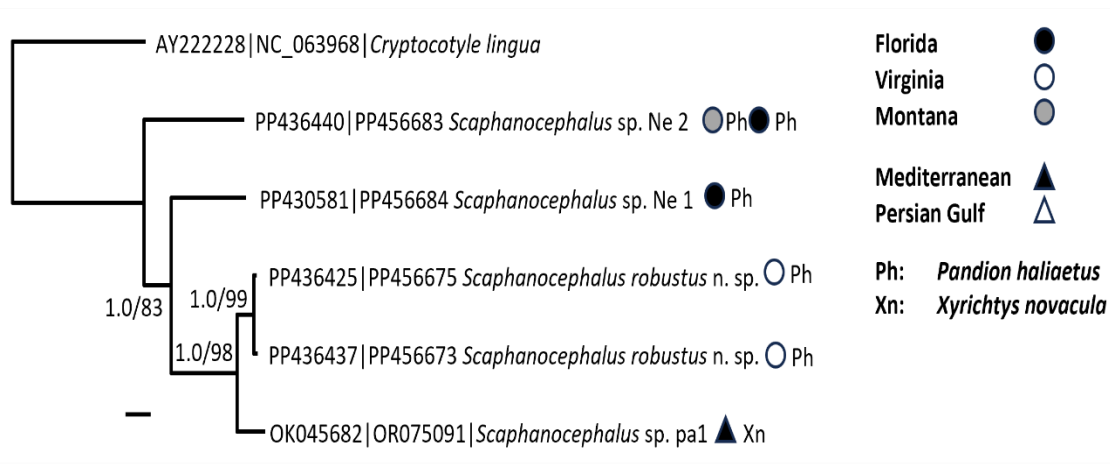
Figure 5. Phylogenetic analysis of concatenated partial 28S (1059 nt) and CO1 (374 nt) sequences of *Scaphanocephalus*. The Maximum Likelihood topology is shown and nodes are annotated with posterior probability (Bayesian Inference) / bootstrap support (1000 replicates in Maximum Likelihood). The ML tree was generated using substitution model GTR+I; the BI tree using HKY+I with unlinked parameters in an alignment partitioned into 28S and CO1.
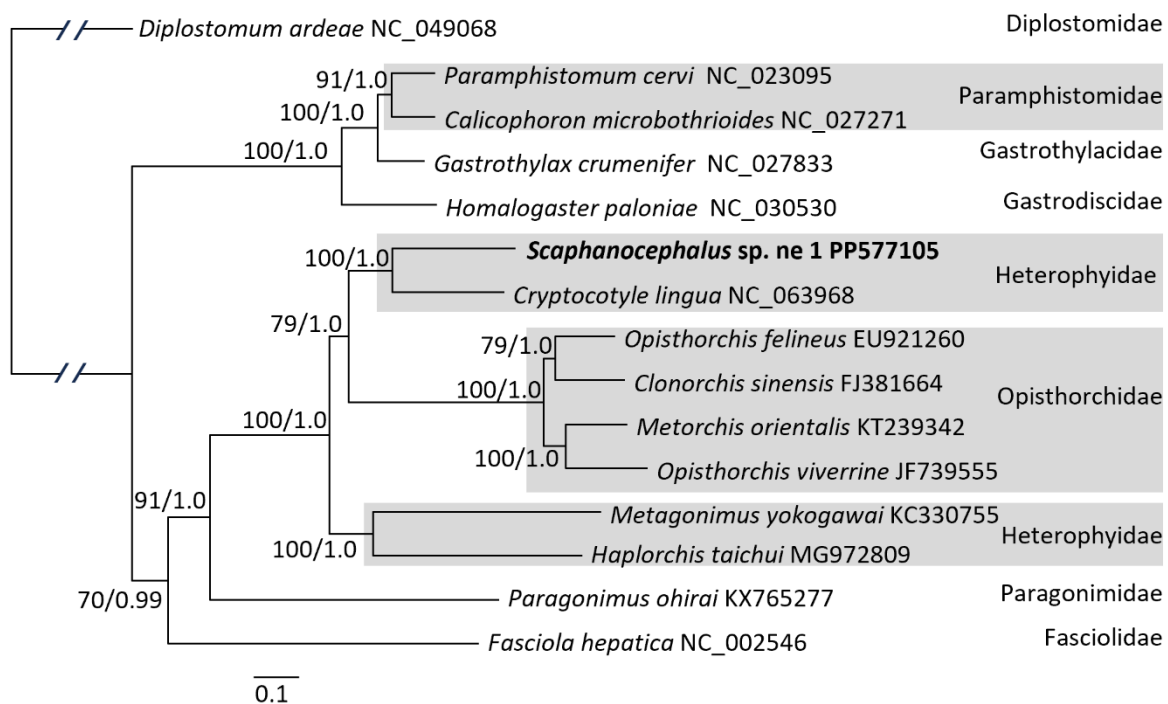
Figure 6. Phylogenetic analysis of complete mitochondrial genomes (1059 nt). The Maximum Likelihood topology is shown and nodes are annotated with posterior probability (Bayesian Inference) / bootstrap support (1000 replicates in Maximum Likelihood). Maximum Likelihood and Bayesian inference trees were both generated using substitution model GTR+G+I

