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Rich but morphologically problematic: an integrative approach to taxonomic resolution of the genus *Neospirorchis* (Trematoda: Schistosomatoidea)

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ABSTRACT

Neospirorchis Price, 1934 is a genus of blood flukes that infect the cardiovascular system, including vessels surrounding the nervous systems of marine turtles. Although the genus comprises just two named species, the available molecular data suggest substantial richness which has not yet been formally described. The lack of description of species of *Neospirorchis* is probably explained by their small, slender, elongate bodies, which allow them to infect numerous organs and vessels in their hosts, such as the heart and peripheral vessels of nervous system, endocrine organs, thymus, mesenteric vessels, and gastrointestinal submucosa. This morphology and site of infection means that collecting good quality, intact specimens is generally difficult, ultimately hampering the formal description of species. Here we supplement limited morphological samples with multi-locus genetic data to formally describe four new species of *Neospirorchis* infecting marine turtles from Queensland, Australia and Florida, USA; *Neospirorchis goodmanorum* n. sp. and *Neospirorchis deburonae* n. sp. are described from *Chelonia mydas*, *Neospirorchis stacyi* n. sp. is described from *Caretta caretta*, and *Neospirorchis chapmanae* n. sp. from *Ch. mydas* and *Ca. caretta.* The four new species are delineated from each other and the two known species based on the arrangement of the male and female reproductive organs, on the basis of cytochrome c oxidase subunit 1 (*cox*1), internal transcribed spacer 2 (ITS2), and 28S ribosomal DNA (rDNA) molecular data, site of infection, and host species. Molecular evidence for three further putative, presently undescribable, species is also reported. We propose that this integrated characterisation of species of *Neospirorchis*, based on careful consideration of host, molecular and key morphological data, offers a valuable solution to the slow rate of descriptions for this important genus. We provide the first known life cycle data for *Neospirorchis* in Australian waters, from Moreton Bay, Queensland; consistent with reports from the Atlantic, sporocysts were collected from a terebellid polychaete and genetically matched to an unnamed species of *Neospirorchis* infecting *Ch. mydas* from Queensland and Florida.

1. Introduction

Turtle blood flukes (TBFs) are an important assemblage of parasitic flatworms that infect the cardiovascular system of marine and freshwater turtles worldwide, currently comprising 97 known species (WoRMS Editorial Board (2022). World Register of Marine Species. Available from https://www.marinespecies.org at VLIZ. Accessed 2022-11-09). TBFs are the causative agent of the highly pathogenic disease spirorchiidiasis in turtles, and have the potential to pose a significant threat to the health of individual turtles and perhaps even overall health of some populations (Chapman et al., 2019). Spirorchiidiasis often produces non-specific clinical signs such as sunken eyes, lethargy, and emaciation, rendering diagnosis difficult (Glazebrook et al., 1981, 1989; Flint et al., 2010; Stacy et al., 2010; Work et al., 2015). Although TBFs infect a multitude of organs (e.g., the heart, major aortae, minor blood vessels, liver, and spleen), only species of *Neospirorchis* Price, 1934 have been reported to infect the vessels surrounding the CNS as adults (together with other blood vessels and organs) (see Stacy et al., 2017). Parasitism of the vessels surrounding the CNS by species of *Neospirorchis* can be associated with neurological issues in severe infections, but this pathology is incompletely understood (Jacobson et al., 2006; Stacy et al., 2010).

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Bullard and Dutton (2022) recently proposed a radical reorganisation of the family-level classification of the Spirorchiidae (previously all the TBFs), recognising six families consistent with the topology of a 28S rDNA phylogenetic analysis. Although this classification resolves the paraphyly of the Spirorchiidae relative to the Schistosomatidae, we have two concerns. First, some of the family group names are seemingly incorrect. The family group name Baracktrematidae Bullard & Dutton, 2022 is one of the six proposed TBF families, comprising the genera *Baracktrema* Roberts, Platt & Bullard, 2016, *Neospirorchis* and *Unicaecum* Stunkard, 1925. However, the authors evidently overlooked existing and available family group names: Unicaecuminae (proposed by Mehra (1934)) and Neospirorchinae (proposed by Skrjabin (1951)). More broadly, we remain unconvinced of the need for six families for this group. In view of these reservations, and pending further work in the area, we refer to the Spirorchiidae sensu lato as TBFs, although we recognise the existing senior synonym of the Baracktrematidae.

Species of *Neospirorchis* are characterised by an elongate, slender body, the absence of a ventral sucker, the possession of simple caeca that fuse in the posterior half of the body, a single tubular ovary and testis, and operculate eggs that lack polar projections (Platt, 2002). Just two species of *Neospirorchis* are currently recognised: *Neospirorchis pricei* Manter & Larson, 1950, known only from *Caretta caretta* Rafinesque, and *Neospirorchis schistosomatoides* Price, 1934, known only from *Chelonia mydas* (Linnaeus). However, there is molecular evidence that the genus is far richer than currently understood. Stacy et al. (2017) conducted a comprehensive study on the molecular diversity of *Neospirorchis* infecting turtles off the Atlantic Ocean and Gulf of Mexico coasts of Florida (USA), identifying 20 distinct corresponding genotypes in the internal transcribed spacer 2 (ITS2) and cytochrome c oxidase subunit 1 (*cox*1) datasets; undoubtedly these represent multiple undescribed species. However, as noted by the authors, collecting specimens of *Neospirorchis* suitable for morphological characterisation is exceptionally difficult, which hampers the formal description of new species.

Similar to the other blood fluke families (the Schistosomatidae Stiles & Hassall, 1898 and Aporocotylidae Odhner, 1912), TBFs have life cycles that involve just one intermediate host (see Corner et al., 2022b); the rest of the Trematoda overwhelmingly use two intermediate hosts (Poulin and Cribb, 2002). Almost all known TBF life cycles (both freshwater and marine) involve gastropods as intermediate hosts, with vermetid gastropods shown to be the intermediate hosts for at least one lineage of marine TBFs (the genera *Amphiorchis* Price, 1934, *Hapalotrema* Looss, 1899, *Learedius* Price, 1934) (see Cribb et al., 2017; Corner et al., 2022b), and ampullariid, physid, and planorbid gastropods for freshwater species (Ciccheto et al., 2021). Remarkably, species of *Neospirorchis* have been shown to use terebellid polychaetes as intermediate hosts, despite falling within a large clade comprising freshwater, gastropod-infecting species (de Buron et al., 2018). To date, it is not known if the infection of terebellid polychaetes is common to all members of *Neospirorchis*.

Here we report on species of *Neospirorchis* infecting marine turtles from off the coasts of southeastern Queensland, and northeastern New South Wales, Australia. We formally describe four new species and provide the first known life cycle data for *Neospirorchis* in Australia. We consider the issues surrounding the morphological description of species of *Neospirorchis* and propose that the description of difficult to collect trematodes need not be based on multiple, entire specimens; instead, careful consideration of host, molecular and key morphological data can be used. Finally, we assess the use of two commonly sequenced, non-overlapping regions of the *cox*1 mtDNA gene, and propose to standardise the *cox*1 data analysed in future TBF studies.

2. Materials and methods

2.1. Collection of hosts and parasites

Terebellid polychaetes were collected in Australia by hand from the surface of seagrass beds in eastern Moreton Bay (27°27′S, 153°24′E) in September 2020, and from under rocks and coral rubble along the shores of North Stradbroke Island (27°29.8′S, 153°23.9′E) and Goat Island (27°31′S, 153°23′E) in Moreton Bay between February 2020 and July 2021, and Heron Island (23°26.7′S 151°55′E) in August, 2019, February, 2020, and April, 2021, and Lizard Island (14°40.8′S 145°26.8′E) on the Great Barrier Reef in November 2020. Specimens were dissected with the aid of a stereomicroscope, with an incision into the body cavity, and examined for the presence of sporocysts and cercariae. Asexual stages were heat-fixed in near boiling 0.85% saline solution and immediately transferred to 70% ethanol.

Adult parasites were opportunistically collected from freshly deceased *Ca. caretta*, *Ch. mydas*, *Eretmochelys imbricata* (Linnaeus) and *Lepidochelys olivacea* (Eschscholtz). All turtles were rescued from locations along the eastern coast of Australia and were euthanised and necropsied at Australia Zoo Wildlife Hospital (Beerwah, Queensland, Australia) or Dolphin Marine Conservation Park (Coffs Harbour, New South Wales, Australia) by qualified veterinarians when deemed to be too sick or injured for rehabilitation. The heart, liver, major aorta, and spleen were removed from the host, cut open and initially grossly examined for the presence of trematodes. After all visible trematodes were collected, each organ was placed separately into a container and washed thoroughly with 0.85% saline solution. Once all suspended sediment had settled to the bottom of the wash container, three-quarters of the supernatant was discarded. If needed, clean 0.85% saline solution was added to the wash container, left to settle, and the supernatant was discarded again. When sufficiently clean, the sediment was examined in a Petri dish under a stereomicroscope. Mesenteries were examined by being separated from the gastrointestinal tract and removed from the host, laid out flat on a dark cutting mat, with each vessel examined by running the blunt side of a scalpel blade along the length of the vessel, forcing out blood and trematodes when present. After all vessels were sufficiently cleared, the contents on the mat were washed into a container, left to settle, and the supernatant discarded. The sediment was then examined in a Petri dish under a stereomicroscope. The brain was grossly examined in a Petri dish under a stereomicroscope, and when feasible, trematodes were removed from vessels. If time did not permit, the brain was stored in 80% ethanol for later dissection. After all organs were removed, the body cavity was filled with 0.85% saline solution, and immediately poured into a large container. The solution was left to settle, and the supernatant was discarded. The sediment was then transferred to a smaller container, and processed as described for the heart, liver, major aorta, and spleen. When found alive, trematodes were fixed without pressure in near boiling 0.85% saline solution and immediately transferred to 70% ethanol for parallel morphological and molecular characterisation. When found dead, trematodes were placed immediately in 70% ethanol.

2.2. Morphological analysis

Specimens for morphological analysis (both adult and asexual stages) were washed with fresh water, stained with Mayer's haematoxylin, destained using 1.0% HCl, and neutralised using 0.5% NH_3 (aq). Specimens were then dehydrated with a graded series of ethanol, cleared with methyl salicylate, and permanently mounted in Canada balsam. Measurements were made using cell-

Sens Standard imaging software (Olympus, Tokyo, Japan) with an Olympus SC50 digital camera mounted on an Olympus BX-53 compound microscope. All measurements are in μm and are presented as a range unless stated otherwise. Drawings of specimens were made with the aid of a drawing tube, attached to the same Olympus BX-53 compound microscope, and digitalised with Adobe Illustrator CC 2022. Type and voucher specimens were lodged in the Queensland Museum (QM), Brisbane, Australia.

Species delineation was based on an integrative interpretation of morphological and genetic data, following, as a starting point, the criteria of trematode species recognition proposed by Bray et al. (2022) (i.e., reciprocal monophyly in the most discriminating available molecular marker (here, ITS2 and *cox*1) + distinction in morphology and/or host range). To comply with the recommendations set out in Article 8.5 of the amended 2012 version of the International Code of Zoological Nomenclature (ICZN, 2012), details of the new species have been submitted to ZooBank and registered with Life Science Identifiers (LSID), which are provided in the taxonomic summaries.

