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Abbreviations and symbols

AFMA – Australian Fisheries Management Authority

AGRF – Australian Genome Research Facility

AUS – Australia

bp – base pair

BLAST – Basic local alignment search tool

c – concentration

CFP – Common Fisheries Policy

dH_2O – distilled water

DNA – deoxyribonucleic acid

dNTP – deoxynucleotide triphosphate

EDTA – ethylendiamine tetra acetic acid

ELISA – enzyme-linked immunosorbent assay

EU – European Union

FDA – Food and Drug Administration

gDNA – genomic DNA

h – hour

IgE – Immunoglobulin subclass E

ITS – internal transcribed spacer

IUPAC – International Union of Pure and Applied Chemistry

kg – kilogram

L_T – longitudinal length

L1 – first stage larva(e)

L2 – second stage larva(e)

L3 – third stage larva(e)

L4 – fourth stage larva(e)

m – meter

MAE – Multilocus allozyme electrophoresis

ml – milliliter

mm – millimeter

mM – millimolar

n – number

NCBI – National Center for Biotechnology Information

ng – nanogram

n. sp. – new species

n/a – not available

PBS – phosphate buffer saline

PCR – Polymerase chain reaction

PCR-RFLP – PCR-linked restriction fragment length polymorphism

pH – potentia hydrogeni

pmol – picomoles

rDNA – ribosomal DNA

SDS – sodium dodecyl sulphate

SeaFIC – The New Zealand Seafood Industry Council Ltd

s.l. – sensu lato

sp. – species

spp. – species (plural)

SPT – skin prick testing

s.s. – sensu stricto

SSCP – Single-strand conformation polymorphism

STH – soil transmitted helminth

Taq – *Thermus aquaticus*

TAE – Tris-acetate-EDTA

TBE – Tris-boric acid-EDTA

™ – trademark

U – unit

UK – United Kingdom

US – United States

UV – ultraviolet light

V – volt

vs. – versus

v/v – volume per volume

µl – microliter

µM – micromolar

µm – micrometer

°C – degree Celsius

°N – latitudinal degree north

°S – latitudinal degree south

% – percent

> – more than

< – less than

Abstract

Background: Nematodes of the family Anisakidae are parasites of marine organisms, such as fish and sea mammals, pose a threat to humans and cause the disease anisakiasis. In Australia, little has been done to evaluate the risk of exposure for consumers of infected fish to these parasites. The aim of the present study is to partially address this gap by a small-scale survey of five local fish species from southern Australia destined for consumption.

Methods: Fish were collected and examined for intestinal worms, including anisakids. The parasite larvae collected were fixed and identified both by morphological/morphometric analysis and by molecular investigation of the PCR-amplified internal transcribed spacer region of nuclear ribosomal DNA. Both datasets were combined and interpreted together with other biological data.

Results: Infection rates of anisakid nematodes in the respective fish species ranged between 20 % and 100 %. Larval stages of seven anisakid species of three different genera, namely *Anisakis*, *Contracaecum* and *Hysterothylacium*, were identified. Some species discovered represent molecularly uncharacterized specimens and await unequivocal identification.

Conclusion: The data show that *Anisakis* and related species are prevalent in southern Australia, in some fish hosts in large numbers. Further research will provide a better understanding of the parasite and other factors linked to anisakiasis.

Zusammenfassung

Hintergrund: Nematoden der Familie Anisakidae sind Parasiten mariner Lebewesen, wie Fische und Meeressäuger, und stellen eine Bedrohung für den Menschen dar durch den Verzehr von infizierten Meerestieren, insbesondere im rohen oder unzureichend gekochten Zustand. Infektiöse dritte Larvenstadien verursachen die Krankheit Anisakiasis, welche sich entweder durch Beschwerden im Gastrointestinalbereich auszeichnet, oder mit allergischen Symptomen von Urtikaria, Angioödem, Bronchospasmus, bis hin zu einem anaphylaktischen Schock einhergehen kann. Besonders in Ländern mit hohem Fischkonsum, wie Japan und Spanien bekannt, ist ein Auftreten auch ausserhalb dieser Länder wahrscheinlich und oftmals unentdeckt. In Australien ist bisher wenig getan worden zur Bewertung des Expositionsrisikos für Konsumenten von Fisch und Meeresfrüchten gegenüber anisakider Parasiten. Ziel dieser Studie ist es, sich teilweise mit dieser Diskrepanz zu befassen anhand einer kleinen Bestandsaufnahme von fünf lokalen Fischarten, die für den Konsum vorgesehen sind.