Table 1. Specimens of *Scaphanocephalus* sequenced in the present and prior studies. CO1 BC= barcode region of cytochrome *c* oxidase I, CO1 JB3= 3' region of cytochrome *c* oxidase I, 28S=partial sequence of 28S nuclear rDNA; MT genome=whole mitochondrial genome; rDNA operon= nuclear rDNA array, with partial external transcribed spacer; MSB=Museum of Southwestern Biology. A more detailed version of the information in this table is in Supplementary Table 1.

Species										
			GenBank Accessions							
	Developmental stage (n)	CO1 BC	CO1 JB3	28S	MT genome	rDNA operon	Museum vouchers	Host	Locality	Source
Scaphanocephalus robustus n. sp.										
	Adult (5)		PP456658-9, PP456666-8	PP436423-4, PP436427-9			MSB:Para:49134-5, 49139-40	*Pandion haliaetus*	Curaçao	Present study
	Adult (11)		PP456660-5, PP456676-9	PP436426, PP436430-4			MSB:Para:49141-2, 49146	*P. haliaetus*	Florida	Present study

Adult (7)	PP456673, PP456675	PP456669-75	PP436425, PP436435-7	MSB:Para:49136-8, 49143-5	*P. haliaetus*	Virginia	Present study
Metacercaria (1)			OR794154		*Mugil curema*	Celestún, Yucatán, Mexico	González-García et al. 2023

Scaphanocephalus sp. ne2

Adult (1)	PP456684	PP456683			*P. haliaetus*	Florida	Present study
Adult (1)	PP456682		PP436440		*P. haliaetus*	Montana	Present study
Metacercariae (10)			MN160569		*Acanthurus bahianus, A.*	St. Kitts	Dennis et al. 2019

coeruleus, A. chirurgus

Scaphanocephalus sp. ne1

Stage					Host	Location	Reference
Adult (1)				PP577105 PP430581	*P. haliaetus*	Florida	Present study
Metacercariae (2)	PP456680	PP456680-1	MN160570		*A. chirurgus*	St. Kitts	Present study, Dennis et al. 2019
Metacercariae (2)			PP436438-9		*A. bahianus*	Curaçao	Present study
Metacercariae* (2)			MK680936		*Sparisoma chrysopterum*	Bonaire	Kohl et al. 2019

Adult (3)*		OR794151-3		*P. haliaetus*	Campeche, Mexico	González-García et al. 2023

Scaphanocephalus sp. pa1

Metacerca riae (8)	OR075091	OK045681-8		*Xyrichtys novacula*	Balearic Islands, Mediterran ean	Present study, Cohen-Sánchez et al. 2023

Scaphanocephalus sp. pa2

Metacerca riae (5)		MT461356		*Siganus argenteus*	Persian Gulf,	Al Salem et al. 2021

Saudi

Arabia

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* Identified as *Scaphanocephalus expansus* by study authors

Table 2. Summary of species distributions in the present and past studies based on morphological and molecular (cytochrome *c* oxidase I, CO1; partial nuclear 28S rDNA, 28S) data. Detailed presentation of morphological and molecular support for species is in the Results and Tables 3,4 and Figures 1-6.