2.3. Molecular sequencing of parasites and hosts

Total genomic DNA was extracted from sporocysts, hologenophores (Pleijel et al., 2008) of adult parasites, and small sections of terebellid tissue using a standard phenol/chloroform extraction technique described by Sambrook and Russell (2001). Following recommendations by Blasco-Costa et al. (2016), two nuclear rDNA regions and one mtDNA region were amplified from parasite material; the partial D1-D3 region of the 28S rDNA region, the complete ITS2 rDNA and two partial regions of the *cox*1 mtDNA. Amplification of the 28S and ITS2 rDNA regions were performed as described by Cutmore et al. (2016), using the primers LSU5 (5′-TAG GTC GAC CCG CTG AAY TTA AGC A-3′; Littlewood, 1994) and 1500R (5′-GCT ATC CTG AGG GAA ACT TCG-3′; Snyder and Tkach, 2001) for the 28S region, and the primers 3S (5′-GGT ACC GGT GGA TCA CGT GGC TAG TG-3′; Morgan and Blair, 1995) and ITS2.2 (5′-CCT GGT TAG TTT CTT TTC CTC CGC-3′; Cribb et al., 1998) for the ITS2 region. Amplification of the partial *cox*1 region was performed following Wee et al. (2017), using the primers Dig_cox1Fa (5′-ATG ATW TTY TTY TTY YTD ATG CC-3′; Wee et al., 2017) and Dig_cox1R (5′-TCN GGR TGH CCR AAR AAY CAA AA-3′; Wee et al., 2017), and Stacy et al. (2017), using the primers JB3 (5′-TTT TTT GGG CAT CCT GAG GTT TTA T-3′; Bowles et al., 1995) and JB4.5 (5′-TAA AGA AAG AAC ATA ATG AAA ATG-3′; Bowles et al., 1995). The partial 16S mtDNA region was amplified for terebellid polychaetes using the primers 16SANNF (5′-GCG GTA TCC TGA CCG TRC WAA GGT A-3′; Sjölin et al., 2005) and 16SBRH (5′-CCG GTC TGAA CTC AGA TCA CGT-3′; Palumbi, 1996) following the cycling procedures described by Lavesque et al. (2021). Sanger sequencing of purified DNA was completed at the Australian Genome Research Facility, using ABI Big Dye™ v.3.1 chemistry following the manufacturers protocols. Sequencing of the ITS2, *cox*1, and 16S regions was conducted with the PCR amplification primers, while sequencing of the 28S regions was performed using the primers 300F (5′-CAA GTA CCG TGA GGG AAA GTT G-3′; Littlewood et al., 2000) and ECD2 (5′-CCT TGG TCC GTG TTT CAA GAC GGG-3′; Littlewood et al., 1997). Geneious® version 10.2.3 (Kearse et al., 2012) was used to assemble and edit contiguous sequences.

2.4. Polychaete identification

Terebellid polychaetes examined during this study were identified through a combination of morphological and molecular data. 16S mtDNA sequence data generated for terebellid polychaetes were analysed relative to sequences available in GenBank. These data corresponded to nine broad genotypes identified as *Lanice* sp. (from Lizard Island), *Loimia* sp. (from Moreton Bay), *Reteterebella* sp. (from Lizard Island), *Thelepus australiensis* Hutchings & Smith, 1997 (from Moreton Bay), *Thelepus* sp. 1 (from Lizard Island), *Thelepus* sp. 2 (from Lizard Island), Terebellidae sp. 1 (from Moreton Bay), Terebellidae sp. 2 (from Heron Island), and Terebellidae sp. 3 (from Heron Island). Specimens identified as *Reteterebella* sp. on the basis of molecular data were identified as *Reteterebella lirrf* Nogueira, Hutchings & Carrerette, 2015 upon morphological analysis. Two additional species were sampled for which no molecular data were generated. These two species were identified as *Loimia ingens* (Grube, 1878) (from Moreton Bay), and *Reteterebella queenslandia* Hartman, 1963 (from Heron Island) on the basis of morphology. Sequence data for genotypes relating to *Lanice* sp., *Loimia* sp., *Reteterebella lirrf*, *T. australiensis*, *Thelepus* sp. 1, *Thelepus* sp. 2, Terebellidae sp. 1, Terebellidae sp. 2, and Terebellidae sp. 3 are lodged in GenBank (OQ732930–38). Morphological vouchers for the specimens identified as *L. ingens* and *R. queenslandia* are lodged at the Queensland Museum (QM G240630and QM G240631, respectively).

2.5. Phylogenetic analyses

Newly generated ITS2 and *cox*1 sequence data were aligned separately with sequence data available on GenBank (Supplementary Table S1) in MEGA X (Kumar et al., 2018) using MUSCLE, with Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering for iterations 1 and 2. Indels greater than three bp and affecting more than 5% of sequences were removed. To determine the correct reading frame in the *cox*1 datasets, the aligned data was translated in MESQUITE v.3.6, using the echinoderm/flatworm mitochondrial translation table, inspected for stop codons and trimmed to start on position one. All three codons were examined for substitution saturation in DAMBE 7 (Xia et al., 2003; Xia and Lemey, 2009; Xia, 2018); significant substitution saturation was not detected in either *cox*1 dataset. Neighbour-joining (NJ) analyses were conducted independently on the aligned and trimmed *cox*1 and ITS2 datasets in MEGA X to determine species identity and boundaries. The parameters for the NJ analyses were: "test of phylogeny = bootstrap", "no. of bootstrap replications = 10,000", "model/method = No. of differences", "substitutions to include = d: Transitions + Transversions" and "rates among sites = Uniform rates". Pairwise distance matrices were generated in MEGA X to determine intra- and interspecific variation within the *cox*1 and ITS2 datasets. The parameters used for the pairwise distances matrices were the same as those used in the NJ analyses. Species of *Hapalotrema* Looss, 1899 and *Learedius* Price, 1934 were designated as functional outgroups in all NJ analyses.

Newly generated partial 28S rDNA sequence data were aligned with those from related taxa available on GenBank (Supplementary Table S1) using MUSCLE version 3.7 (Edgar, 2004) on the CIPRES portal (https://www.phylo.org/), with ClustalW sequence weighting and UPGMA clustering for iterations 1 and 2. The resulting alignments were refined by eye in MEGA X by removing indels greater than three base positions and affecting more than 5% of sequences. The aligned and trimmed 28S rDNA dataset was analysed for the estimated best fit nucleotide substitution model using jModelTest 2.1.10 (Darriba et al., 2012). The model TMV + I + Γ was predicted to be the best estimator by both the Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC), and the closest approximation of this model was used in both the Bayesian inference (BI) and maximum likelihood (ML) analyses. The BI analysis was completed using Mr Bayes version 3.2.7a (Ronquist et al., 2012), while the ML analysis was completed using RaxML vesion 8.2.12 (Stamatakis, 2014), both run on the CIPRES portal. The BI analysis was run with the following parameters: 10,000,000 generations (ngen = 10,000,000) with two runs (nruns = 2) containing

four Markov Chain Monte Carlo chains (MCMC chains) (nchains = 4), sampling every 1,000th tree (samplefreq = 1,000), "nst = 6", "rates = invgamma", "ngammacat = 4" (default), and "ratepr = va riable". The first 30% of sampled trees were disregarded, "sumt burnin value = 3,000" and "sump burnin value = 3,000". Nodal support for the ML analysis were generated by performing 1,000 bootstrap pseudoreplicates. *Elopicola bristowi* Orélis-Ribeiro & Bullard, 2017 and *Elopicola franksi* Orélis-Ribeiro & Bullard, 2017 were designated as the functional outgroup, following the relationships inferred by Cutmore et al. (2023).

2.6. Data accessibility

The aligned and trimmed ITS2, *cox*1 and 28S rDNA datasets used in this study have been uploaded to the Mendeley data repository with the https://doi.org/10.17632/4sm28btz2w.2.

3. Results

3.1. General results

A total of 106 marine turtles were examined from southeastern Queensland and northeastern New South Wales, Australia. Of these, 29 were infected with adult TBFs which morphologically conform to the genus *Neospirorchis*: 27 of 82 *Ch. mydas* and two of 10 *Ca. caretta*. No adult specimens of *Neospirorchis* were recovered from the single *L. olivacea* individual examined or from the 13 individuals of *E. imbricata*. From the newly collected adult *Neospirorchis* specimens, 35 ITS2, 61 (Dig_cox1 primers) / 26 (JB primers) *cox*1 and 13 28S rDNA sequences were generated.

A total of 560 terebellid polychaetes relating to 11 putative species were examined for asexual stage infections from Moreton Bay, Heron Island and Lizard Island (Table 1). Of these, just one of eight *L. ingens* examined from Moreton Bay was infected. From this single infected polychaete, large numbers of immature sporocysts (>1,000) were recovered from the haemocoel but no cercariae were recovered; on this basis we interpret the infection as not yet patent. One ITS2, two *cox*1 (one of each primer set) and one 28S rDNA sequence were generated for the polychaete infection.

3.2. Molecular taxonomy

Two separate *cox*1 datasets were generated. The first used the general trematode primers Dig_cox1Fa and Dig_cox1R, published by Wee et al. (2017) (Fig. 1A). No comparable *cox*1 data are available on GenBank for *Neospirorchis* for this primer set. In the phylogenetic analysis of this dataset, the 62 sequences formed 11 major lineages of which nine were replicated, differing at 32–100 bp (6.8%–21.1%). These 11 lineages were interpreted initially as operational taxonomic units (OTUs). Intra-lineage variation was ≤15 bp (≤3.2%) and nodal support for the nine replicated lineages was >99. These nine OTUs, represented by more than one sequence, showed intra-clade variation: 0–15 bp (0–3.2%) in OTU1; 2 bp (0.4%) in OTU2; 14 bp (3%) in OTU3; 14 bp (3%) in OTU4; 1 bp (0.2%) in OTU5; 10 bp (2.1%) in OTU6; 1 bp (0.2%) in OTU7; 5 bp (1.1%) in OTU8; and 3 bp (0.6%) in OTU10. The distinction recognised between inter- and intra-lineage variation was at least 17 bp. OTUs 1, 2, 4, 6, 7, 8, 9, 10, and 11 infect *Ch. mydas*, while OTUs 3 and 5 infect *Ca. caretta*. No OTU was found to infect more than one species of turtle.

To enable comparison with existing *cox*1 data on GenBank, a second *cox*1 dataset was generated using the primers JB3/JB4.5 of Bowles et al. (1995) (Fig. 1B); 27 sequences were generated from the same DNA extractions used in the amplification of the sequences mentioned above. The 11 OTUs identified above differed by 20–71 bp in this dataset, whereas intra-lineage variation (based on significantly fewer sequences) was at 1–12 bp. The distinction between inter- and intra-lineage variation for this dataset was at least 8 bp. On this basis, a criterion of an intra-clade variation of <16 bp (midway between intra- and inter-lineage distinction) was used to assign lineages in the entire JB3/JB4.5 dataset. Using this criterion, a total of 24 lineages were identified, including the 11 identified above and an additional 13 for sequences generated by Stacy et al. (2017) and de Buron et al. (2018). Nodal support for all lineages with replication was >90. The 27 new sequences generated from turtles and terebellids did not match *cox*1 data from GenBank, however, four of the OTUs recognised in this study also contained sequence data of Stacy et al. (2017): OTU1 included sequence data representing *Neospirorchis* sp. Neogen3 of Stacy et al. (2017); OTU3 included sequence data representing *Neospirorchis* sp. Neogen2 of Stacy et al. (2017), differing by 6–7 bp (1.7–2%); OTU5 included sequence data representing *Neospirorchis* sp. Neogen6 of Stacy et al. (2017), differing by 5 bp (1.4%); and OTU10 included sequence data representing *Neospirorchis* sp. Neogen15 of Stacy et al. (2017), differing by 3–5 bp (0.9–1.4%). The phylogenetic topologies generated from the two *cox*1 datasets were identical, but nodal support was generally lower in the dataset generated using the JB3/JB4.5 primer set. No intra-clade variation was seen in OTU2. The remaining OTUs with more than one replicate all showed intra-clade variation: 1–16 bp (0.3–4.6%) in OTU1: 6–9 bp (1.7–2.6%) in OTU3; 10 bp (2.9%) in OTU4; 5 bp (1.4%) in OTU5; 2 bp (0.6%) in OTU6; 1 bp (0.3%) in OTU8; 1–5 bp (0.3–1.4%) in OTU10; 6 bp (1.7%) in OTU13; 1 bp (0.3%) in OTU16; 1–2 bp (0.3–0.6%) in OTU19; 8 bp (2.3%) in OTU20; 15 bp (4.3%) in OTU22; and 1 bp (0.3%) in OTU23.

