Occurrence and abundance of zoonotic nematodes in snapper *Chrysophrys auratus*, a popular table fish from Australian and New Zealand waters

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ABSTRACT

In Australia and New Zealand (NZ), snapper *Chrysophrys auratus* is known for delicate mild flavoured flesh and is a favoured species to serve raw as sashimi or in sushi. The diet of snapper includes a variety of intermediate hosts of larval nematodes, and as a result, snapper has potential to become highly infected with zoonotic/non-zoonotic nematodes. The aims of this study were to survey nematodes in snapper from Australia and New Zealand waters and to identify nematode species using combined morphological and molecular methods. The zoonotic potential of nematodes identified in this study are discussed. A total of 112 snapper were purchased from the Sydney fish market, New South Wales, Australia. Fish were dissected and only the visceral content and digestive tract were examined for nematode infection. Parasites were initially identified by the microscopic method as four different types belonging to the families Anisakidae (*Anisakis* types I & III, and *Terranova* type II) and Cucullanidae (*Dichelyne* spp.). All Anisakidae nematodes were at infective stages. Species-level identification was actualised through sequencing of the internal transcribed spacer (ITS–1, 5.8S, ITS–2) regions. The *Anisakis* types I & III were confirmed as *Anisakis pegrefi* and *A. brevispiculata*, respectively of which *A. pegrefi* is considered globally as a zoonotic nematode. The specific identification of *Terranova* type II and *Dichelyne* spp. was not possible as no comparable sequence data were available in GenBank. The phylogenetic tree clustered *Anisakis* types I & III with *A. pegrefi* and *A. brevispiculata*, respectively; *Terranova* type II sequences as a separate clade with previously identified larval and adult *Terranova* and *Pseudoterranova* species. Based on phylogenetic analyses the present Cucullanid specimens were assigned herein as *Dichelyne* cf. *pleuronectidis* and *Dichelyne* sp. 1. This study represents the first host record globally for zoonotic Anisakid nematodes in this popularly consumed table fish and a new region record for *D. cf. pleuronectidis* and *Dichelyne* sp. 1. Further investigation is required, using more comprehensive parasite detection and recovery methods, to assess the health risk these nematodes may pose to human and fish health in Australia/NZ.

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1. Introduction

The snapper *Chrysophyrs auratus* (Perciformes: Sparidae) is important to Australian recreational, commercial (Kailola et al., 1993; Norriss and Crisafulli, 2010), and charter industries. Snapper is also an important food species for indigenous Australians (FRDC, 2019) and is a revered fish within their spirituality and traditional knowledge (Prober et al., 2011). It is considered one of the nation’s most important and iconic fish (Fowler et al., 2017; Norriss and Crisafulli, 2010) and is a popular and well-known table species that returns high prices at the retail level (FRDC, 2019). Australia/New Zealand (NZ), as multicultural nations, have embraced a range of imported cultural cuisines, including sushi and sashimi. According to the Kobe Jones Blog (Kobe Jones, 2019) snapper in Australia is considered suitable for sashimi and is regularly recommended by Australian chefs as compatible to use in raw fish dishes (Allen, 2019; Kwong, 2015).

Snapper is distributed in warm to temperate Indo-Pacific waters which occur off Australia/NZ (Paulin, 1990). Snapper is a long-lived predatory fish (Norriss and Crisafulli, 2010), and feed predominantly on crustaceans and small fish which are the intermediate hosts of many nematode species (Godfriaux, 1969; Gregori et al., 2015; Hurst, 1984). As a result, snapper have the potential to become highly parasitised. There have been no contemporary studies using combined morphological and molecular tools to accurately describe nematode parasites of snapper in Australian/NZ waters (Table 1). The most detailed parasitological examination of snapper nematodes was conducted by Johnston and Mawson (1945) and Sharples and Evans (1995a, 1995b, 1995c) in Australia and NZ waters, respectively. Both studies relied on morphological methods for nematode identification. However, few morphological markers of taxonomic significance for reliable species identification of larval nematodes are available (McManus and Bowles, 1996). During the last two decades, the application of molecular tools for the identification of nematodes has greatly advanced their taxonomy. Therefore, the primary aim of this study was to investigate the potential of snapper from the waters of Australia/NZ to be infected with nematode parasites. The secondary aim was to accurately identify these nematode parasites using combined morphological and molecular tools.

2. Materials and methods

2.1. Fish collection

A total of 112 fish were purchased from the Sydney fish market, New South Wales (NSW), Australia. The fish had been sourced from three separate localities: off the coast of NSW ($n = 44$; 11/10/2018), off the coast of NZ ($n = 20$; 28/07/2018), and ($n = 30$; 16/09/2019), and an unknown location ($n = 20$; 29/08/2018). Fish were transferred to the Parasitology Laboratory of Charles Sturt University, Wagga Wagga Campus in an insulated ice-filled box.

2.2. Parasite collection

All fish from each batch were examined on the day of arrival at the University. Fish were dissected and examined for the presence of nematodes according to the method described in Shamsi and Suthar (2016b) using both visual examination and incubation method to ensure maximum recovery of nematodes. Firstly, the surface of all inner organs was thoroughly inspected for the presence of nematodes under a dissecting microscope (Leica EZ4 Stereo Microscope, China). Encysted nematodes were removed and placed in sterile Eppendorf tubes containing 70% ethanol. The alimentary canal was then split from mouth to anus and other internal organs gently separated and placed in a petri dish containing a small amount of ambient temperature water before examining again under a dissecting microscope for the presence of parasites. The internal organs were placed in containers with water and after vigorous shaking were left to incubate overnight at room temperature. Splitting the digestive tract and separating internal organs allows trapped nematodes an opportunity to be released into the water and provides greater parasite recovery. All collected parasites were washed in ambient temperature physiological saline and preserved in 70% ethanol and stored at room temperature for further morphological and genetic study. Laboratory temperature was kept at a constant 25 °C.