Methoden: Fische fünf verschiedener Arten aus unterschiedlichen Habitaten wurden gesammelt und auf intestinale Würmer untersucht, einschliesslich Anisakiden. Entnommene Parasitenlarven wurden fixiert und einerseits durch morphologische/morphometrische Analyse, andererseits durch molekulare Erforschung der PCR amplifizierten „Internal transcribed spacer" Region aus nuklearer ribosomaler DNA identifiziert. Beide Datensätze wurden kombiniert und mit Hilfe vorhanderer biologischer Daten interpretiert.

Ergebnis: Die Infektionsraten anisakider Nematoden in den jeweiligen Fischspezies schwankten zwischen 20 % und 100 %. Larvale Stadien von sieben Arten von Anisakiden aus drei verschiedenen Gattungen, nämlich *Anisakis*, *Contracaecum* und *Hysterothylacium*, wurden identifiziert. Zu den vorkommenden Arten gehören *Anisakis simplex sensu lato*, *Contracaecum multipapillatum D*, *Contracaecum ogmorhini sensu stricto* und *Hysterothylacium aduncum sensu lato*. Einige entdeckte Spezies repräsentieren molekular unbeschriebene Exemplare und erwarten ihre eindeutige Identifizerung.

Fazit: Die Ergebnisse zeigen, dass *Anisakis* und verwandte Spezies im südlichen Australien weit verbreitet sind, in manchen Fischwirten in hoher Anzahl. Die molekulare Auswertung anhand genetischer Marker in Kombination mit morphologischer Einordnung hat sich als effizient erwiesen zur Bestimmung anisakider Arten. Auch konnten Variationen bzw. Punktmutationen von individuellen Markersequenzen innerhalb der Art erkannt werden. Da diese Studie sich auf fünf verschiedene Fischarten beschränkt, ist mit einer hohen Artendiversität von Anisakiden in australischen Gewässern zu rechnen. Schliesslich wird die weitere Erforschung zu einem besserem Verständnis des Parasiten und anderer Faktoren, die mit Anisakiasis verbunden sind, führen.

Chapter 1

Literature review

1.1 Introduction

Infectious and parasitic diseases are one of the major causes of mortality in the world, despite the fact that most of these diseases are preventable or treatable (May 2007). Parasitic helminths – like flukes, roundworms, hookworms or whipworms – are one major group of parasites which are responsible for many of these fatalities. Important helminths include Schistosoma, the cause of the water-borne disease schistosomiasis (bilharziasis) and the soil transmitted helminthes (STH), also referred as intestinal worms. The most prevalent STH are *Ascaris lumbricoides*, *Trichiuris trichiura*, *Necator americanus*, *Ancylostoma duodenalis* and *Strongyloides stercoralis*, of which nearly two billion people were infected with in 2001 (De Silva *et al.* 2003). Symptoms of infection normally range from nausea, tiredness, loss of appetite, anaemia and abdominal pain. Though they are rarely life-threatening, the resultant nutrition deficiencies can impair growth and brain development (Hesham *et al.* 2004).