Phylogenetic analyses of the combined *Neospirorchis* ITS2 dataset resulted in 21 lineages (Fig. 2). Lineages were recognised where the intra-lineage variation was <5 bp and where, if lineages were represented by more than one sequence, the nodal support for the clade was >75. Six of the 21 lineages correspond to seven of the 11 *cox*1 OTUs identified in the Dig_cox1Fa/Dig_cox1R dataset (OTUs 1–7). ITS2 sequence data generated here corresponding to the previously identified *cox*1 OTUs1, 6, and 7 also incorporate data identical to those from GenBank, which represent *Neospirorchis* sp. neospirgen3/neospiregen1 of Chapman et al. (2016) for OTU1, and *Neospirorchis* sp. neospirgen2 of Chapman et al. (2016) for OTUs 6 and 7. The remaining ITS2 lineages incorporate highly similar sequence data from GenBank: *Neospirorchis* sp. Neogen2 of Stacy et al. (2017) was similar to data representing OTU3 (new sequences differ by 2 bp; 0.66%); *Neospirorchis* sp. Neogen6 of

Table 1
Host, location, and infection prevalence data for terebellid species examined during this study.

Host	Location	TBF species	Prevalence
Lanice sp.	Lizard Island, GBR	Uninfected	0 of 2
Loimia ingens	North Stradbroke Island, MB	*Neospirorchis* sp. Neogen16	1 of 8 (12.5%)
Loimia sp.	Dunwich, MB	Uninfected	0 of 40
	Goat Island, MB	Uninfected	0 of 28
Reteterebella lirrf	Lizard Island, GBR	Uninfected	0 of 3
Reteterebella queenslandia	Heron Island, GBR	Uninfected	0 of 283
Thelepus australiensis	Dunwich, MB	Uninfected	0 of 4
	Goat Island, MB	Uninfected	0 of 178
Thelepus sp. 1	Lizard Island, GBR	Uninfected	0 of 5
Thelepus sp. 2	Lizard Island, GBR	Uninfected	0 of 1
Terebellidae sp. 1	Dunwich, MB	Uninfected	0 of 1
	Goat Island, MB	Uninfected	0 of 5
Terebellidae sp. 2	Heron Island, GBR	Uninfected	0 of 1
Terebellidae sp. 3	Heron Island, GBR	Uninfected	0 of 1

TBF, turtle blood fluke; GBR, Great Barrier Reef, Queensland, Australia; MB, Moreton Bay, Queensland, Australia.

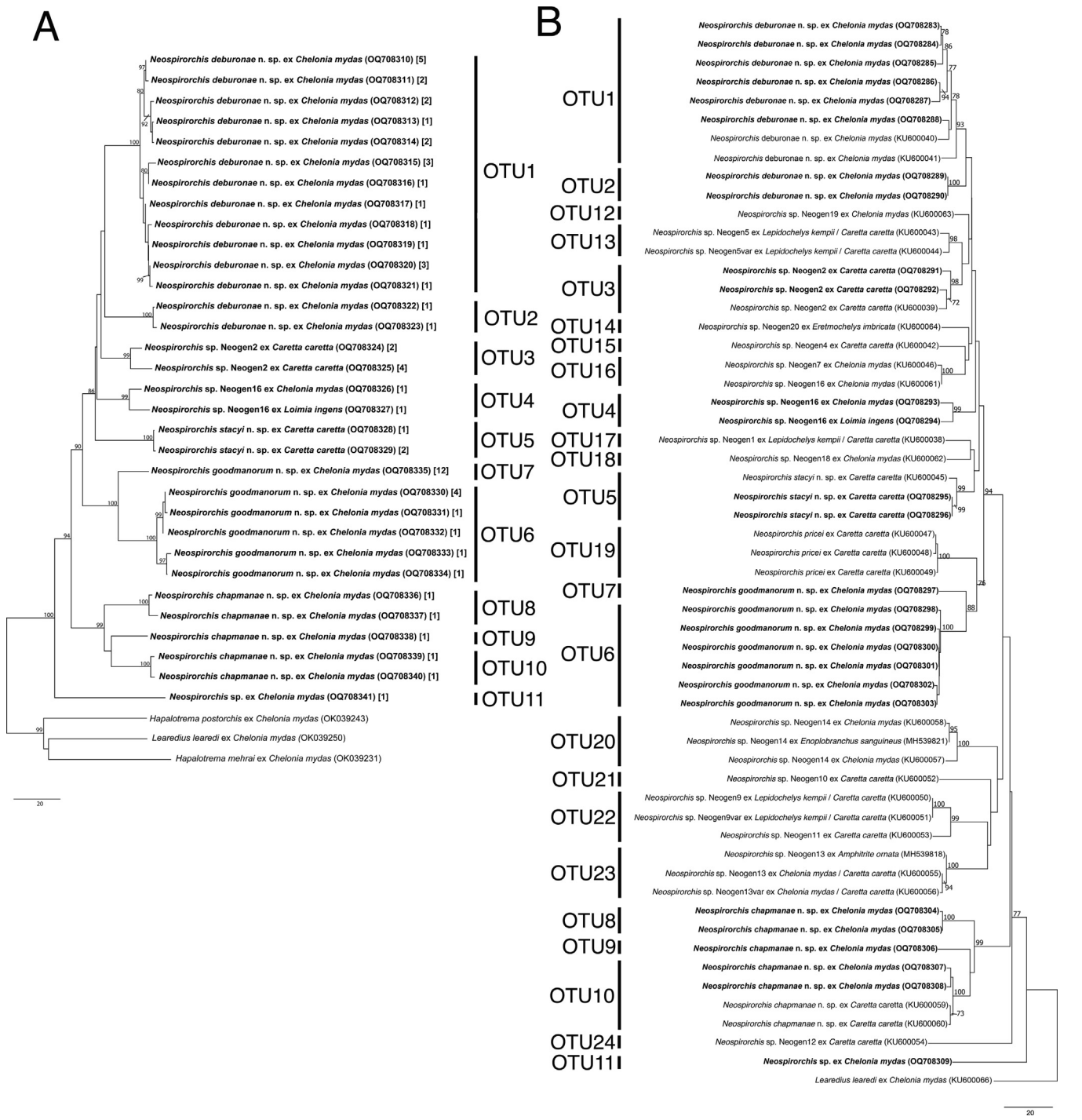

Fig. 1. Phylogenetic relationships between *Neospirorchis* spp. sequenced during this study inferred from rooted neighbour-joining analysis of the cytochrome c oxidase subunit 1 (*cox*1) datasets. Taxa in bold represent sequences generated during this study. (A) Data generated using the primers Dig_cox1Fa and Dig_cox1R. (B) Data generated using the primers JB3 and JB4.5. Nodal support < 70 are not shown. Numbers in square brackets denote numbers of replicates for individual genotypes. The scale bars indicate the number of base pair differences. OTU, Operational taxonomic unit.

Stacy et al. (2017) (new sequences differ by 2–3 bp; 0.66–0.99%); *Neospirorchis* sp. Neogen16 of Stacy et al. (2017) was similar to data representing OTU4 (new sequences differ by 2 bp; 0.66%); *Neospirorchis* sp. Neogen3 of Stacy et al. (2017) was similar to data representing OTU2 (new sequences differ by 2 bp; 0.66%). Interestingly, despite representing two lineages in the *cox*1 datasets (differing by 33–36 bp; 7–7.6%), sequences representing OTUs 6 and 7 were identical in the ITS2 dataset. No ITS2 data representing OTUs 8, 9, 10, or 11 were able to be generated.

3.3. Integrated taxonomy

Morphological examination of specimens revealed four relatively easily distinguishable morphospecies, which can be differentiated principally on the basis of genital pore position relative to the ovary and testis (see Discussion). The four morphospecies correspond to eight *cox*1 OTUs identified in the Dig_cox1Fa/Dig_cox1R dataset: i) OTUs 1 + 2; ii) OTU5; iii) OTUs 6 + 7; and iv) OTUs 8 + 9 + 10. In each case, where multiple OTUs correspond to a single

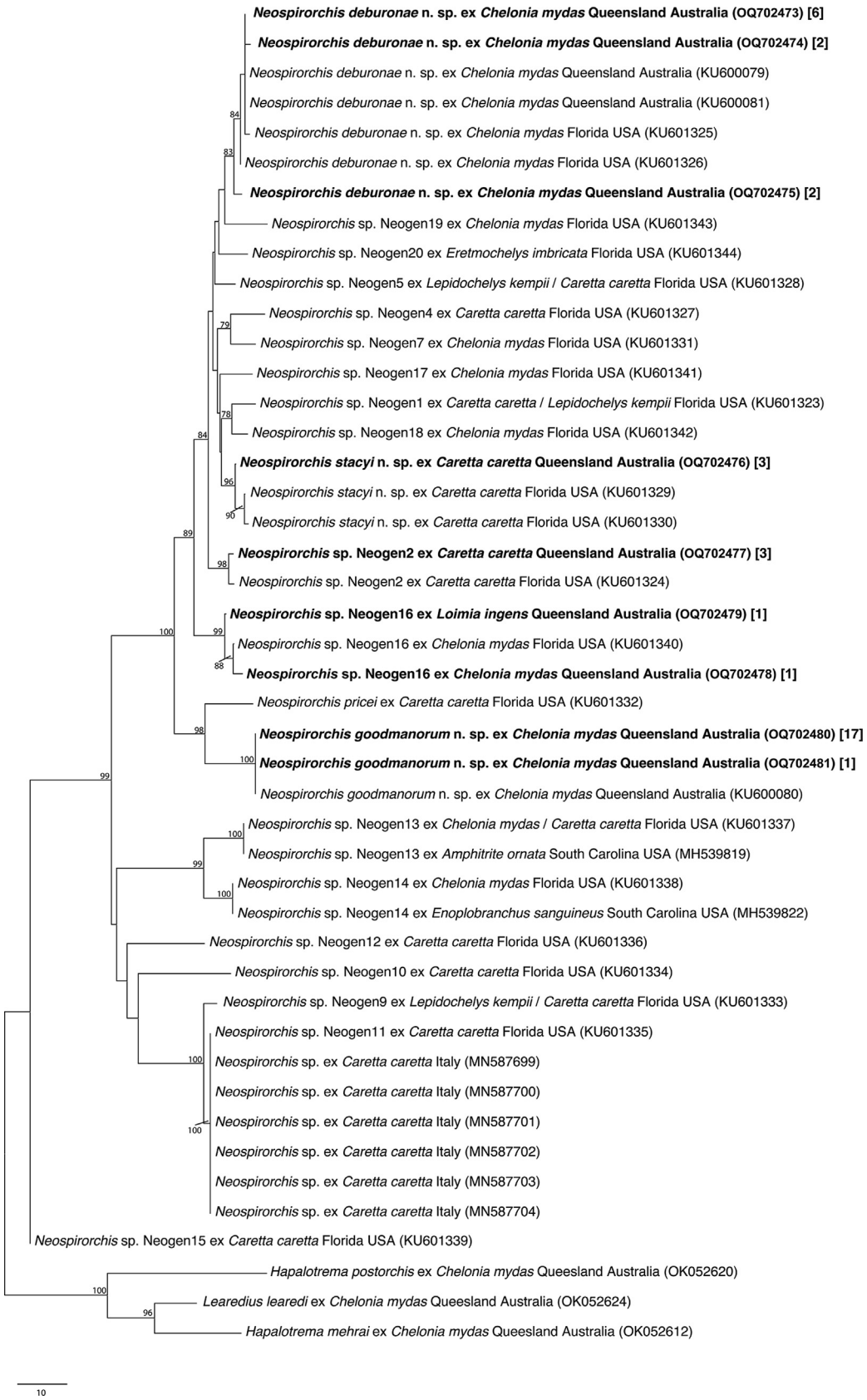

Fig. 2. Phylogenetic relationships of *Neospirorchis* spp. sequenced during this study and those available on GenBank inferred from a rooted neighbour-joining analysis of the internal transcribed spacer 2 (ITS2) dataset. Taxa in bold represent sequences generated during this study. Nodal support < 70 not shown. Numbers in square brackets denote numbers of replicates for individual genotypes. The scale bar indicates the number of base pair differences.

morphotype, they ultimately form clades in the *cox*1 or ITS2 analyses. No suitable morphological specimens were available for the remaining OTUs to assign them to morphospecies. Although they cannot be formally described, the finding of OTUs 3 and 4 (*Neospirorchis* Neogen2 and Neogen16 of Stacy et al. (2016), respectively) in this study extends their known range, from Florida, USA to Queensland, Australia. Additionally, the finding of OTU11, which did not match any available sequence data on GenBank, shows that there still remains uncharacterised genetic diversity of *Neospirorchis*. Notably, specimens of OTUs 1 + 2 (classified as the same morphospecies) were only found in the vessels around brain of *Ch. mydas*, while specimens of OTUs 8 + 9 + 10 (classified as the same morphospecies) were only found in the mesenteric vessels of *Ch. mydas*. The four morphospecies identified have sufficient material for formal descriptions, which are provided below. Each species is delineated using an integrated approach, considering distinctions in morphology, host, infection site and molecular data.