	Hosts	Geographic distribution	Morphological and distributional support for species	Molecular support for species	Source
Scaphanocephalus robustus n. sp.	*Pandion haliaetus*	Curaçao; Florida, Virginia, USA; Yucatán, Mexico	Smaller and with greater hindbody width/total length ratio greater than in *S. expansus* from type region. Eggs slightly longer, narrower than *S. australis*. Unlikely to be conspecific with Old World species.	Divergence in CO1 from all other species; divergence in 28S from all other species except *Scaphanocephalus* sp. pa1; phylogenetically separate lineage from *Scaphanocephalus* sp. pa1 (Fig. 5).	Present study; Gonzalez-Garcia *et al.*, 2023

| *Scaphanocephalus* sp. ne2 | *P. haliaetus, Acanthurus tractus, A. coerulus, A. chirurgus* | St. Kitts; Montana, USA | Unlikely to be conspecific with Old World species. | Divergence in 28S from all other species except *Scaphanocephalus* sp. pa2. Divergence in CO1 from all other species (CO1 not available from *Scaphanocephalus* sp. pa2). Phylogenetically separate lineage according to CO1, but not 28S (identical in *Scaphanocephalus* sp. pa2). | Present study, Dennis *et al*., 2019 |
| *Scaphanocephalus* sp. ne1 | *P. haliaetus, Acanthurus tractus, A. coerulus, Sparisoma chrysopterum* | Florida, USA; St. Kitts; Curaçao; Campeche, Mexico | Egg length smaller than in *S. robustus* n. sp. Body total length, anterior width and posterior width generally smaller than in Old World descriptions of *S. expansus*. Unlikely to be conspecific with Old World species. | Divergence and phylogenetic lineage independence in CO1 and 28S from all other species. | Present study, Kohl *et al*., 2019; Dennis et al. 2019; Gonzalez-Garcia *et al*., 2023 |

Scaphanocephalus sp. pa1	*Xyrichtys novacula*	Mediterranean	Unlikely to be conspecific with New World species.	Divergence in CO1 from all other species; divergence in 28S from all other species except *S. robustus* n. sp.; phylogenetically separate lineage from *S. robustus* n. sp. according to concatenated 28S+CO1, but not if 28S/CO1 analyzed separately.	Present study; Cohen-Sánchez *et al.*, 2023b
Scaphanocephalus sp. pa2	*Siganeus argentus*	Persian Gulf	Unlikely to be conspecific with New World species.	Divergence in 28S, and phylogenetically independent lineage from all other species except *Scaphanocephalus* sp. ne2, which shares identical 28S. No CO1 available from *Scaphanocephalus* sp. pa2.	Al Salem *et al.*, 2021

Table 3. Morphometrics of *Scaphanocephalus* described in the present and prior studies, as mean (range), ± standard deviation, n measured, in μm.

	Scaphanocephalus robustus n. sp.	*Scaphanocephalus* sp. ne1 (as *S. expansus*)	*S. expansus*	*S. expansus*	*S. expansus*	*S. expansus*	*S. australis*	*S. adamsi*	*S. adamsi*
	Current study	González-García et al. 2023	Creplin, 1842	Jägerskiöld, 1904	Dubois et al., 1960	Foronda et al., 2009	Johnston, 1916	Tubangui, 1933	Kifune and Kugi, 1979
Location	Virginia, Florida, USA; Curaçao	Campeche, Mexico	Germany	El Tor, Egypt	Sète, France	Canary Islands, Spain	Terrigal, Australia	Manilla, Philippines	Oita, Kyushu, Japan