2.3. Morphological examination

A small piece from the mid-body of each nematode was excised for molecular study and the rest of the body (head and tail) were cleared with lactophenol for morphological study. This study was conducted through a microscope (Upright Motorized Microscope ECLIPSE Ni-E, Nikon, Japan) fitted with a computer screen. Anisakid nematodes were initially identified to genus level using morphology and morphometry of anterior and posterior ends, oesophagus, ventriculus, the position of excretory pore and nerve ring (Murata et al., 2011; Shamsi and Suthar, 2016a). Cucullanid nematodes were morphologically identified based on the presence of anterior pseudobuccal capsule, spicules, number and organisation caudal papillae, precloacal ventral sucker, and tail according to instructions in Li et al. (2014); Moravec et al. (2019); Yamaguti (1935); Yamaguti (1941). The morphometric and meristic characteristics of systematic importance were measured directly with an eyepiece micrometre (BX-43 Olympus Microscope, Olympus Corporation, Japan). All measurements were recorded in both micrometres and millimetres as the mean, followed by the range in parentheses. A dash (−) indicates that measurements could not be made or were not available. All drawings were made to scale with the aid of a drawing tube of the compound microscope (BX-43 Olympus Microscope,
Olympus Corporation, Japan). The prevalence, mean intensity, and mean abundance of nematodes were calculated according to Bush et al. (1997).

2.4. Sequencing

Genomic DNA from nematodes was extracted by DNeasy Blood & Tissue Kits (QIAGEN, Germany) and eluted by 40 μl of elution buffer. A volume of 25 μl PCR reaction was conducted to amplify the whole ITS (ITS–1, 5.8S, and ITS–2) regions of Anisakid nematodes using the primer sets of forward (SS1): 5′–GTTCCTCCGCTTATGCAGGC–3′ and reverse (NC2): 5′–TTCCTCCCTGCTTCTTG–3′. The cycling condition was followed according to Hossen et al. (2021); Shamsi et al. (2020). A similar volume (25 μl) of PCR for the Cucullanid nematodes was conducted to amplify the whole ITS regions using another primer set, which included forward ITS–F: 5′–CCTTAAAAAGCCCTAACGGGTG–3′ and reverse ITS–R1: 5′–GCATACGACTGAGACGAGCC–3′ with the cyclic conditions of initial 95 °C for 2 min, then 95 °C for 30 s, 60 °C for 45 s, 72 °C for 1 min × 40 cycles following extension at 72 °C for 10 min and finally at 4 °C. An aliquot (3 μl) of each amplicon from both Anisakid and Cucullanid nematodes was examined on a 1.5% w/v agarose gel after staining with GelRed™ and photographed using a gel documentation system.

Representative samples were chosen from each group to send for sequencing to the Australian Genome Research Facility (AGRF). Identical primers set as for PCR were used to prepare the samples to send for sequencing. Sequence data including chromatogram were observed initially through Sequence Scanner software (Applied Biosystems® Genetic Analysers). Subsequently, sequences were aligned by MUSCLE (in MEGA v. 7) (Kumar et al., 2016) and then adjusted manually wherever necessary. Evolutionary analyses were conducted in MEGA v. 7 (Kumar et al., 2016).

2.5. Construction of phylogenetic tree

Two phylogenetic trees were constructed (based on two groups identified: Anisakids and Cucullanids) from the sequences generated in this study along with the representative sample sequences from GenBank (Table 2). GenBank sequences were sorted based on the BLAST searches and from the available publications. The phylogenetic relationships among specimens were calculated by the Bayesian method using MrBayes v 3.2 (Ronquist and Huelsenbeck, 2003). The GTR + G model was applied for both trees as suggested by jModelTest2 (Darriba et al., 2012). *Anisakis marina* (GenBank accession: KP699576) and *Hysterohylacium aduncum* (GenBank accession: KY909270) were used as outgroups for Anisakid and Cucullanid nematodes, respectively. For the construction of both phylogenetic trees, the sample frequencies were set at 1000, and calculated for 10,000,000 generation until the p value reached <0.01. After the mcmc run, the first 30% samples were discarded, and the sum command was used to summarise the phylogenetic trees. The phylogenetic tree was visualised using Figtree v 1.4.3 (Rambaut, 2014).

Table 1

Previous records of nematodes identified from the snapper *Chrysophrys auratus* in Australia and New Zealand.

| Nematode | Microhabitat | Family | Location | Reference |
|----------|--------------|--------|----------|-----------|
| *Anisakis* sp. larva | Encapsulated on viscera, mesenteries, and peritoneum of the body cavity | Anisakidae | Hauraki Gulf (Okakari Point and Kawau Bay), NZ | Sharples and Evans (1995a, 1995b) |
| *Cucullanus* sp. | Intestine | Cucullanidae | Hauraki Gulf (Okakari Point and Kawau Bay), NZ | Sharples and Evans (1995a, 1995b) |
| *Philometra latalelabracis* | Gonads | Philometridae | Hauraki Gulf (Okakari Point and Kawau Bay), NZ | Sharples and Evans (1995a, 1995b) |
| *P. latalelabracis* | – | Philometridae | NZ | Hine and Anderson (1981) |
| *Anisakis* sp. larva | – | Anisakidae | NZ | Brunsdon (1956) |
| *Dichelyne* cnidoglanis | – | Cucullanidae | NZ | Brunsdon (1953) |
| *Hysterothylacium* sp. | – | Raphidascarididae | NZ | Brunsdon (1953) |
| *Dichelyne* sheardi | – | Cucullanidae | Outer harbour, SA | Johnston and Mawson (1949) |
| *Dichelyne* sheardi | – | Cucullanidae | Glenelg, SA | Johnston and Mawson (1945) |
| *Echinocephalus* uncinatus larva | Mesentery or omentum | Gnathostomatidae | Glenelg, SA | Johnston and Mawson (1945) |
| *Anisakis* larval type* | – | Anisakidae | Glenelg and Cape Jervis, SA | Johnston and Mawson (1945) |

*’–’ indicates no information available; Abbreviations: NZ = New Zealand, SA = South Australia.