3.4. Taxonomy

Family Unicaecidae Mehra, 1934 **(emend.)**

Synonyms: Baracktrematidae Bullard & Dutton, 2022; Neospirorchinae Skrjabin, 1951.

Type-genus: *Unicaecum* Stunkard, 1925

Other accepted genera: *Baracktrema* Roberts, Platt, and Bullard, 2016; *Neospirorchis* Price, 1934.

Remarks: On the basis of the principle of priority (Article 23.1 of the ICZN), Unicaecuminae is the senior synonym of Baracktrematidae and Neospirorchinae, and is here recognised at the family level in line with the proposal of Bullard and Dutton (2022) who, however, proposed a new junior synonym at the family group level. The subfamily name as proposed by Mehra (1934) is malformed, as the case ending of the genitive singular was not deleted (see article 29.3 of the ICZN). Here we propose to formally emend the family-level name (as per article 33.2 of the ICZN) and recognise it as Unicaecidae Mehra, 1934 (emend.).

Adult stages

Genus *Neospirorchis* Price, 1934

3.4.1. *Neospirorchis goodmanorum* n. sp. (Fig. 3)

Type-host: *Chelonia mydas* (Linnaeus), Green sea turtle (Cheloniidae).

Type-locality: Off Fraser Coast (25°13′S 152°42′E), Queensland, Australia.

Other localities: Off Noosa (26°22′S 153°05′E); off Peregian Beach (26°28′S 153°06′E); off Rainbow Beach (25°54′S 153°06′E); off Urangan (25°17′S 152°55′E) Queensland, Australia. Unknown locations along the Queensland coast, Australia (see Chapman et al., 2016).

Site in host: Heart and body wash.

Prevalence: In one of two off Fraser Coast; in two of six off Noosa; in one of three off Peregian Beach; in one of three off Rainbow Beach; in one of two off Urangan. In two of 12 from unknown locations along the coast of southeast Queensland (see Chapman et al., 2016).

Type-material: Holotype (hologenophore QM G240589) and 18 paratypes including four hologenophores (QM G240590–240607).

Representative DNA sequences: *cox*1 mtDNA generated using Dig_cox1Fa/Dig_cox1R: 20 replicates (six submitted to GenBank OQ708330–35). *cox*1 mtDNA generated using JB3/JB4.5: seven replicates (all submitted to GenBank OQ708297–303). ITS2 rDNA: 18 replicates (two submitted to GenBank OQ702480–81); KU600080 of Chapman et al. (2016). 28S rDNA: three replicates (all submitted to GenBank OQ702482–84).

ZooBank registration: The Life Science Identifier (LSID) for *Neospirorchis goodmanorum* is urn:lsid:zoobank.org:act:234C1B4E-2246-4F7B-A99D-4DD4BFFF68C3.

Etymology: The specific name *goodmanorum* is in honour of and in gratitude to the Goodman family for their continued support of the Goodman Foundation. The Goodman Foundation generously supported this research, directly enabled this study, and continues to support conservation focussed research in Moreton Bay.

Description

[Based on 10 complete, whole-mounted, unflattened, gravid worms and 5 hologenophores; measurements in Table 2.] Body slender, elongate, weakly dorsoventrally flattened, aspinous, with minute transverse ridges. Oral sucker small, unspecialised, terminal. Ventral sucker absent. Oesophagus slightly sinuous, thick-walled, generally widening posteriorly. Oesophageal glands enveloping oesophagus posterior to dorsal nerve commissure. Intestine bifurcating anterior to vitelline field. Caeca sinuous, sometimes with irregular swellings, reuniting just posterior to anterior margin of testis, with common caecum terminating near posterior end of body, often overlapping excretory vesicle.

Testis long, compactly coiled, sinistral to caeca, reaching from just anterior to level of caecal reunion to just posterior to anterior margin of ovary. Vas deferens short, slightly sinuous, passing from posterior margin of testis, entering cirrus-sac anteriorly. Cirrus-sac thick-walled, slender, straight to slightly sinuous. External seminal vesicle absent. Internal seminal vesicle an elongate sac, sometimes convoluted, occupying approximately half of cirrus-sac. Transition between internal seminal vesicle and eversible cirrus difficult to detect. Eversible cirrus usually straight, but can be convoluted, muscular, with distinct muscular striations. Common genital pore sub-medial, ventral, just anterior to posterior margin of ovary.

Ovary long, convoluted or loosely coiled, with anterior portion overlapping posterior end of testis and posterior portion terminating just posterior to common genital pore. Oviduct sinuous, arising from posterior margin of ovary, reaching posteriorly to level of vitelline reservoir before turning anteriorly to enter oötype. Uterus thin-walled, initially slender, slightly sinuous, with distinct egg-filled swelling beginning just posterior to posterior margin of ovary in gravid specimens, extending just anterior to common genital pore, before turning posteriorly leading to common genital pore. Metraterm absent. Laurer's canal and canalicular seminal receptacle absent. Mehlis' gland anterior to vitelline reservoir. Vitellarium comprising numerous, densely packed follicles, from just posterior to intestinal bifurcation, somewhat symmetrically distributed in anterior half of body, asymmetrically distributed in posterior half of body, dextral to testis and ovary, mostly ventral to caeca and testis, terminating just anterior to common genital pore. Vitelline duct slender, long, extending from posterior margin of vitelline field to join anterior margin of vitelline reservoir. Vitelline reservoir irregularly shaped, with short, straight duct connecting to oötype. Eggs ovoid, lacking polar processes.

Excretory pore sub-terminal, dorsal. Excretory vesicle Y-shaped with short, straight median stem, anterior extent not observed.

3.4.2. *Neospirorchis deburonae* n. sp. (Fig. 4)

Synonyms: *Neospirorchis* sp. neospirgen1 of Chapman et al. (2016); *Neospirorchis* sp. neospirgen3 of Chapman et al. (2016); *Neospirorchis* sp. Neogen3 of Stacy et al. (2017).

Type-host: Chelonia mydas (Linnaeus), Green sea turtle (Cheloniidae).

Type-locality: Off Buddina (26°42′S 153°09′E), Queensland, Australia.

Other localities: Off Coolum Beach (26°32′S 153°06′E); off Hervey Bay (25°16′S 152°51′E); off Manly (27°27′S 153°12′E); off Margate Beach (27°15′S 153°07′E); off Mooloolaba (26°40′S 153°07′E); off Moreton Island (27°06′S 153°24′E); off Noosa (26°22′S

Table 2
Morphometric data from previous studies and the present collection for species of *Neospirorchis* expressed as a range and mean in micrometres or as percentages.

	Neospirorchis pricei	*Neospirorchis schistosomatoides*	*Neospirorchis goodmanorum*	*Neospirorchis chapmanae*	*Neospirorchis deburonae*	*Neospirorchis stacyi*
Source	Manter and Larson (1950)	Price (1934)	Present study	Present study	Present study	Present study
Length	6,100–8,100	7,450–9,500	4,216–9,438 (7,131)	28,047	>2,628	>8,992
Width	360–540	140–220	185–384 (320)	103–163 (125.2)	228	89–111 (99)
OS Length	52–57	32–40	30–57 (44)	26–29 (27.5)	–	–
OS Width	52–57	32–40	32–58 (48)	24–35 (29.5)	–	–
Pre-nerve commissure distance	–	–	101–160 (129)	94	–	–
Oesophagus length	320–340	595–680	342–529 (435)	–	–	–
Oesophagus width	–	–	47–67 (56)	–	–	–
Pre-vitelline field distance	–	–	427–965 (644)	480–610 (545)	–	–
Distance between intestinal bifurcation and reunion	–	–	1,893–4,766 (3,222)	–	–	–
Right caeca width	–	–	53–141 (101)	–	–	–
Left caeca width	–	–	60–134 (102)	–	–	–
Post-reunion intestine length	–	–	1,871–4,576 (3,374)	–	–	–
Post-reunion intestine width	–	–	36–112 (89)	–	–	–
Post intestinal distance	–	–	25–76 (49)	–	84	–
Testis length	–	–	4,377–7,753 (5,633)	9272–15,746 (12,509)	–	4739
Testis width	–	–	95–191 (140)	43–77 (61.6)	116	64–98 (81)
Testis field length	–	–	1,519–3,319 (2,546)	6,598–11,704 (9,151)	–	4714
Ovary length	–	–	1,674–2,924 (2,304)	1,831–2,310 (2,002)	–	1,726–2,497 (2,180)
Ovary width	–	–	50–107 (76)	54–86 (66.3)	92	51–70 (60)
Ovary field length	–	–	500–1,387 (1,009)	795–1396 (1,077)	–	1523–1,906 (1,731)
Vas deferens length	–	–	83–189 (125)	251–280 (261)	486	684–777 (704)
Post-genital pore distance	900–1,100	1,000–1,200	486–1,098 (742)	1652–2746 (2,118)	716	569–832 (696)
Post-testis distance	~25% of body length	–	–	–	–	–
Cirrus sac length	423	–	409–756 (596)	391–1,249 (726)	1462	1091–1,538 (1,256)
Cirrus sac width	76	–	52–80 (66)	52–56 (54.6)	100	44–47 (45)
Cirrus sac wall thickness	23	–	–	–	–	–
Internal seminal vesicle length	224	–	–	–	–	–
Eversible cirrus length	200	–	–	–	–	–
Mehlis' gland length	–	–	30–44 (38)	77–159 (109)	39	51–54 (53)
Mehlis' gland width	–	–	45–83 (58)	68–90 (78)	41	41–42 (42)
Oviduct length	513	–	–	–	–	–
Vitelline reservoir length	190	–	53–100 (77)	88–131 (103)	176	92–188 (13)
Vitelline reservoir width	152	–	47–87 (59)	58–88 (69)	103	52–76 (62)
Egg number	200+	7–15	13–40	–	–	–
Egg length	36–40	44	35–47 (39)	–	47	35–45 (41)
Egg width	23–24	32	23–34 (26)	–	23	28–39 (33)

OS, oral sucker.

153°05′E); off Nudgee (27°20′S 153°07′E); off Peregian Beach (26°28′S 153°06′E); off Rainbow Beach (25°54′S 153°06′E); off Scarborough (27°12′S 153°07′E); off Urangan (25°17′S 152°55′E), Queensland, Australia. Unknown locations off Queensland, Australia (see Chapman et al., 2016). off Florida, USA (see Stacy et al., 2017).

Site in host: cerebral blood vessels.

Prevalence: In one of one off Buddina; in one of one off Coolum Beach; in one of five off Hervey Bay; in one of four off Manly; in one of one off Margate Beach; in one of three off Mooloolaba; in two of four off Moreton Island; in one of six off Noosa; in one of two off Nudgee; in two of three off Peregian Beach; in one of three off Rainbow Beach; in two of four off Scarborough; in one of two off Urangan.

Type-material: Holotype (hologenophore QM G240608) and five paratypes (hologenophores QM G240609–613).