Host species	P. haliaetus	P. haliaetus	P. haliaetus	P. haliaetus	P. haliaetus	P. haliaetus	Icthyophaga leucogaster	Bodianus mesothorax	Buteo buteo burmanicus
Life stage	Adult	Adult	Adult	Adult	Adult	Adult	Adult	Metacercaria	Adult
N	15	19	4		3	43?	3		≈10
Length	3340 (2178-4178) ±646, 9	(2256-5063)	5569 (4794-6204)	5000	(4650-4760)	3261 ± 449	(3000-3250)	(3020-3070)	(3500-3840)
Anterior width	2725 (2376-3248) ±302, 7	(1177-2792)	3948	3200 (1000-1500 unextended)	(2730-3290)	1690 ± 455	2200	(2150-2370)	(2920-3560)
Posterior length	2391 (1386-3406) ±599, 11		4230	-	-	-	-	-	-

Posterior width	1047 (634-1366) ±238, 14	(643-1375)	1410	1000	-	926 ± 126	1170	(1170-1210)	(1510-1760)
Oral sucker length	98 (77-117) ±13, 6	(64-129)	-	128	138	101 ± 6	134	120	(110-150)
Oral sucker width	108 (71-133) ±27, 4	(74-116)	-	96	125	86 ± 3	107	(110-130)	(100-140)
Prepharynx length	22 (8-40) ±16, 4	(14-43)			(37-42)	84 ± 12			
Pharynx length	76 (55-85) ±11, 6	(54-88)		80	(81-95)	76 ± 7	96	80-90	(95-110)
Pharynx width	61 (38-69) ±12, 6	(40-87)		60	(78-85)	58 ± 12	75	80	(70-100)
Esophagus	167 (83-250) ±66, 6	(151-309)		272	180	215 ± 52	270	60-110	(80-140)
Ventrogenital length	288 (198-359) ±55, 10	(214-415)	-	270	-	-	276	180-210	

Ventrogenital width	198 (109-273) ±52, 11	(178-279)	-	-	-	-	214	130-190	
Ovary length	159 (55-396) ±98, 13	(105-263)	-	160	(270-380)	171 ± 37	155	100-110	(160-180)
Ovary width	388 (203-535) ±109, 13	(226-454)	-	400	(700-870)	298 ± 75	407	270-280	(600-660)
Seminal receptacle length	120 (47-178) ±50, 15					105 ± 37			
Seminal receptacle width	381 (139-625) ±154, 15					1498 ± 227			
Anterior testis length	399 (234-772) ±141, 15	(221-465)	-	480-560	440-690	375 ± 74	-	190-230	(420-630)
Anterior testis width	734 (422-970) ±183, 15	(442-912)	-	740-860	1270-1300	593 ± 72	-	740-760	(1060-1220)

Posterior testis length	421 (198-609) ±123, 15	(276-495)	-	480-560	580-840	428 ± 89	-	200-270	(560-780)
Posterior testis width	739 (453-1069) ±182, 15	(424-951)	-	740-860	1150-1270	634 ± 86	-	720-800	(1200-1340)
Egg length	32 (28-34) ±2.2, 13	(22-24)	-	27	29, 22-40	25 ± 5.5	29, 24-32	-	(34-36)
Egg width	18 (14-20) ±1.9, 13	(18-21)	-	16	17, 16-20	18 ± 3.8	20, 19-21	-	(17-18)
Excretory pore to posterior margin	65 (38-102) ±22, 6						70-130		

Table 4. Average genetic distances (range in parenthesis) within and among species of *Scaphanocephalus* in cytochrome *c* oxidase I (CO1) and 28S rDNA (uncorrected p expressed as percent difference among all sites in alignments of 29 sequences of CO1, 33 of 28S)