*’Anisakis marina (= *Capsularia marina*; *Stomachus marinus*) has been reported from various hosts across Australian coasts (Johnston and Mawson, 1944, 1945, 1949). In these reports “marina” mostly refers to larval stage of the nematode. Therefore, *A. marina* is not considered a valid taxon but regarded as Anisakis larval type. Both *Capsularia* and *Stomachus* have later been synonymised with Anisakis (Shamsi, 2014).
Table 2
Details of the specimens used to construct the phylogenetic trees.

| Nematode specimen     | GenBank accession number | Host scientific name | Host common name | Geographical origin of the specimen | Reference                      |
|-----------------------|--------------------------|----------------------|------------------|-------------------------------------|----------------------------------|
| Anisakis pegreffii     | AY821740                 | Lissodelphis borealis| Northern right whale dolphin | Drakes Beach, California, USA Cantabrian Sea, Spain | Nadler et al. (2005) |
| A. simplex S. S.      | AY826723                 | Trachurus trachurus  | Atlantic horse mackerel |                                      | Nadler et al. (2005) |
| A. typica             | AY826724                 | Stenella longirostris| Spinner dolphin    | Coast of Brazil                      | Nadler et al. (2005) |
| A. physeteris         | AY826721                 | Physeter catodon     | Sperm whale       | Tyrrenhenian Sea, Italy              | Nadler et al. (2005) |
| A. brevispiculata     | KM325199                 | Kogia breviceps      | Pygmy sperm whale  | Southeast of Melbourne, Australia    | Shamsi et al. (2019b) |
| A. berlandi           | KM325187                 | K. breviceps         | Pygmy sperm whale  | Southeast of Melbourne, Australia    | Shamsi et al. (2019b) |
| A. paggiae            | KM325218                 | K. breviceps         | Pygmy sperm whale  | Southeast of Melbourne, Australia    | Shamsi et al. (2019b) |
| A. nascetti           | JQ912692                 | Mesoplodon gravi     | Gray’s beaked whale | Off the NZ coast                     | Mattiucci et al. (2014) |
| A. ziphidurum         | JQ912691                 | Zhiphus cavirostris  | Cuvier’s beaked whale | Off the South African coast         | Mattiucci et al. (2014) |
| Contra Garcem rodulphi D | FM210251+FM210261       | Phalacrocorax carbo  | Great cormorant    | NSW and Victoria, Australia          | Shamsi et al. (2009a) |
| C. rudulphi E         | FM210257+FM210269        | Phalacrocorax varius | Pied cormorant     | NSW and Victoria, Australia          | Shamsi et al. (2009a) |
| C. pyripapillatum     | AM940062+AM940066        | Pelecanus conspicillatus | Australian pelican | Victoria, Australia                  | Shamsi et al. (2008) |
| C. multipapillatum D  | AM940056+AM940060        | Pelecanus conspicillatus | Australian pelican | Victoria, Australia                  | Shamsi et al. (2008) |
| C. bancrofti          | EU839566+FM177880        | Pelecanus conspicillatus | Australian pelican | Victoria, Northern Territory, NSW, Australia | Shamsi et al. (2009b) |
| C. microcephalum      | FM177524+FM177528        | Phalacrocorax melanoleucos | Little pied cormorant | Lara and Healesville, Victoria, Australia | Shamsi et al. (2009b) |
| C. variegatum         | FM177531+FM177541        | Anthisa melanogaster | Australian darter and | Melbourne, Victoria.                | Shamsi et al. (2009b) |
| C. eudyptulae         | FM177550+FM177578        | Eudyptula minor      | Arctocephalus psullus | Victoria, Australia                  | Shamsi et al. (2009b) |
| C. ognomorhini        | FM177542+FM177549        | Arctocephalus forsteri | Australian and New Zealand fur seals | Victoria, Australia                  | Shamsi et al. (2009b) |
| Mawson carcini        | MK476521                 | Pastinachus ater     | Cowtail stingray   | Queensland, Australia                | Shamsi et al. (2019c) |
| Terranova type I      | MT633348                 | Platyccephalus bassensis | Sand flathead      | NSW, Australia                       | Hossen et al. (2021) |
| Pelchrascaris australis | MK890749               | Sphyraena lewini     | Scalloped hammerhead shark | Off Cairns, Australia | Shamsi et al. (2020) |
| Terranova type II     | MT633350                 | Platyccephalus richardsoni | Tiger flathead       | NSW, Australia                       | Shamsi et al. (2021) |
| T. pectinolabata      | MK542878                 | S. mokarran          | Great hammerhead shark | NSW, Australia                       | Shamsi et al. (2019a) |
| Pseudoterranova azorii | AJ413973+AJ413974       | Eumetopias jubatus   | Steller sea lion   | Iwani, Japan                         | Zhu et al. (2002) |
| P. bulbosa            | AJ413970+AJ413971        | Eringnathus barbatus | Bearded seal       | Newfoundland, Canada                 | Zhu et al. (2002) |
| P. cattani            | AJ413982+AJ413984        | Otaria byronia       | South American sea lion | Conception, Chile           | Zhu et al. (2002) |
| P. decipiens          | AJ413967+AJ413968        | Phoca vitulina       | Harbour seal       | Newfoundland, Canada                 | Zhu et al. (2002) |
| P. krabbei            | AJ413965+AJ413980        | Halichoerus grypus   | Gray seal          | Froya Island, Norway                 | Zhu et al. (2002) |
| Raphidascaris acus    | AJ40633537               | Anguilla anguilla    | European eel       | Vistula Lagoon, Poland               | Kijewska et al. (2008) |
| Dichelyne pleuronectolis | KF470872–83           | Pleuronichthys cornutus | Rugged-eye flounder | East China Sea                       | Li et al. (2014) |
| D. romani             | KF699576                 | Notacanthus chemmitii | Snub-nosed spiny eel | Northeast Atlantic, Argentina       | Isbert et al. (2015) |
| D. szidatii           | KM131264                 | Acanthistius paticentricus | Argentine sea bass | Off the coast of Otago, NZ   | Unpublished |
| Hysterothyacium aduncum | KY090270             | Pelethurampus novaezeelandiae | New Zealand sole | Port Augusta, South Australia  | Shamsi et al. (2016) |
| H. australis           | HE862216+HE862223        | Seriola lalandi      | Yellowtail amberjack | Port Augusta, South Australia  | Shamsi et al. (2016) |
| H. brucei             | HE862222+HE862230        | Kajikia audax        | Striped marlin     | Nelson Bay, NSW, Australia          | Shamsi et al. (2016) |
| H. kajikiae           | HE862220+HE862226        | Kajikia audax        | Striped marlin     | Nelson Bay, NSW, Australia          | Shamsi et al. (2016) |
| Anisakis type I       | MT791088–103             | Chrysophyrs auratus  | Striped marlin     | Australia and NZ                    | Present study with voucher numbers 169, 177, 231–2, 232–1, 235, 236–1, 239, 246–1, 247, 252–2, 258–1, 285, 291, 420, 427, and 440 |
Larvae were found in the digestive tracts, gonads, and liver. All Cucullanid nematodes were extracted only from the digestive system of the examined fish and were morphologically identified as Dichelyne (Cucullanellus) spp. which included larvae and adults (Figs. 2a–l). Among all nematode morphotypes identified in this study, Anisakis type I occurred in all samples/batch and had the highest prevalence of infection. The second most prevalent nematode morphotype was identified as Dichelyne spp. Anisakis type III and Terranova type II larvae were the least prevalent nematode morphotypes identified in this study. The general epidemiological data are represented in Table 3.