Representative DNA sequences: *cox*1 mtDNA generated using Dig_cox1Fa/Dig_cox1R: 25 replicates (14 submitted to GenBank OQ708310–323). *cox*1 mtDNA generated using JB3/JB4.5: eight replicates (all submitted to GenBank OQ708283–290); KU600040 and KU600041 of Stacy et al. (2017). ITS2 rDNA: 10 replicates (three submitted to GenBank OQ702473–75); KU600079 and KU600081 of Chapman et al. (2016); KU601325 and KU601326 of Stacy et al. (2017). 28S rDNA: three replicates (all submitted to GenBank OQ702488–490).

ZooBank registration: The Life Science Identifier (LSID) for *Neospirorchis deburonae* is urn:lsid:zoobank.org:act:BD4BCA43-A30B-410C-9487-0264718A8D80.

Etymology: This species is named in honour of Prof. Isaure de Buron, in recognition for her contribution to understanding of the life cycles of *Neospirorchis*.

Description

[Based on one partial-mounted, posterior-end hologenophore, unflattened, gravid worm; measurements in Table 2.]

Body elongate, cylindrical. Anterior section, oral sucker, oesophagus, and intestinal bifurcation not observed. Ventral sucker presumed absent. Posterior end of caeca sinuous, with common caecum terminating near posterior end of body.

Testis large, cylindrical, terminating posterior to anterior margin of ovary, anterior extent not observed. Vas deferens long, straight to slightly sinuous, passing from posterior margin of testis, entering cirrus-sac anteriorly. Cirrus-sac long, thick-walled, slen-

der, mostly straight, containing prominent eversible cirrus. External seminal vesicle absent. Internal seminal vesicle an elongate sac. Transition between internal seminal vesicle and eversible cirrus difficult to detect. Eversible cirrus everted in examined specimen, unspined, muscular. Common genital pore opening midway between posterior margin of ovary and oötype.

Ovary long, tightly coiled, with anterior portion overlapping at least some of posterior end of testis and posterior portion terminating anterior to common genital pore. Oviduct mostly straight, arising from posterior margin of ovary, reaching posteriorly to level of vitelline reservoir before turning anteriorly to enter oötype. Uterus thin-walled, slender, slightly sinuous, extending anterior to posterior margin of ovary, before turning posteriorly leading to common genital pore. Metraterm absent. Laurer's canal and canalicular seminal receptacle absent. Mehlis' gland immediately anterior to vitelline reservoir. Vitellarium comprising numerous, densely packed follicles, anterior extent not determined, mostly ventral to caeca and testis, leading to and joining anterior margin of vitelline reservoir. Vitelline reservoir ovoid, connected by short, slightly sinuous duct to oötype. Eggs ovoid, lacking polar processes.

Excretory pore and vesicle not observed.

3.4.3. Neospirorchis stacyi n. sp. (Fig. 5)

Synonym: *Neospirorchis* sp. Neogen6 of Stacy et al. (2017).

Type-host: Caretta caretta (Linnaeus), Loggerhead turtle (Cheloniidae).

Type-locality: Off Bargara (24°50′S 152°29′E), Queensland, Australia.

Other localities: Off Florida, USA (see Stacy et al., 2017).

Site in host: Blood vessels within liver.

Prevalence: In one of one off Bargara.

Type-material: Holotype (hologenophore QM G240619) and five paratypes (hologenophores QM G240620–24).

Representative DNA sequences: *cox*1 mtDNA generated using Dig_cox1Fa/Dig_cox1R: three replicates (two submitted to GenBank OQ708328–29). *cox*1 mtDNA generated using JB3/JB4.5: two replicates (both submitted to GenBank OQ708295–96); KU600045 of Stacy et al. (2017). ITS2 rDNA: three replicates (one submitted to GenBank OQ702476); KU601329–30 of Stacy et al. (2017). 28S rDNA one sequence (GenBank OQ702485).

ZooBank registration: The Life Science Identifier (LSID) for *Neospirorchis stacyi* is urn:lsid:zoobank.org:act:402B8BF6-120C-4385-A6FA-1798EE87B40A.

Etymology: This species is named in honour of Dr Brian Stacy, in recognition for his contribution to the systematics of turtle blood flukes.

Description

[Based on six partial mounted, unflattened, hologenophore, gravid worms; measurements in Table 2.] Body thread-like, cylindrical, aspinous. Oral sucker and oesophagus not observed. Ventral sucker presumed absent. Anterior extent of caeca not observed. Caeca largely obscured by vitelline follicles, testis, ovary, and cirrus-sac. Common caecum terminating near posterior end of body.

Testis long, with anterior half mostly straight and posterior half loosely coiled, occupying most of body width, reaching from just posterior to level of main vitelline field to just posterior to anterior margin of ovary. Vas deferens long, straight to slightly sinuous, passing from posterior margin of testis, entering cirrus-sac anteriorly. Cirrus-sac thick-walled, long, slender, straight. External seminal vesicle absent. Internal seminal vesicle an elongate sac, occupying approximately two-thirds of cirrus-sac length. Transition between internal seminal vesicle and eversible cirrus difficult to detect. Eversible cirrus straight, muscular, with distinct muscular striations. Common genital pore well posterior to posterior margin of ovary.

Ovary long, convoluted or loosely coiled, anterior portion occupying most of body width, slightly overlapping posterior portion of testis, noticeably narrower posteriorly, terminating just anterior to anterior margin of uterus. Oviduct sinuous, arising from posterior margin of ovary, reaching posteriorly to level of vitelline reservoir before turning anteriorly to enter oötype. Uterus thin-walled, initially slender, mostly straight, widening anteriorly, extending to posterior margin of ovary, before turning posteriorly to common genital pore. Metraterm absent. Laurer's canal and canalicular seminal receptacle absent. Mehlis' gland not observed. Vitellarium comprising numerous, densely packed follicles, filling body width anterior to testis, asymmetrical at level of testis, mostly ventrosinistral to testis, terminating just anterior to common genital pore. Vitelline duct slender, long, extending from posterior margin of vitelline field entering vitelline reservoir anteriorly. Vitelline reservoir irregularly shaped. Eggs ovoid, lacking polar processes.

Excretory pore and vesicle not observed.

3.4.4. Neospirorchis chapmanae n. sp. (Fig. 6)

Synonym: *Neospirorchis* sp. Neogen15 of Stacy et al. (2017).

Type-host: Chelonia mydas (Linnaeus), green sea turtle (Cheloniidae).

Other hosts: Caretta caretta (Linnaeus), loggerhead turtle (Cheloniidae) (see Stacy et al., 2017).

Type-locality: Off Alexandra Headland (26°40′S 153°07′E), Queensland, Australia.

Other localities: Off Margate Beach (27°15′S 153°07′E); off Moreton Island (27°06′S 153°24′E); off Peregian Beach (26°28′S 153°06′E), Queensland Australia. Unknown locations off the coast of southeast Queensland, Australia (see Chapman et al., 2016). Off Florida, USA (see Stacy et al., 2017).

Site in host: Mesenteries.

Prevalence: In one of one off Alexandra Headland; in one of one off Margate Beach; in one of four off Moreton Island; in one of three off Peregian Beach. In one of 11 from unknown locations along the southeast coast of Queensland.

Type-material: Holotype (hologenophore QM G240614) and four paratypes (QM G240615; hologenophores QM G240616–18).

Representative DNA sequences: *cox*1 mtDNA generated using Dig_cox1Fa/Dig_cox1R: five replicates (all submitted to GenBank OQ708337–341). *cox*1 mtDNA generated using JB3/JB4.5: five replicates (all submitted to GenBank OQ708304–08); KU600059 and KU600060 of Stacy et al. (2017). ITS2 rDNA: KU601339 of Stacy et al. (2017). 28S rDNA: five replicates (all submitted to GenBank OQ702491–95).

ZooBank registration: The Life Science Identifier (LSID) for *Neospirorchis chapmanae* is urn:lsid:zoobank.org:act:3084DD66-46DE-477F-B75D-5773B8607E7F.

Etymology: This species is named in honour of Dr Phoebe Chapman, in recognition for her contribution to the systematics of turtle blood flukes.

Description

[Based on one whole-mounted, and four hologenophore, unflattened, worms; measurements in Table 2.] Body thread-like, cylindrical, aspinous. Oral sucker small, unspecialised, terminal. Ventral sucker absent. Dorsal nerve commissure just posterior to oral sucker. Oesophagus straight. Oesophageal glands not observed. Level of intestinal bifurcation undetermined. Caeca often obscured by vitelline follicles, sinuous, caecal reunion anterior to anterior margin of testis, with common caecum terminating near posterior end of body.

Testis long, loosely coiled, anterior extremity in posterior half of body, terminating posteriorly just posterior to posterior margin of vitelline field. Vas deferens long, slightly sinuous, passing from posterior margin of testis, entering-sac anteriorly. Cirrus-sac thick-walled, slender, straight to slightly sinuous. External seminal vesicle absent. Internal seminal vesicle an elongate sac, occupying approximately half of cirrus-sac length. Transition between inter-

nal seminal vesicle and eversible cirrus difficult to define. Eversible cirrus usually straight, muscular, minute spines present immediately posterior to a distinct thickening of the cirrus wall. Common genital pore anterior to anterior margin of ovary.

Ovary long, convoluted or tightly coiled, distinctly separated from testis, anterior margin posterior to common genital pore, posterior margin anterior to Mehlis' gland. Oviduct sinuous, filled with sperm, arising from posterior margin of ovary, reaching posteriorly to level of vitelline reservoir before turning anteriorly to enter oötype. Uterus thin-walled, slender, slightly sinuous. Metraterm absent. Laurer's canal and canalicular seminal receptacle absent. Mehlis' gland anterior to vitelline reservoir. Vitellarium comprising numerous follicles, terminating just anterior to posterior margin of testis; follicles oblong in anterior of body, gradually becoming spherical to irregular shaped in posterior of body, ventral to caeca and testis. Vitelline duct slender, long, extending posteriorly from posterior margin of vitelline field to join anterior margin of vitelline reservoir. Vitelline reservoir spherical to irregular shaped, with short, straight to slightly sinuous duct connecting to oötype. Eggs not observed.

Excretory pore and vesicle not observed.

Remarks

Here, we report the presence of apparent, minute spines on the cirrus in *N. chapmanae*. This is significant, as cirrus spines have not been reported in any TBF species. We note, however, that the spines are posterior to a thickening of the part of the male duct which best interpreted as the pars-prostatica. This apparent arrangement of features strongly suggests that spines would not be useful, as the pars-prostatica of trematodes does not evert with eversible cirri, and thus the spines of *N. chapmanae* would not be on the external surface of an everted cirrus. Examination of additional specimens is needed to determine if cirrus spines are truly present in this species.

Asexual stages

3.4.5. Neospirorchis sp. Neogen16 of Stacy et al. (2017) (Fig. 7)

Definitive host: *Chelonia mydas* (Linnaeus), green sea turtle (Cheloniidae).

Locality: Florida, USA.

New material

Intermediate host: *Loimia ingens* (Grube, 1878)

Locality: off North Stradbroke Island (27°27′S 153°24′E), Moreton Bay, Queensland, Australia.

Site in host: Haemocoel.

Deposition of specimens: Voucher specimens deposited in the Queensland Museum (QM G240625–29).

Representative DNA sequences: *cox*1 mtDNA generated using Dig_cox1Fa/Dig_cox1R: one submitted to GenBank OQ708327. *cox*1 mtDNA generated using JB3/JB4.5: one submitted to GenBank OQ708294; KU600061 of Stacy et al. (2017). ITS2 rDNA: one submitted to GenBank OQ702479; KU601340 Stacy et al. (2017). 28S rDNA: one submitted to GenBank OQ702487.

Description

Sporocyst

[Based on 40 immature, unflattened sporocysts] Body oblong, 238–510 (407) × 95–194 (156), containing up to 19 developing cercariae or sporocysts, none close to fully developed. Birth pore terminal, conspicuous.