Besides these prominent examples, humans may be infected by numerous helminth species which employ animals as their natural host – termed zoonotic infection. In 1960, a novel zoonotic infection was discovered by Van Thiel in a patient who had eaten raw herring: the intestinal roundworm *Anisakis simplex*. This case from the Netherlands soon gained the attention of parasitologists from Japan, where the habit of eating raw fish is widespread. A research group was established in 1964 to investigate the nature of this disease, today known as anisakiasis. Since then, thousands of cases have been reported in Japan (Ishikura *et al.* 1988; Kark and McAlpine 1994) and hundreds throughout the world, predominantly in countries of high fish consumption like Spain and Italy (Guijarro Huertas *et al.* 2000; Lopez Penas *et al.* 2000a; Pampiglione *et al.* 2002). Outside these countries, it is highly likely that cases of anisakiasis have remained undetected due to a lack of awareness among physicians. Today it is

known that also other genera within the subfamily Anisakinae can inflict the disease (Pellegrini *et al.* 2005).

1.2 Biology of anisakid nematodes

Anisakis spp. belong to the subfamily Anisakinae, family Anisakidae, superfamily Ascaroidea, suborder Ascaridina, order Ascarida, subclass Secernentrea, class Nematoda (Nadler and Hudspeth 1998). Other genera of the subfamily Anisakinae, collectively known as anisakids, include *Pseudeterranova*, *Contracaecum* and *Hysterothylacium*. They all share a similar life cycle as they usually parasitize animals of the marine environment throughout their different developmental stages. Figure 1.1 illustrates the general principle of the anisakids' life cycle. Embryonated eggs (L1) enter the water column through the faeces of its definitive host, and moult further inside the egg to the second stage larvae (L2). The eggs or L2, respectively, get ingested by little crustaceans, generally copepods, which act as first intermediate hosts. When infected crustaceans get consumed by second intermediate hosts, such as fish, cephalopods and larger crustaceans, larvae reach the third larval stage (L3) through moulting. Larger fish may become infected by predation of smaller fish, leading to an accumulation of infectious L3's in their body cavity (paratenic host). All kinds of L3-contaminated seafood can cause anisakiasis when eaten by humans. As human beings and other terrestrial mammals are accidental hosts of the parasite, they cannot complete their life cycle and die within days. Within the natural terminal host, which are aquatic mammals, birds or reptiles, the L3 develop into the fourth larval stage (L4) and subsequently to its adult form. A more specific view for each genera discussed is given below.

1.2.1 *Anisakis*

Adult *Anisakis* spp. – sometimes referred as "whale worms" – are parasites of the stomach of pinnipeds (eared seals, true seals, walruses) and cetaceans (dolphins, porpoises, whales). Euphausiids or other marine crustaceans are first intermediate hosts (Smith and Snyder 2005).

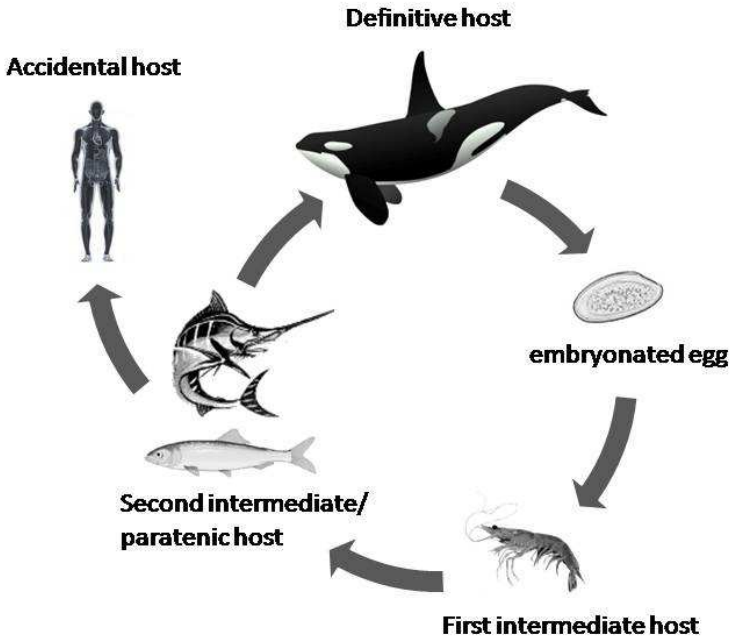

Figure 1.1. Life cycle of anisakid nematodes.