### 3.2. Molecular identification

Representative samples from each morphotype were subjected to sequencing to confirm the specific identity of nematodes as detailed below.

Sixteen specimens belonging to Anisakis type I were subjected to sequencing. The length of ITS regions was 857 bp long and identical. Our sequences were 100% identical to those belonging to adult A. pegreffii (accession number AY821740) identified from the Northern right whale dolphin Lissodelphis borealis in the Drakes Beach, California, USA (Nadler et al., 2005). A single specimen belonging to Anisakis type III had the ITS sequence of 804 bp long and was 100% identical with the adult A. brevipliculata (accession number MK325199) reported from a pygmy sperm whale Kogia breviceps in the Southeast of Melbourne, Australia (Shamsi et al., 2019b).

Two specimens belonging to Terranova type II were subjected to sequencing. The ITS sequences of both specimens were 893 bp long and identical. There was no identical or highly similar sequence available in the GenBank. A search in GenBank showed 99% similarity with D. pleuronectidis (accession numbers KF470872–KF470883) identified from ridged-eye flounder Pleuronichthys cornitus in the East China Sea (Li et al., 2014). The length of the ITS sequence for the other specimen (voucher number 282; a gravid female Cucullanid) was 813 bp long which had no closest similarity with the registered GenBank sequences. The pairwise comparison between the first four ITS sequences and the latter, generated in the present study, revealed a substantial nucleotide variability at 0–24.10% and considered herein as interspecific genetic variation (Fig. 5).

### 3.3. Phylogenetic analyses of the nematodes

The Bayesian inference phylogenetic tree clustered Anisakid nematodes found in this study with the members of Anisakidae identified previously in the literature (Fig. 3A). Anisakis type I independently grouped with members of A. pegreffii and Anisakis type III with A. brevipliculata demonstrating 100% posterior probabilities, respectively. Terranova type II larva found in the present study clustered into a separate clade and revealed a clear distinction with previously identified larval and adult Terranova and Pseudoterranova specimens in Australian waters and elsewhere with 100% posterior probability value. The Cucullanid nematodes found in the present study grouped with closely related GenBank Dichelyne specimens. Four sequences explored in this study clustered with the D. pleuronectidis with 100% posterior probability value (Fig. 3B). A single sequence obtained from a gravid

### Table 2 (continued)

| Nematode specimen | GenBank accession number | Host scientific name | Host common name | Geographical origin of the specimen | Reference |
|-------------------|--------------------------|----------------------|------------------|--------------------------------------|-----------|
| Anisakis type III | MT791104                 | C. auratus           | Snapper          | Australia                            | Present study with voucher number 413 |
| Terranova type II | MT791105–06              | C. auratus           | Snapper          | NZ                                   | Present study with voucher numbers 168 and 252–5 |
| Dichelyne cf. pleuronectidis | MT791107–10 | C. auratus           | Snapper          | Australia and NZ                     | Present study with voucher numbers 91–4, 93–1, 281–1, and 283 |
| Dichelyne sp. 1  | MT791111                 | C. auratus           | Snapper          | Australia                            | Present study with voucher number 282 |

Abbreviations: NZ = New Zealand, NSW = New South Wales.