3.5. Phylogenetic results

Partial 28S rDNA sequence data were generated from 14 samples relating to five clades identified in the ITS2 dataset, and 10 clades identified in the *cox*1 datasets. The final, trimmed alignment yielded 1,332 characters for analysis. ML and BI analyses produced identical phylograms (Fig. 8) and are consistent with the findings of Snyder (2004), Pinto et al. (2015), Corner et al. (2022b), and Bullard and Dutton (2022) in that the Spirorchiidae sensu lato is paraphyletic relative to the Schistosomatidae. All sequences representing species of *Neospirorchis* formed a well-supported clade, sister to Spirorchiidae sp. 3 (an unidentified freshwater species), within a large, mostly freshwater TBF clade. The *Neospirorchis* clade comprises two smaller, well-supported clades, one consisting of *N. goodmanorum*, *N. stacyi*, *Neospirorchis* Neogen16 of Stacy et al. (2017) (including the infection from a terebellid), and *N. deburonae*, and the other consisting of *N. chapmanae*, and three undescribed species [one from *Ca. caretta* from Italy (LT882716), and two from terebellid polychaetes from South Carolina, USA (MH539820 and MH539823)].

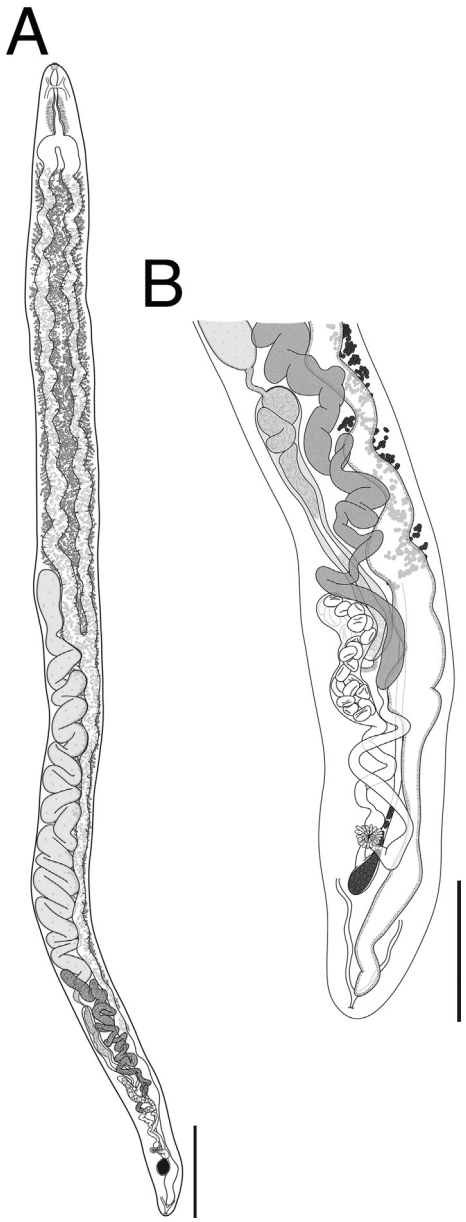

Fig. 3. *Neospirorchis goodmanorum* ex *Chelonia mydas*. (A) Whole worm, dorsally mounted, paratype. (B) Dorsal view of terminal genitalia, holotype. Scale bars: A, 500 μm; B, 250 μm.

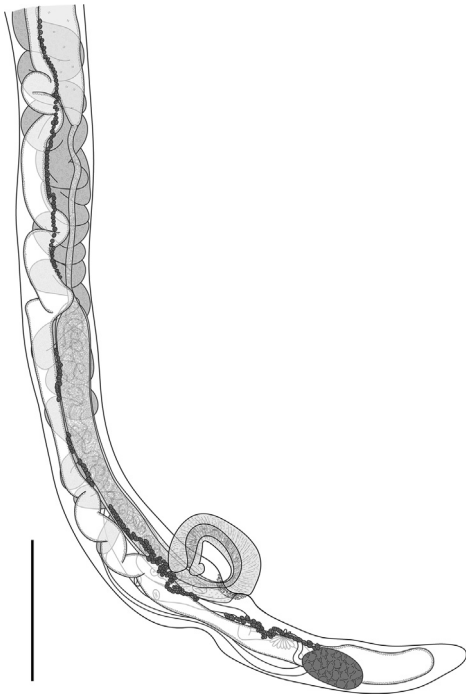

Fig. 4. Posterior end of *Neospirorchis deburonae* ex *Chelonia mydas*, holotype. Scale bar: 100 μm.

3.6. Comparison of cox1 markers

Pairwise distance matrices were generated for all sequences present in both *cox*1 datasets and base pair differences between sequences were plotted relative to each other to assess the difference in inferred intra- (Fig. 9A) and inter-specific (Fig. 9B) variation between the two datasets. Both markers behaved similarly, and no significant anomalies were present. 47 of the 59 pairwise comparisons showed higher intra-specific variation in the Dig_cox1Fa/Dig_cox1R dataset than in the JB3/JB3.5 dataset, and 274 of the 289 pairwise comparisons showed higher inter-specific variation for that dataset.

4. Discussion

4.1. Recognition of Neospirorchis spp.

The description of new helminth species typically involves the examination of multiple whole specimens, with recent trematode studies often including sequenced hologenophores that allow sequence data to further support proposed species hypotheses (e.g., Hernández-Orts et al., 2019; Faltýnková et al., 2020; Cutmore et al., 2021; Corner et al., 2022a; Duong et al., 2022; Wee et al., 2022). While we agree that the use of multiple whole and hologenophored specimens is desirable and should be encouraged when possible, the present study is an example of when this is not feasible.

Several helminth taxa were characterised by elongate form, difficulty of collection from non-gastrointestinal tissues, and paucity of morphological characters which renders them especially taxonomically challenging. Among nematodes, species of the genus *Huffmanela* Moravec, 1987 of fishes are typically described on the basis of only morphology of trapped eggs and host identity (e.g., Justine, 2004; Justine and Iwaki, 2014). Several filarioid genera are proving rich in cryptic species with highly limited morphological distinctions (see Cháves-González et al., 2022). Among trematodes, threadlike didymozoids and species of *Trichobilharzia* Skrjabin & Zakharow, 1920 (Schistosomatidae) are similarly problematic (see Davis et al., 2021; Louvard et al., 2022). The genus *Neospirorchis* can be considered strongly in this category of taxonomic difficulty. Good quality specimens are exceptionally difficult to obtain, partly due to problems in the examination of fresh hosts (e.g., Werneck et al., 2016; Marchiori et al., 2017; Santoro et al., 2020) and partly due to their tiny, fragile nature. As a result, despite the apparent richness demonstrated first by Stacy et al. (2017), just two species had been described prior to this work. There is evidently a taxonomic impediment to the characterisation of species of this genus. Here we have adopted what we consider to be a pragmatic integrated approach which takes advantage of what morphological information is available (and always requires some such evidence at least as the basis for type specimens) together with host identity, site in host and, critically, molecular data. This approach has enabled the description of four new species of the minimum of seven species that we infer as present in marine turtles off the Queensland coast. Even though three of the four novel species described here are based on just a few incomplete specimens, despite thorough examination of nearly 110 marine turtles, all are presently distinguishable on the basis of a combination of morphology, host identity, and site in host, which appears to confer some discriminatory capacity. However, it is clear that molecu-

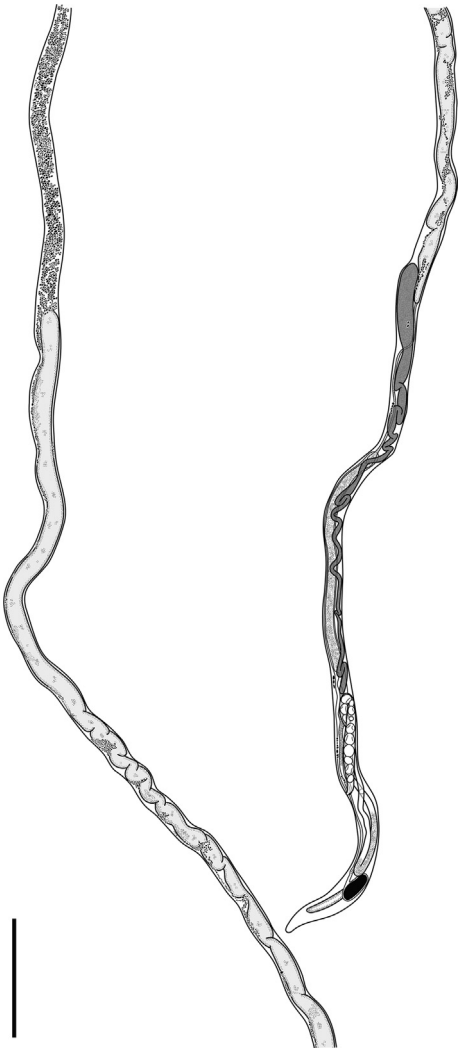

Fig. 5. Posterior end of *Neospirorchis stacyi* ex *Caretta caretta*, paratype. Scale bar: 400 μm.

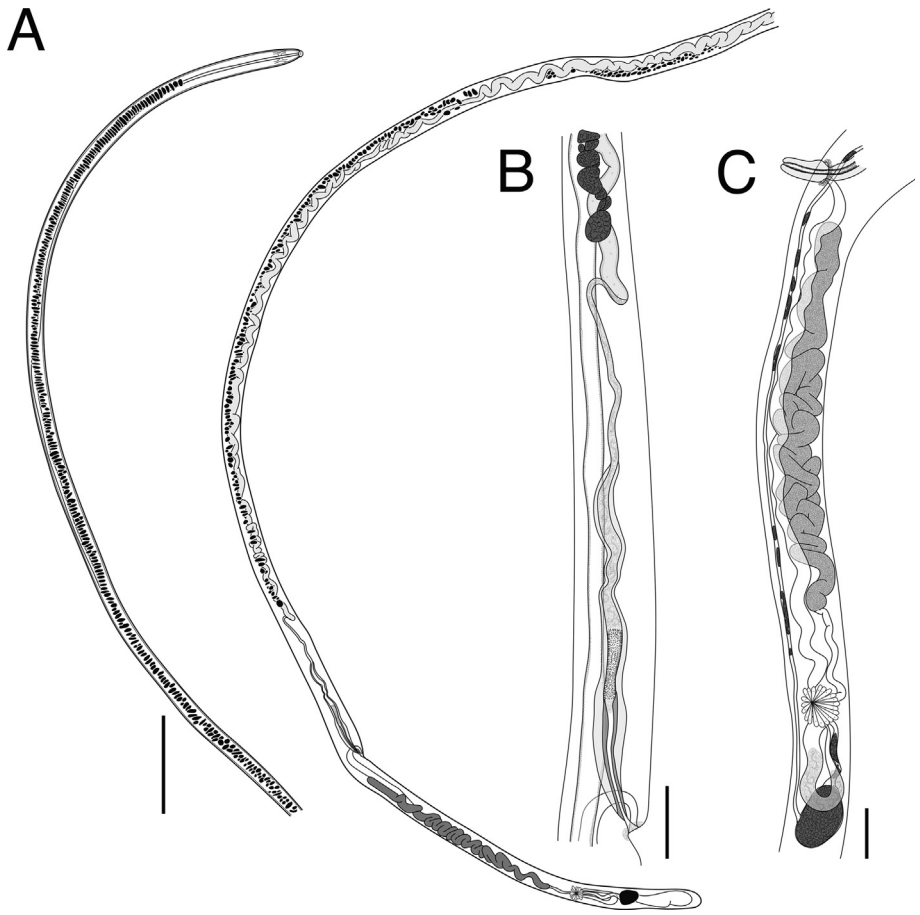

Fig. 6. *Neospirorchis chapmanae* ex *Chelonia mydas*. (A) Anterior and posterior ends of worm, middle of body missing, holotype. (B) Ventral view of male genitalia, holotype. (C) Ventral view of egg-forming complex, paratype. Scale bars: A, 500 μm; B, C, 100 μm.

lar data, particularly *cox*1 data, provide the clearest basis for distinction. Our view is that species should continue to be described on the basis of some sort of integrated approach as employed here, but that routine identification will be most reliably done by sequencing which, importantly, can be done successfully with partial specimens, or samples that are significantly morphologically degraded. In addition to identifying adult worms, molecular delineation lends itself to being useful in non-invasive surveys such as molecular-based faecal examinations. A comprehensive molecular dataset, with most or all species of interest delineated on the basis of molecular data, would allow the identification of samples from host faeces, fast-track life cycle studies with the use of environmental DNA, and enable improved understanding of infections recovered from stranded, decomposing hosts.