Figure 1.2. Larvae of anisakid species extracted from the visceral cavity of a tiger flathead. Petri dish = 90 mm diameter.

The L3 are found encapsulated on the surface of visceral organs or muscle tissue of a wide range of second intermediate hosts of marine fish, cephalopods and crustaceans (Smith 1984; Pascual *et al.* 1995). Paratenic hosts can accomodate huge numbers of parasite larvae (Fig. 1.2) during their lifespan. Most anisakiasis cases are traced back to this genus as etiological agent (Szostakowska *et al.* 2005; Umehara *et al.* 2007).

1.2.2 *Contracaecum*

Piscivorous birds (e.g. cormorants and pelicans) and aquatic mammals (seals) have been shown to be final hosts for *Contracaecum* spp. (Liu and Edward 1971; Kijewska *et al.* 2002). The range of first intermediate hosts of aquatic invertebrates is very diverse, including cephalopods, chaetognaths, coelenterates, crustaceans, ctenophores, echinoderms, gastropods and polychaetes (Semenova 1979), but the further transmission from these hosts to the next is not known in detail (Anderson 2000). Freshwater, brackish and marine fish are the principal second intermediate and paratenic hosts. Reports of human infection are documented (Ruitenberg *et al.* 1979).

1.2.3 *Hysterothylacium*

Unlike the other anisakid genera discussed here, *Hysterothylacium* spp. utilize predatory fish as definitive hosts. Crustaceans like copepods, amphipods, euphausiids and isopods act as first intermediate host (Marcogliese 1995), followed by larger crustaceans as second intermediate host. Various predatory fish species serve as paratenic and final host, respectively – occasionally both at the same time. Larval size seems to be one of the determining factors for the developmental status within the fish host (Koie 1993). Human infections with *Hysterothylacium* larvae have been reported (Chai *et al.* 2005).

1.3 Public Health significance of *Anisakis* species

Living L3 of the Anisakidae family pose a health hazard to humans by causing the disease anisakiasis. The source of infection is usually raw, pickled or smoked fish and squid, as well as undercooked seafood (Nawa *et al.* 2005). Table 1.1 indicates some of the most popular seafood dishes that have a significant risk to harbor L3 of anisakids. Besides anisakiasis from ingesting contaminated seafood, an additional risk has been shown in the domestic and occupational environment, where allergens from the parasite can enter the immune system *via i*ngestion, skin contact or inhalation, causing an allergic response in sensitized persons (Scala *et al.* 2001; Nieuwenhuizen *et al.* 2006).

The disease has global occurrence with strongest impact in Western Europe and northern Asia. Of the approximately 20,000 cases reported worldwide yet, 90% originated from Japan. The majority of remaining cases came from European countries like the Netherlands, Germany, France, Spain and Italy (Van Thiel 1976; Lorenz and Warzok 1988; Hubert *et al.* 1989; Sabater and Sabater 2000; Pampiglione *et al.* 2002; Repiso Ortega *et al.* 2003). But also other regions are believed to harbor high incidence rates of this zoonosis mostly undetected, for example Russia (Serdyukov 1993) or the Americas (Mercado *et al.* 1997; Laffon-Leal *et al.* 2000; Cabrera *et al.* 2004). Currently, annual reported cases of anisakiasis exceed 2000 in Japan (Takabe *et al.* 1998), of which *Anisakis* spp. account for an estimated 90 %, followed by *Pseudoterranova decipiens* (4 to 5 %) and other species of the Anisakidae family (5 %) (Ishikura 1989). However, based on the geographic locality, strong fluctuations in the proportion of the disease-causing species have been recorded (Ishikura *et al.* 1995; Konishi and Sakurai 2002). While anisakiasis has occurred in New Zealand (Paltridge *et al.* 1984), little epidemiological information is available for Australia. A first case of anisakiasis has been confirmed from a patient in Adelaide, South Australia, caused by an L3 of the *Contracaecum* genus (Shamsi 2007).