NB: Single GenBank accession indicates the whole ITS (ITS–1, 5.8S, ITS–2) sequence. Two ITS sequences connected with ‘+’ represent ITS-1 and ITS-2 sequences, respectively.
female Cucullanid was isolated from the existing GenBank Dichelyne species with a 100% posterior probability value. The isolated specimen in this study was assigned herein as Dichelyne species 1 (Fig. 3B).

4. Discussion

This study confirmed the presence of A. pegreffii, A. brevispiculata, Terranova type II, D. cf. pleuronectidis, and Dichelyne sp. 1 infection in snapper from coastal waters of NSW and NZ. This is the first time that infectious stage larvae of the zoonotic A. pegreffii and potentially zoonotic Terranova type II have been identified in this species of snapper. New host records for A. brevispiculata, D. cf. pleuronectidis, and Dichelyne sp. were also established.

The individual prevalence of Anisakid and Cucullanid nematodes were 18% and 13%, respectively and for zoonotic A. pegreffii, the overall prevalence was 15%. This differs from Sharples and Evans (1995a) where Anisakis sp. was found to be rare with limited prevalence and low abundance. In the same study, Cucullanus sp. was reported moderately abundant in snapper. Differences between the present study and Sharples and Evans (1995a) may be due to the epidemiological profile of nematodes changing over time and differences in genera identified in the previous study. A further investigation with a greater sample size and conducted at different time points is required to clarify nematode infection trends in snapper.

In the present study, Anisakis larval types were morphologically identified as types I & III (Figs. 1a–d). In morphological identification, Anisakis type I could not be distinguished from larvae of A. pegreffii, A. simplex S. S., A. berlandi, and A. typica (Mattiucci et al., 2018; Murata et al., 2011). Similarly, Anisakis type III could be the larvae of A. brevispiculata, A. physeteris, and A. paggiae (Mattiucci et al., 2018; Murata et al., 2011). Previously, Anisakis sp. larva was found from the same host in Australia (Johnston and Mawson, 1945) and NZ (Sharples and Evans, 1995b, 1995c). However, specific identification of the larva was uncertain at the time due to a lack of molecular techniques. The phylogenetic tree showed a clear distinction between Anisakis types I & III found in this study and grouped them with previously identified adult A. pegreffii and A. brevispiculata, respectively (Fig. 3A). Therefore, the present study confirms snapper as a host of A. pegreffii and A. brevispiculata.

Another Anisakid nematode morphologically identified in this study was Terranova type II. Terranova types I and II have been previously identified and reported from other marine fish in Australia and New Caledonia (Hossen et al., 2021; Jabbar et al., 2012; Shamsi et al., 2018a; Hossen and Shamsi, 2019; Shamsi et al., 2018b; Shamsi and Suthar, 2016a) as Pulchrascaris australis and
Fig. 2. Morphology of Cucullanid nematodes identified as Dichelyne (Cucullanellus) spp. from snapper Chrysophrys auratus. a) Anterior end and b) Posterior end of mature male Dichelyne cf. pleuronectidis; c) Anterior end and d) Posterior end of gravid female Dichelyne cf. pleuronectidis; e) Anterior end and f) Posterior end of gravid female Dichelyne sp. 1; g) A typical pseudobuccal capsule; h–j) Posterior end of immature female specimens; k) Posterior end of immature male specimen; l) Eggs.
T. pectinolabiata, respectively (Shamsi et al., 2019a; Shamsi et al., 2020). Although, the morphological and morphometric data of the present specimens either partially or completely matched with previously identified Terranova larval types (Table 4) the molecular data did not match with existing sequences registered in GenBank and revealed 20.70–20.90% nucleotide variability (Fig. 4).

Very little is known about Terranova larval types compared to other Anisakid nematodes (Moravec and Justine, 2020). The larvae belonging to the genera Pulchrascaris, Terranova, and Pseudoterranova look morphologically similar and are all referred to as Terranova larval types (Shamsi and Suthar, 2016a). The species belonging to the later genus are considered to be the second most common zoonotic nematode causing human illness after Anisakis spp. (Arizono et al., 2011; McClelland, 2002; Menghi et al., 2020; Torres et al., 2007; Weitzel et al., 2015). Although, Terranova type A larva has been reported for two cases of human infection in Korea (Lee et al., 1985; Seo et al., 1984), however this requires further investigation (Moravec and Justine, 2020). The phylogenetic tree grouped Terranova type II sequences obtained in the present study independently from the registered sequences for Ascaridoid nematodes larvae and adults in GenBank with 100% posterior probability value (Fig. 3A). The new sequences did not cluster with earlier larval and adult Terranova and Pseudoterranova species identified in the Australian waters and abroad. Therefore, the specific identification of the present Terranova type II requires clarification. Further parasitological examination with greater sample size is required to explore more genetic information for the specific identification of Terranova larval infection in snapper.

Although other nematodes (Dichelyne spp.) found in this study are not of zoonotic significance, they impact fish health and can significantly damage the intestinal wall and disrupt functional nutrient absorption leading to deficits in host growth, health and death (Dunn et al., 1983; Li et al., 2014; Rezaei et al., 2013). Until now, two Dichelyne (Cucullanellus) spp. have been reported from the Australian waters, D. shearidi from the silver spot Chironeurus maculosus and snapper C. auratus as well as D. cnioglanis from the estuarine catfish Cnidoglanis macrocephalis (Johnston and Mawson, 1944, 1945, 1949). In NZ, a single species D. cnioglanis has been reported (Brunsdon, 1953; ex. from Sharples and Evans in 1995c) with reports of unidentified species as Cucullanus (not Dichelyne species) from snapper (Sharples and Evans, 1995c). The morphometric and meristic data suggest our specimens are different from those previously described from the Australian and NZ waters.