The clear problem in the unusually heavy reliance on molecular data for the distinction of species of *Neospirorchis* is the extensive genetic variation between *cox*1 sequences. For three of the four species described here (*N. chapmanae*, *N. deburonae*, and *N. goodmanorum*) there are at least two well-separated (although ultimately monophyletic) clades of *cox*1 sequences differing by at least 33 bp, and at most 49 bp. All such distinctions were represented by both *cox*1 markers explored. These differences are comparable to some interspecific differences in the genus (as low as 34 bp). The basis for considering these disparate sequences to relate to single species is in our use of objective species recognition criteria, specifically reciprocal monophyly in the most discriminating available molecular marker (here, ITS2 and *cox*1) and a clear similarity in morphology, host species infected, and site within host (discussed below). The level of *cox*1 variation reported here is comparable to that between some species of teleost infecting trematodes, both across geographic range (i.e., *Preptetos luguncula* (54 bp); *Bivesicula claviformis* (17–23 bp); and *Elphrobates chaetodontis* (15–24 bp) (see Bray et al., 2022; Cribb et al., 2022; Cutmore and Cribb, 2022)) and in complete sympatry (i.e., *Preptetos luguncula* (22–23 bp) and *Preptetos zebravaranus* (22–28 bp) (see Bray et al., 2022)). However, these examples all occur in site-attached hosts. In contrast, marine turtles have wide ranging movements, evidenced by their complex population structures (Jensen et al., 2019). This wide distribution likely results in turtles acquiring TBF infections from multiple localities, subsequently carrying them to novel regions during migrations between breeding and foraging grounds, where they may cycle with limited gene flow. Alternatively, the observed *cox*1 variation may have arisen from historical host separation events, causing parasites to also form the distinct populations seen in the mitochondrial data. These characteristics are consistent with the substantial genetic variation for trematodes in marine turtles, amplified in *cox*1 datasets because it is a non-recombining region (see below for discussion regarding the usage of the different *cox*1 markers).

ITS2 and *cox*1 data generated in this study provides strong evidence that at least five species of *Neospirorchis* (three named and two unnamed) have distributions encompassing at least the western Atlantic Ocean or Gulf of Mexico and the far Western Pacific Ocean. These wide distributions agree with what is described for other species of marine turtle trematodes, including *Amphiorchis* sp. of Cribb et al. (2017), *Hapalotrema mistroides* (Monticelli, 1896) Stiles & Hassall, 1908, *Hapalotrema postorchis* Rao, 1976, *Learedius learedi* Price, 1934, and *Plesiochorus cymbiformis* Looss,

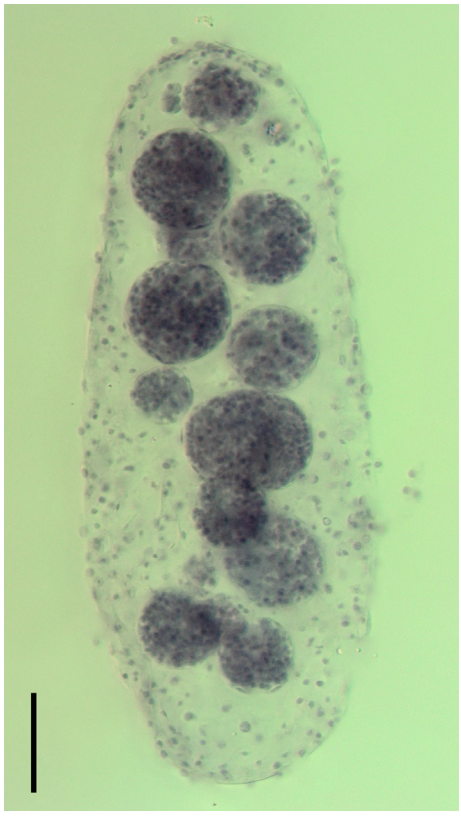

Fig. 7. Immature sporocyst of *Neospirorchis* sp. Neogen16 of Stacy et al. (2017) ex *Loimia ingens*. Scale bar: 50 μm.

1901, all of which have been shown to have a near global distribution on the basis of molecular data (see Chapman et al., 2015; Corner et al., 2022a, 2022b). Other than for turtle parasites, few species of marine trematodes have been convincingly shown to have global distributions. *Cardicola forsteri* Cribb, Daintith & Munday, 2000 (Aporocotylidae), which infects *Thunnus* spp. (tuna), has been shown to have a cosmopolitan distribution (Aiken et al., 2007). It seems likely that the distribution of marine trematodes is closely linked to the vagility of at least one of their hosts. Hosts with wide distributions and high vagility, such as pelagic fishes (e.g., tunas) and marine turtles, can be predicted to have parasite fauna with near global distributions. The distribution of marine parasites, particularly those with complex life cycles, may also be heavily driven by the distribution of suitable intermediate hosts. Both the definitive hosts and intermediate hosts must occur in sympatry, in at least some regions, in order to facilitate the geographic distribution of turtle and pelagic fish parasites.

4.2. Comparison of Neospirorchis spp.

The two previously described species of *Neospirorchis* are easily morphologically differentiated on the basis of oesophagus length (shorter in *N. pricei*), vas deferens length (shorter in *N. pricei*), cirrus-sac morphology (longer and thicker-walled in *N. pricei*), and overall body shape (*N. schistosomatoides* is far more slender) (see Manter and Larson, 1950). Of the four novel species described in this study, *N. chapmanae* is the most morphologically distinct. Unlike *N. pricei*, *N. schistosomatoides*, and the other three species newly described, the ovary and testis are distinctly separated in *N. chapmanae*, with the cirrus-sac occupying the space between them. Additionally, the genital pore is anterior to the ovary in *N. chapmanae*, whereas in *N. pricei*, *N. schistosomatoides*, and the other three new species, the genital pore is always at least posterior to the anterior margin of the ovary. *Neospirorchis deburonae* and *N. stacyi* can be differentiated from *N. pricei*, *N. schistosomatoides*, and *N. goodmanorum* by the position of the genital pore relative to the ovary. In both *Neospirorchis deburonae* and *N. stacyi* the genital pore is always entirely posterior to the ovary, whereas the genital pore in *N. pricei*, *N. schistosomatoides*, and *N. goodmanorum* is anterior to the posterior margin of the ovary. Additionally, *N. stacyi* can be differentiated from *N. pricei* and *N. goodmanorum* in being significantly more slender, and from *N. schistosomatoides* in possessing a distinctly wider and less coiled testis and ovary. *Neospirorchis stacyi* can be differentiated from *N. deburonae* in possessing a less coiled ovary and a larger distance between the ovary and genital pore. Additionally, *N. deburonae* is distinct from all known and new species of *Neospirorchis* in that it is the only species, and indeed the only genotype reported by Stacy et al. (2017), to infect the vessels surrounding the brain of *Ch. mydas*. Stacy et al. (2017) reported multiple genotypes of *Neospirorchis* infecting the cerebral blood vessels of marine turtles, but only the genotype matching *N. deburonae* was found in *Ch. mydas*.

Neospirorchis goodmanorum is easily distinguished from *N. schistosomatoides* in possessing a shorter oesophagus (342–529 versus 595–680), a less slender body, and in infecting the heart, rather than the visceral blood vessels. *Neospirorchis pricei* and *N. goodmanorum* are, however, far more difficult to differentiate on the basis of morphology alone. *Neospirorchis goodmanorum* tends to have a narrower body than *N. pricei* (185–384 versus 360–540, respectively), however Manter and Larson (1950) noted the specimens examined were likely fixed under pressure, possibly affecting overall body shape. Additionally, *N. goodmanorum* possesses a marginally longer oesophagus than *N. pricei* (342–529 versus 320–340, respectively) and has significantly fewer eggs (13–40 versus >200). Although *Neospirorchis goodmanorum* was sister to *N. pricei* in both the ITS2 and *cox*1 analyses, the ITS2 interspecific variation (19 bp (6.29%)) is comparable to that seen between other closely related but distinct species of marine TBFs; for example. *Hapalotrema mehrai* Rao, 1976 and *Hapalotrema synorchis* Luhman, 1935 differ by 17 bp (3.4%), and *H. postorchis* and *H. mistroides* differ by 16 bp (3.5%) (see Corner et al., 2022b). It is also plausible that further genetic collections from localities between Queensland, Australia and Florida, USA may render the differences between *N. goodmanorum* and *N. pricei* as insignificant geographic genetic variation. However, there is little intraspecific variation seen in *N. chapmanae* and *N. stacyi* between Queensland and Florida (5 bp (1.27%) for both), suggesting that geographic intraspecific genetic variation is not always present for species of *Neospirorchis*. Host exploitation offers additional evidence for recognising two morphologically similar species, as *N. goodmanorum* is so far restricted to *Ch. mydas*, while *N. pricei* has only been reported from *Ca. caretta*. Conceivably, however, further sampling of these two host species may reveal lower host specificity for either species; *N. chapmanae* and other undescribed genotypes of *Neospirorchis* have been reported from both *Ch. mydas* and *Ca. caretta* (Stacy et al., 2017). Based on the current data as a whole (morphology, molecular and host range), we think the best interpretation is to treat the two forms as distinct species.

Morphological data generated here shows that entire specimens are not necessary for the differentiation of species of *Neospirorchis*. The position of the ovary, testis, and genital pore alone can differentiate most species; in this case single specimens used as holo-genophores are highly effective for species delineation.

4.3. Global richness of Neospirorchis

Our findings, and those of Stacy et al. (2017), suggest that *Neospirorchis* is significantly richer than currently understood. How-