Clinical symptoms of anisakiasis are distinguished according to the worm's location inside the body, namely gastric, intestinal and extragastrointestinal anisakiasis, the latter occurring infrequent. Etiopathology may be acute or chronic, with symptoms starting usually between

Table 1.1. Popular seafood dishes at risk of harboring infectious anisakid larvae.

Country/Region of origin	Dish
Japan	*Sushi* or *sashimi*
South America	*Ceviche* (raw fish marinated in lemon juice)
Spain	*Boquerones en vinagre* (anchovies in vinegar sauce)
Italy	*Alici marinate* (pickled anchovies)
Netherlands	*Matjes* (salted herring)
Germany	*Rollmops* (pickled herring)
Scandinavia	*Gralax* (dry, cured salmon)
Hawaii	*Lomi lomi* (raw salmon)
UK	Smoked salmon
Italy, UK, US	Seared tuna
Russia	*Caviar*
Malaysia	Fish tripe (undercooked)

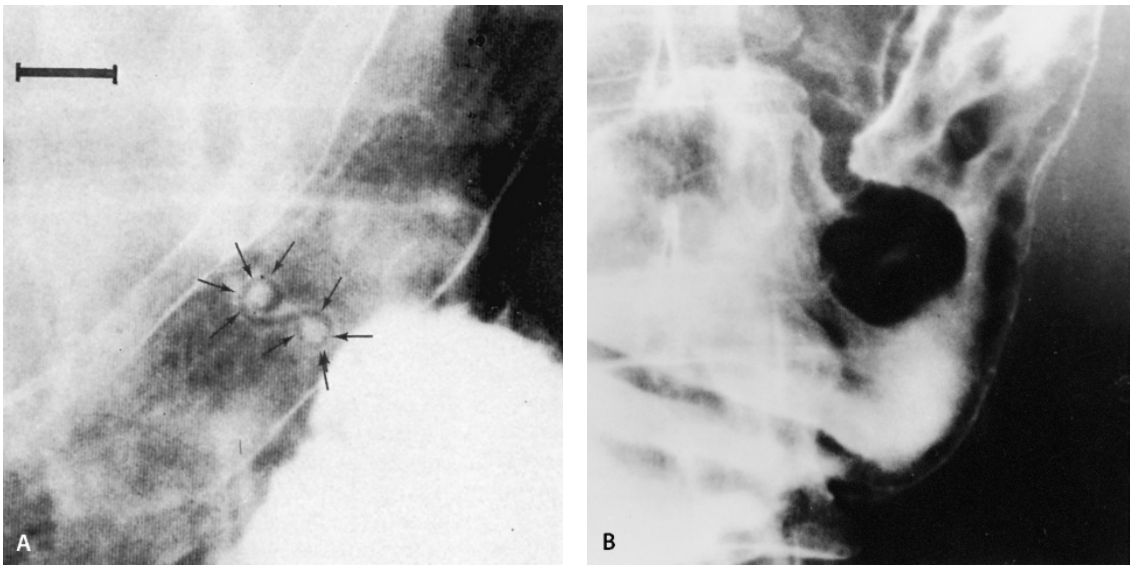

Figure 1.3. Radiologic image sections of the stomach of two Japanese patients, showing (A) *Anisakis* worm and (B) a well-defined eosinophilic infiltrate that caused an intramural inflammatory mass. (Courtesy of Dr. Masayoshi Namiki, Japan; reprinted from Cockshott and Middlemiss (1979)).

several hours to several days after ingestion of the live larvae. Single or double infection is common, even though multiple infections are possible (Alonso *et al.* 1999). Moreover, gastroallergic anisakiasis has been defined for clinical conditions mimicking an allergic reaction after the exposure to secreted proteins from the parasite (De Corres *et al.* 1996). Compared with *Anisakis* spp., the leading cause of diagnosed anisakiasis, intestinal worms of the *Hysterothylacium*, *Contracaecum* or *Pseudoterranova* genus create milder symptoms, transient progression and are less invasive (Valdiserri 1981; Ishikura *et al.* 1995; Jofre *et al.* 2008).