Globally, a total of three Dichelyne (Cucullanellus) spp. have been identified from the Sparid hosts (Ishert et al., 2015), D. adriaticus (Petter, 1974); D. jialaris (Moravec et al., 2018); and D. pleuronectidis (Li et al., 2014; Yamaguti, 1935; Yamaguti, 1941) of which our specimens most closely resembled D. pleuronectidis (Table 5). However, specimens in our study were different from previous descriptions in Li et al. (2014); Yamaguti (1935) and Yamaguti (1941) with respect to: i) markedly thick cuticle with transverse striations; ii) muscular oesophagus expanded at the anterior end to form a distinct pseudobuccal capsule; iii) anterior oesophageal region being much-expanded/wider than the posterior region and clearly distinguishable. The pairwise genetic comparison between the four ITS sequences generated in this study and those available in GenBank demonstrated 0–0.60% nucleotide variability (Fig. 5) with D. pleuronectidis thus the Dichelyne nematode identified in this study was assigned as D. cf. pleuronectidis. Further investigation is required to reach a solid conclusion if the observable morphological variations in D. cf. pleuronectidis found in the present specimens are due to geographical differences of host origin. In the present study, a single gravid female Dichelyne species herein named as Dichelyne sp. 1 remains unidentified due to the lack of comparable mature male specimens and revealed 0.10–24.40% nucleotide variability with those sequences registered in GenBank (Fig. 5).

### Table 3

Occurrence and abundance of nematodes in snapper Chrysophrys auratus examined in the present study.

| Source of fish (number examined) | Nematode | Number of fish infected | Range in infected fish | Prevalence (%) | Total number of parasites found | Mean intensity | Mean abundance |
|----------------------------------|----------|-------------------------|------------------------|----------------|---------------------------------|---------------|---------------|
| SFM, NSW, Australia Date: 29-08-2018 (n = 20) | Anisakis pegreffii' | 3 | 1–2 | 15 | 4 | 1.33 ± 0.59 | 0.20 |
| | Dichelyne cf. pleuronectidis | 4 | 1–1 | 20 | 4 | 1 ± 0.61 | 0.20 |
| | Dichelyne sp. 1* | 1 | 1–1 | 5 | 1 | 1 ± 0.62 | 0.05 |
| Off the coast of NSW, Australia Date: 11-10-2018 (n = 44) | Anisakis pegreffii | 3 | 1–1 | 7 | 3 | 1 ± 0.66 | 0.10 |
| | Anisakis brevispiculata, Dichelyne cf. pleuronectidis | 4 | 1–4 | 9 | 8 | 2 ± 0.69 | 0.18 |
| Off the coast of NZ Date: 28-07-2018 (n = 20) | Anisakis pegreffii | 2 | 1–1 | 10 | 2 | 1 ± 0.75 | 0.10 |
| | D. cf. pleuronectidis | 3 | 1–1 | 15 | 3 | 1 ± 0.78 | 0.15 |
| | Terranova type II* | 1 | 1–1 | 5 | 1 | 1 ± 0.81 | 0.05 |
| Off the coast of NZ Date: 16-09-2019 (n = 28) | Anisakis pegreffii | 9 | 1–6 | 32 | 25 | 2.78 ± 0.86 | 0.89 |
| | D. cf. pleuronectidis | 3 | 1–1 | 11 | 3 | 1 ± 0.78 | 0.11 |
| | Terranova type II | 1 | 1–1 | 4 | 1 | 1 ± 0.82 | 0.04 |

Abbreviations: SFM = Sydney Fish Market, NSW = New South Wales, NZ = New Zealand. * Asterisk indicates the new host records of these nematodes.
Fig. 3. Phylogenetic relationship among the nematodes identified from snapper *Chrysophrys auratus* and those closely related species in GenBank (see Table 2 for details) inferred using Bayesian method with ITS (ITS-1, 5.8S, ITS-2) sequences. A) Phylogenetic tree for Anisakid nematodes; B) Phylogenetic tree for Dichelyne spp. nematodes. * indicates the ITS sequences generated in this study. Bayesian posterior probability values (%) were shown on the node.
The phylogenetic tree clustered four (out of five) *Dichelyne* nematode sequences obtained in the present study with the sequences registered for *D. pleuronectidis* in GenBank. A single sequence from the present study grouped in isolation with 100% posterior probability value. The isolated specimen was herein named as *Dichelyne* sp. 1 (Fig. 3B). Further genetic analyses are required, for the morphologically identified *Dichelyne* nematodes, to verify their specific position.

The zoonotic potential of all *Anisakis* spp. are not yet clear (Abinu et al., 2019; Mattiucci et al., 2018). In most cases nematode larvae infecting humans are often damaged on removal (Mattiucci et al., 2013) and consequently consumed (Mattiucci and D’Amelio, 2014). Given that a range of genera within the family can frequently infect humans, it is likely that all members of the *Anisakis* genus are potentially zoonotic. Therefore, further research on the specific identification of these larvae in human cases of infection is required.

According to Bao et al. (2017a) anisakiasis (human infection caused by *Anisakis* nematodes) is an emerging zoonosis which is underestimated globally. These zoonotic nematodes are medically important and cause intestinal (Kojima et al., 2013), and less commonly extra-intestinal anisakiasis as well as allergic reactions (Mattiucci et al., 2017). *Anisakis pegreffii* has increasingly been identified in human cases of allergic (Mattiucci et al., 2013), intestinal (Moschella et al., 2004), and extra-intestinal anisakiasis (Guardone et al., 2018; Bao et al., 2019).