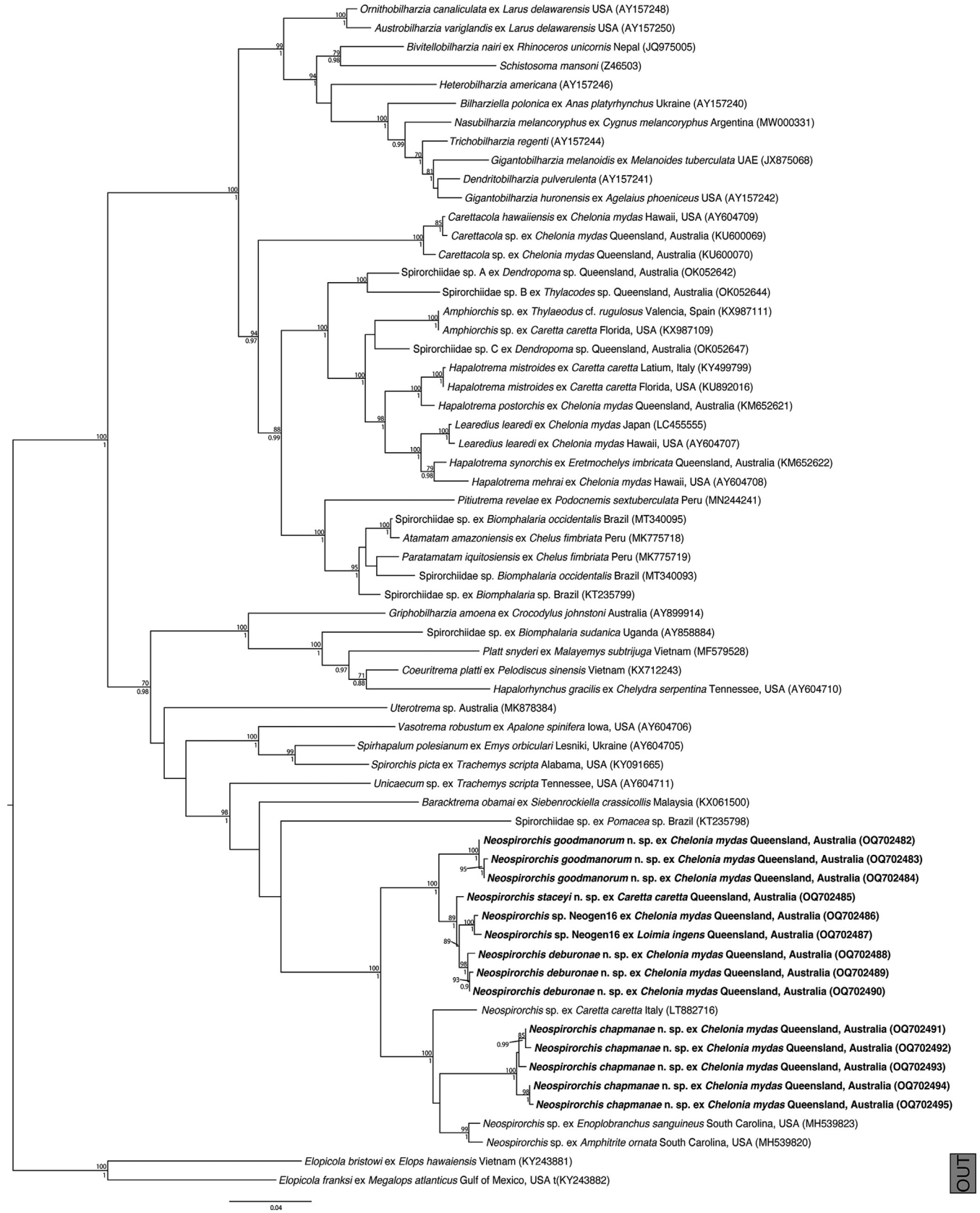

Fig. 8. Phylogenetic relationships between species of the Schistosomatidae and the turtle blood flukes inferred from 28S rDNA maximum likelihood (ML) and Bayesian inference (BI) analyses. Taxa in bold represent sequences generated during this study. Bootstrap support (ML) and posterior probability (BI) values are shown above and below the nodes, respectively. Nodal support < 80 (ML) and < 0.85 (BI) are not shown. OUT denotes functional outgroup. The scale bar indicates the expected number of substitutions per site.

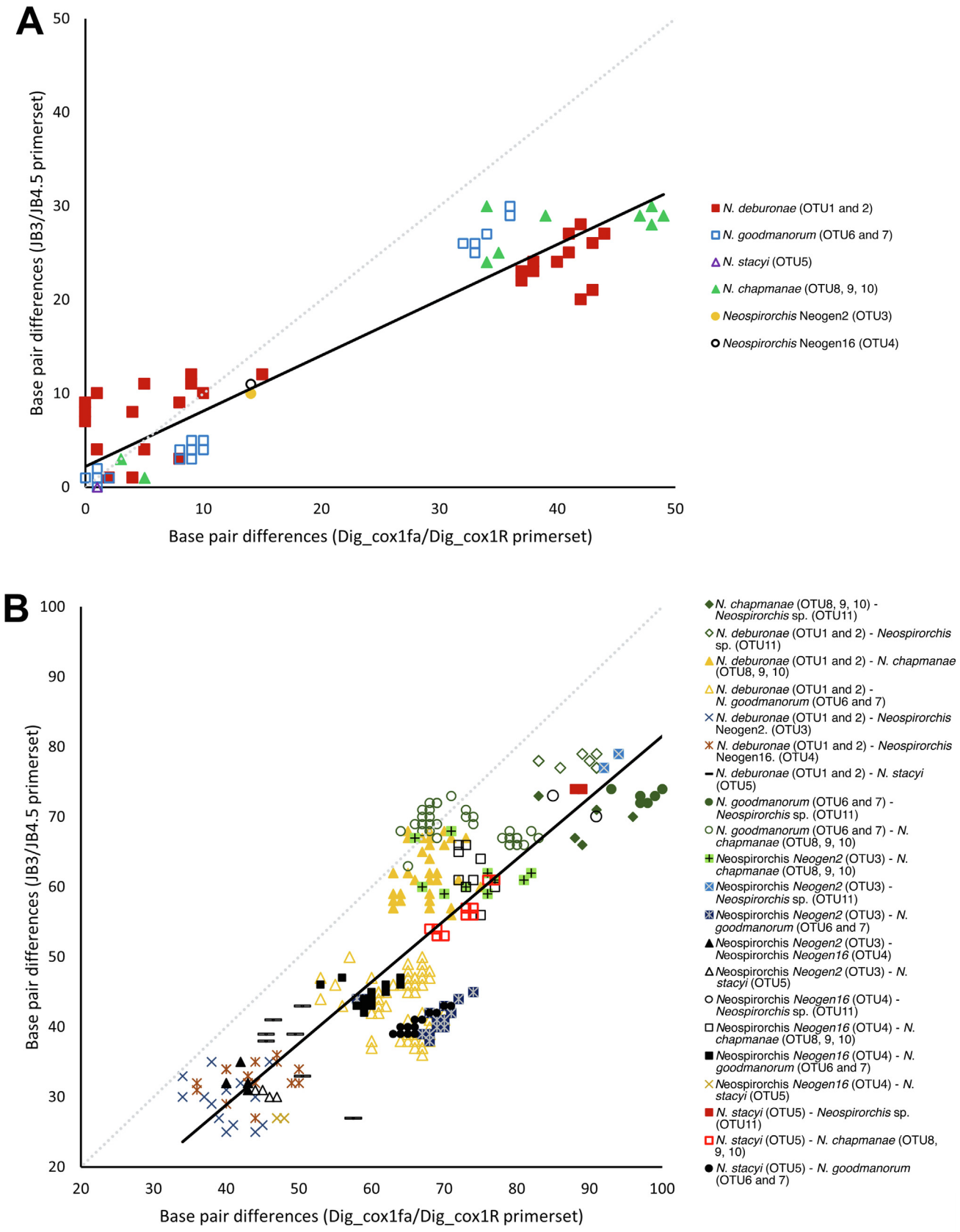

Fig. 9. Pairwise differences from two separate cytochrome c oxidase subunit 1 (*cox*1) sequences generated during this study. (A) Intra-specific pairwise distances. (B) Inter-specific pairwise distances. Key on right side of figure identifies species being compared. Black line represents trend line. Dashed grey line represents equality. OTU, Operational taxonomic unit.

ever, the diversity found during the present study, five ITS2 and 11 *cox*1 genotypes from turtles and terebellid polychaetes, is significantly lower than was reported by Stacy et al. (2017), who identified 20 ITS2/*cox*1 genotypes. It should be noted, however, that the multiple genotypes identified by Stacy et al. (2017) were detected from eggs, whereas the current study incorporated only adult trematodes. Additionally, Stacy et al. (2017) found a rich diversity of *Neospirorchis* genotypes infecting the submucosal layer of the gastrointestinal tract, and endocrine organs. These sites were not examined in the current study, and likely also hold a rich diversity of *Neospirorchis* in Australian turtles. Adult *Neospirorchis* spp. can be easily overlooked during necropsies, as they infect small vessels in hard to examine body sites whereas their eggs may be more obvious and persistant. Generating sequence data from ova provides evidence of the presence of these hard-to-find species. Additionally, the two studies had distinctly different representations of turtle species; during the present study we examined more *Ch. mydas* (82 versus 23), and fewer *Ca. caretta* (10 versus 61) than Stacy et al. (2017). It is plausible that *Ca. caretta* harbours a more diverse assemblage of *Neospirorchis* than *Ch. mydas*. Future studies examining the diversity of *Neospirorchis* should pool data from multiple geographic regions, target multiple host species, genetically examine both ova and adults, and generate molecular datasets that can be compared across studies.

4.4. cox1 markers for species discrimination

In recent years *cox*1 mtDNA data have been increasingly incorporated as a part of species descriptions and population structure studies for TBFs (e.g., Stacy et al., 2017; Kitayama et al., 2019; Corner et al., 2022b) and a range of other trematode families (e.g., Huston et al., 2021; Bray et al., 2022; Cribb et al., 2022). The *cox*1 region varies in length between trematode groups, but typically occupies at least 1,500 bp of the mitochondrial genome (see Suleman et al., 2021). Among TBF studies, perhaps unfortunately, two sets of primers have been employed to amplify partial fragments of the *cox*1 region, Dig_cox1Fa/Dig_cox1R (Wee et al., 2017) and JB3/JB4.5 (Bowles et al., 1995). These two primer sets produce data that are entirely non-overlapping, preventing comparison between studies. Here, we generated *cox*1 datasets using both primer sets for the same samples of *Neospirorchis* and demonstrated that there are subtle differences between the two datasets. Although the resultant phylograms had identical topologies, the phylogram generated using the JB3/JB4.5 primers generally had lower support at major nodes than that produced using the Dig_cox1Fa/Dig_cox1R primer set. The reduced nodal support probably relates to the shorter sequence length produced from the JB3/JB4.5 primers than the Dig_cox1Fa/Dig_cox1R primers (377 bp, on average versus 474 bp, respectively). Additionally, the data produced from the JB3/JB4.5 primers shows generally lower variation than that produced from Dig_cox1Fa/Dig_cox1R primers. When conducting population structure studies, or using *cox*1 data to delineate species, it is desirable to capture the variation present at the highest resolution possible. Finally, of the two primer sets, Dig_cox1Fa/Dig_cox1R has been used to generate sequence data representing more TBF genera and species, which allows for intergeneric comparisons in future studies. In light of these considerations, we recommend future authors studying TBFs generate *cox*1 sequence data using the Dig_cox1Fa/Dig_cox1R primer set, until perhaps sequencing of the entire *cox*1 gene or entire mitochondrial genome becomes standard. We do note, however, that future studies specifically examining *Neospirorchis* will need to generate data using the JB3/JB4.5 primers, so as to include all currently available data for the genus.

4.5. Neospirorchis life cycles

Previous work on marine TBF life cycles has shown the use of two distinct intermediate host lineages. Cribb et al. (2017) and Corner et al. (2022b) provided evidence that a clade of nine marine TBF species, consisting of species of *Amphiorchis* Price, 1934, *Hapalotrema* Looss, 1899, and *Learedius* Price, 1934, consistently infect vermetid gastropods as intermediate hosts. However, de Buron et al. (2018) provided evidence that two unidentified species of *Neospirorchis* infect terebellid polychaetes as intermediate hosts, and hypothesised that a previous report by Martin (1952) of an unknown cercaria from *Lanicides vayssierei* (Terebellidae) was likely a TBF. The positive identification of *Neospirorchis* sp. Neogen16 infecting *L. ingens* from Moreton Bay is just the third life cycle report for this genus. Significantly, rather than being closely related to the two previously reported terebellid infections, the novel infection reported here falls within a separate major clade within *Neospirorchis*, suggesting that all species of *Neospirorchis* infect terebellid polychaetes as intermediate hosts. As *Neospirorchis* is nested within a large, freshwater TBF clade (which infect freshwater planorboid snails as intermediate hosts (see Corner et al., 2022b)), the most parsimonious interpretation is that the switch to terebellid polychaetes occurred when the common ancestor of all members of *Neospirorchis* switched to the marine environment. Interestingly, de Buron et al. (2018) noted that the *Neospirorchis* cercariae recovered from terebellids possessed a prominent ventral sucker, despite ventral suckers being absent in adults, suggesting it is secondarily lost as adults.

Predicting the identity of intermediate hosts for genera where intermediate hosts have not yet been identified is difficult, given the apparent ability for some TBFs to host-switch between major groups of invertebrates. Marine TBF genera such as *Carrettacola* Manter & Larson, 1950, *Cheloneotrema* Simha & Chattopadhyaya, 1980, and *Monticellius* Mehra, 1939 have no life cycle data. For genera not yet genetically characterised, such as *Cheloneotrema* and *Monticellius*, it is difficult to predict what group of invertebrates their species infect. Genetic data are available for species of *Carettacola*, but, as discussed by Corner et al. (2022b), the identity of intermediate hosts for this genus remains enigmatic, given that species of *Carettacola* are phylogenetically related to freshwater TBFs, and distinct from the vermetid-infecting TBFs. In order to completely understand the evolution of intermediate exploitation by TBFs, the intermediate hosts of *Carettacola* need to be elucidated, and the remaining marine genera need sequence and life cycle data.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2023.03.005.

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