	Scaphanocephalus sp. ne1	*Scaphanocephalus* sp. pa1	*Scaphanocephalus* sp. pa2	*Scaphanocephalus robustus* n. sp.	*Scaphanocephalus* sp. ne2	Overall
CO1 intraspecific				0.92 (0-2.54)	0 (0-0)	0.92 (0-2.54)
CO1 interspecific	15.04 (13.49-16.62)	0.066 (5.0-16.74)		12.68 (5.0-17.57)	17.2 (16.32-17.76)	12.84 (5.0-17.76)
28S intraspecific	0.0489 (0-0.2318)	0.1507 (0-0.3897)		0.2205 (0-0.5208)	0 (0-0)	0.1835 (0-0.5208)
28S interspecific	1.3541 (1.1008-2.0481)	0.7203 (0-2.0796)	1.5805 (0-1.9531)	1.0169 (0-2.0735)	1.6998 (0-2.0796)	1.1264 (0-2.0796)

Table 5. Position and characteristics of protein-coding and non- coding sequences from the mt genome of *Scaphanocephalus* sp. ne 1 (GenBank accession: PP577105)

| Name | Type | Coding Position | | | Codon |
		Start	Ends	Length	Start/Stop
cox3	CDS	1	648	648	ATG/TAG
trnH	tRNA	657	727	71	
Cytb	CDS	739	1848	1110	ATG/TAG
nad4l	CDS	1853	2119	267	GTG/TAG
nad4	CDS	2080	3366	1287	ATG/TAG
trnQ	tRNA	3375	3437	63	
trnF	tRNA	3490	3555	66	
trnM	tRNA	3555	3625	71	
atp6	CDS	3626	4141	516	ATG/TAG
nad2	CDS	4213	5082	870	ATG/TAA
trnV	tRNA	5094	5167	74	
trnA	tRNA	5208	5269	62	
trnD	tRNA	5300	5364	65	
nad1	CDS	5365	6267	903	GTG/TAG
trnN	tRNA	6274	6344	71	
trnP	tRNA	6346	6413	68	
trnI	tRNA	6418	6482	65	
trnK	tRNA	6493	6559	67	
nad3	CDS	6562	6918	357	GTG/TAG
trnS	tRNA	6970	7030	61	
trnW	tRNA	7043	7111	69	

cox1	CDS	7118	8656	1539	TTG/TAG
trnT	tRNA	8657	8722	66	
16S rrnL	rRNA	8723	9706	984	
trnC	tRNA	9707	9770	64	
12S rrnS	rRNA	9778	10527	750	
cox2	CDS	10528	11199	672	ATG/TAG
nad6	CDS	11183	11644	462	ATG/TAG
trnY	tRNA	11647	11711	65	
trnL1	tRNA	11713	11779	67	
trnS2	tRNA	11777	11846	70	
trnL2	tRNA	11861	11926	66	
trnR	tRNA	11961	12030	70	
nad5	CDS	12031	13614	1584	GTG/TAG
trnE	tRNA	13654	13721	68	
trnG	tRNA	14027	14095	69	

Table 6. Comparison of mitochondrial protein-coding genes from the Opisthorchiidae.

Species	GenBank accession number	Base composition (%)					Total bp length	Total number of codons
		A	C	G	T	A+T		
Scaphanocephalus sp. ne 1	PP577105	16.0	12.7	26.8	44.5	60.5	10,215	3,405
Cryptocotyle lingua	NC_063968	20.5	11.5	22.2	45.8	66.3	10,173	3,391
Haplorchis taichui	MG972809	17.0	11.9	27.9	43.1	60.2	10,164	3,360
Metagonimus yokogawai	KC330755	15.3	14.3	29.7	40.7	56	10,245	3,415
Opisthorchis felineus	EU921260	15.3	12.1	27.2	45.3	60.7	10,218	3,106
Metorchis orientalis	KT239342	14.4	11.4	26.9	47.2	61.6	10,176	3,392
Clonorchis sinensis	FJ381664	15.7	11.9	27.3	45.1	60.8	10,209	3,403