Identification of zoonotic and/or potentially zoonotic larvae from a popular Australian/NZ table fish such as the iconic snapper, considered a suitable species for consuming raw, is of concern for human health (Shamsi, 2020). According to Shamsi and Sheorey (2018), anisakidosis (human infection caused by the larval *Anisakid* nematodes) in Australia is of emerging importance and may correspond with the adoption of novel cuisines including the popular sushi and sashimi. Very recently, the first human anisakiasis, caused by *Anisakis* spp. larvae was diagnosed in NZ after consumption of a sushi rolls (Beig et al., 2019).

All anisakid nematodes found in this study were viable infectious third-stage larvae and may cause human illness if accidentally consumed in raw or partially cooked fish (Bao et al., 2017a; Bao et al., 2018; Buchmann and Mehrdana, 2016; Cipriani et al., 2016; Caldeira et al., 2021; D’Amico et al., 2014). *Anisakis pegreffii* as the dominant species in the present study and recent reports of other infected fish in the Asia Pacific region may support a southward shift in parasite distribution (Palm et al., 2017; Chen et al., 2018; Zhang et al., 2018). *Anisakis pegreffii* has been identified as the dominant species in pelagic blue mackerel *Scomber australasicus* (Taiwanese waters) (Chen and Shih, 2015), pelagic scombrid bullet tuna *Auxis rochei* (Indonesian waters) (Palm et al., 2017), white spotted Conger *Conger myriaster* (South China Sea) (Chen et al., 2018) and the yellow goosefish *Lophius litulon* (East China Sea) (Zhang et al., 2018). Further monitoring of fish in Australian waters, therefore, seems warranted.

Further research is required to determine the human health risks using a greater sample size covering both a greater temporal and spatial range. In addition, more advanced methods of parasite isolation should be considered in future studies by examining only the fish viscera in the present study limited the strength of the results. Candling of fish fillets is recommended in Codex *Alimentarius ‘Code of practice for fish and fishery products’* for identification of parasites (Codex *Alimentarius*, 2020) in fish muscle. However, this method is less effective in detection of *Anisakis* spp. and darker *Pseudoterranova* spp. larvae (Levsen et al., 2005; McGladdery, 1986; Petrie et al., 2007; Mercken et al., 2020a). Candling combined with pressing may be more effective
to detect nematode parasites in the musculature of fish (Karl and Leinemann, 1993). Levsen et al. (2005) found that in blue whiting with an average thickness of 11 mm, the detection of nematodes using UV light was only 10–15% however Gómez-Morales et al. (2018) found the UV press method had a high sensitivity for detection of nematodes in fish musculature and viscera. Artificial pepsin digestion of fish musculature is also recommended in the Codex fishery code of practice for recovery of parasites in high-risk fish species (Codex Alimentarius, 2020) and fillets (Mercken et al., 2020b). This results in the total de-struction of the fish and for commercial purposes is impractical. However, with the optimisation of this technique described in Llarena-Reino et al. (2013) the pepsin digestion method in the experimental setting would be an effective, low-cost, and accessible alternative to advanced methods such as Magnetic Reso-nance Imaging (MRI) described in Bao et al. (2017b). Press method candling in combination with artificial digestion of fish fillets in future studies will provide a more accurate indication of the potential of snapper fillets to be infected with zoonotic nematodes larvae.

Also, of importance is the potential for cross-contamination as a human health concern. Anisakis simplex has been identified as an important hidden allergen in food (Anibarro et al., 2007) and Bao et al. (2019) considers all Anisakis spp. as potential and important food allergens. As A. pegreffii has been identified as the causative agent in cases of human allergic anisakiasis the potential for cross-contamination of viable larvae from viscera onto fillets should be considered as a human health concern.

Seafood borne parasitic disease in Australia/NZ is little recognised or acknowledged. The Australia New Zealand Food Standards Code (ANZFSC, 1997) has not included information on fish borne parasites in local fish. The 'The Compendium of Microbiological Criteria for Food (2018)' from Food Standards Australia and NZ in Appendix 1 (FSANZ, 2018) mentioned parasites as a possible pathogenic microorganism that can cause foodborne illness only once.

In conclusion, snapper sourced from the waters of Australia and NZ was identified infected with zoonotic, potentially zoonotic, and non-zoonotic nematodes. Further research into zoonotic nematodes in snapper using advanced detection methods will identify if current Australian food safety regulations regarding zoonotic or potentially zoonotic nematodes in fish and fishery products in Australia/NZ require updating. Fish is an excellent source of lean protein and contains many beneficial fatty acids essential for early development as well as eye, brain and cardiovascular health (Aadland et al., 2015). The identification of zoonotic and potentially zoonotic parasites should not be a deterrent to regular consumption of this healthy protein. If intended for consumption raw or lightly processed, it is recommended that fish be frozen at $-20 \degree C$ for a minimum period of one day (EC, 2004). Ade-quately cooking, where the internal temperature of fish reaches $\geq 55 \degree C$, for a minimum period of 5 min will largely negate the risks to human health (De Marval et al., 2013).

Animal ethics

Not applicable.
Table 5
Comparative measurements of *Dichelyne cf. pleuronectidis* and *Dichelyne* sp. 1 from snapper *Chrysophrys auratus*, for specimens collected in the present study and previous studies.

| Parasite                  | Present study | Present study | Li et al. (2014) | **Yamaguti (1935)** | **Yamaguti (1941)** |
|---------------------------|---------------|---------------|------------------|---------------------|---------------------|
| *Dichelyne cf. pleuronectidis* | **Host**      | *Snapper C. auratus* | **Host** | *Snapper C. auratus* | **Host** | *Snapper C. auratus* |
| **Locality**              | Australia: SFM New Zealand: Off the coast of NZ | China: The East China Sea Japan: Toyama Bay; the Inland Sea; and Mutu Bay Japan: The Inland Sea | Australia: SFM New Zealand: Off the coast of NZ | China: The East China Sea Japan: Toyama Bay; the Inland Sea; and Mutu Bay Japan: The Inland Sea |
| **Specimen type (number measured)** | Mature male $(n = 3)$ Immature male $(n = 3)$ Gravid female $(n = 2)$ Immature female $(n = 5)$ | Mature male $(n = 15)$ Gravid female $(n = 1)$ | Male $(n = not specified)$ Female $(n = not specified)$ Male $(n = not specified)$ Female $(n = not specified)$ |
| **Body length (mm)**      | 4.76 (4.13–5.63) 3.14 (3.00–3.38) 7.22 (6.63–7.80) 5.56 (2.8–7.43) 4.03 | 4.37 (3.68–5.08) 3.18 (3.00–3.38) 7.22 (6.63–7.80) 5.56 (2.8–7.43) 4.03 | 6.67 (5.49–7.94) 7.40 (5.80–8.70) 3.15–8.00 5.50–11.00 3.70–4.40 4.00–11.00 |
| **Maximum body width**    | 460 (380–500) 523 (450–640) 975 (850–1100) 776 (500–950) 520 | 450 (380–500) 523 (450–640) 975 (850–1100) 776 (500–950) 520 | 328 (225–421) 391 (294–471) 175–350 300–650 270–320 260–700 |
| **Oesophagus length**     | 800 (700–850) 100 (80–140) 210 (200–220) 184 (120–220) 100 | 800 (700–850) 100 (80–140) 210 (200–220) 184 (120–220) 100 | 809 (735–882) 915 (833–980) 500–910 800–1120 600–700 740–1000 |
| **Minimum oesophagus width** | 73 (60–80) 55 (40–75) 90 | 73 (60–80) 55 (40–75) 90 | 107 (88–137) – – – – – |
| **Ratio of oesophagus length to body length (%)** | 17 (15–21) 17 (15–19) 13 (13–14) 14 (12–18) 13 | 17 (15–21) 17 (15–19) 13 (13–14) 14 (12–18) 13 | 12 (10–15) 13 (11–15) – – – – – |
| **Pseudobuccal capsule length** | 290 (240–350) 215 (180–275) 365 (330–400) 310 (190–400) 200 | 290 (240–350) 215 (180–275) 365 (330–400) 310 (190–400) 200 | 70 (49–88) 64 (49–78) – – – – – |
| **Pseudobuccal capsule width** | 217 (160–260) 157 (120–200) 270 (240–300) 232 (190–270) 130 | 217 (160–260) 157 (120–200) 270 (240–300) 232 (190–270) 130 | 83 (69–108) 97 (78–118) 110–220 150–200 135–155 150–240 |
| **Intestinal caecum length** | 250 (200–300) 180 (150–180) 350 (320–380) 153 (80–200) 130 | 250 (200–300) 180 (150–180) 350 (320–380) 153 (80–200) 130 | 239 (98–394) 262 (69–415) 180–450 110–530 320–380 200–500 |
| **Intestinal caecum width** | 100 (80–100) 50 (40–50) 130 | 100 (80–100) 50 (40–50) 130 | 58 (39–69) 58 (49–69) – – – – – |
| Measurement Description                        | Mean (Range) |
|-----------------------------------------------|--------------|
| Excretory pore to anterior end                 | 480 (430–530) |
| Nerve ring to anterior end                    | 433 (400–500) |
| Deirids to anterior end                       | 650 (520–750) |
| Ventral precloacal sucker length              | 215 (200–230) |
| Ventral precloacal sucker width               | 377 (350–400) |
| Ventral precloacal sucker to cloaca           | 570 (530–600) |
| Ventral precloacal sucker to posterior end    | 933 (900–1000) |
| Spicule length                                | 975 (735–1176) |
| Ratio of spicule length to total body length (%) | 20 (17–24) |
| Gubernaculum length                           | 40 (40–45) |
| Number of precloacal papillae (pairs)         | 4 |
| Number of parcloacal papillae (pairs)         | 3 |
| Total number of caudal papillae (pairs)       | 10 |
| Tail length                                   | 173 (150–200) |
| Phasmids to posterior end                     | 233 (200–250) |
| Eggs count                                    | 23 |
| Eggs length                                   | – |
| Eggs width                                    | – |

All measurements are given in micrometres unless otherwise stated; mean followed by range in parentheses. ‘–‘ indicates no measurement/data available; Abbreviations: SFM = Sydney Fish Market, NZ = New Zealand.

* Some of the measurements have been converted into micrometres.
The ITS (ITS-1, 5.8S, ITS-2) sequence alignments of the present Dichelyne specimens and closely related species from GenBank. Sample's information is provided in Table 2. The dots represent identical bases and dashes indicate alignment gaps. The numbers at the right of alignments indicate the alignment position.

Fig. 5. The ITS (ITS-1, 5.8S, ITS-2) sequence alignments of the present Dichelyne specimens and closely related species from GenBank. Sample's information is provided in Table 2. The dots represent identical bases and dashes indicate alignment gaps. The numbers at the right of alignments indicate the alignment position.
Declarations of Competing Interest

Md. Shafaet Hossen, Sky Wassens, and Shokoofeh Shamsi declare that they have no conflict of interest.

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