Gastric anisakiasis: This is the most common form of this disease in Japan (Ishikura *et al.* 1988). It has been frequently misdiagnosed as peptic ulcer (Sakanari and McKerrow 1989; Barros *et al.* 1992). In its acute progression, symptoms start normally within 12 h and range from abdominal pain, nausea, vomiting, diarrhea, chest pain, urticaria and anorexia (Sakanari and McKerrow 1989; Sugano *et al.* 1993; Daschner *et al.* 1998). Generally, treatment by parasite removal brings immediate relief for the patient (Akasaka *et al.* 1979; Sugano *et al.* 1993). Sometimes the disease remains asymptomatic until it reaches the chronic stage. The chronic stage is characterized by an inflammatory response in the intestine's submucosa, which can result in bloating, loss of appetite, dyspepsia and even gastric bleeding (Bouree *et al.* 1995; Takeuchi *et al.* 2000; Kim *et al.* 2006). In Japan, an association with gastric cancer has been demonstrated (Tsutsumi and Fujimoto 1983; Petithory *et al.* 1990). Therapy includes anti-inflammatory but no anti-helminthic medication, together with endoscopy.

Intestinal anisakiasis: Most cases in Europe originate from intestinal anisakiasis (Jimenez-Saenz *et al.* 2003). It is speculated, however, that gastric anisakiasis may be underreported by European physicians and wrongly diagnosed as food poisoning (Ishikura and Kikuchi 1983; Lopata and Potter 2000; Castan *et al.* 2002). Another common misdiagnosis for intestinal anisakiasis is appendicitis or peritonitis (Perez-Naranjo *et al.* 2003). The acute phase, normally occurring within 8 to 48 h, include symptoms like abdominal pain, nausea, diarrhea, vomiting and fever (Matsui *et al.* 1985), whereas the chronic phase can exhibit abdominal cramps, constipation, diarrhea, weight loss and sometimes a bloody stool (Gani *et al.* 2001). In general, treatment requires surgery plus anti-helminthic and anti-inflammatory drugs (Lopez Penas *et al.* 2000b; Couture *et al.* 2003).

Extragastrointestinal anisakiasis: On very rare occasions, the larvae penetrate the mucosa of the patient and migrate to different locations of the body (Matsuoka *et al.* 1994; Cancrini *et al.* 1997; Yeum *et al.* 2002). Reported localities have been the liver, spleen, lungs, oral cavity, pancreas, ovaries, peritoneum, and lymph nodes. Either an inflammatory response develops or the symptoms remain dormant.

Gastroallergic anisakiasis: In infected consumers, anisakiasis symptoms may be accompanied by allergic symptoms such as urticaria, angioedema, bronchospasm and anaphylaxis (Daschner *et al.* 1998, 2000). An association with symptoms of gastric anisakiasis does occur, but is not frequent (Daschner *et al.* 1998; Fraj Lazaro *et al.* 1998; Lopez-Serrano *et al.* 2000). Patients, who suffer from gastroallergic anisakiasis denote *Anisakis* specific-IgE in their sera during the infection. In contrast to the temporal occurrence of specific-IgE in gastric anisakiasis after the infection, where it is believed that it is a memory response of the immune system to a prior, possibly asymptomatic infection, in conjunction with a primary response to previously unrecognized antigens (Daschner *et al.* 1998). Symptoms typically start within hours up to one day after parasite exposure and are short lived (Lopez-Serrano *et al.* 2000). In Spain, where *Anisakis simplex sensu lato* is the main causative agent, the disease is considered as one of the most common sources for anaphylaxis cases (Del Pozo *et al.* 1997; Anibarro *et al.* 2007). Whether the live parasite is a prerequisite for the reaction still is a matter of debate (Audicana *et al.* 2002; Alonso-Gomez *et al.* 2004). Although several studies indicate that subjects tolerate oral challenges of killed *Anisakis* larvae doses (Alonso *et al.* 1999; Ortega *et al.* 2000; Alonso-Gomez *et al.* 2004), other groups report *Anisakis*-related reactions in patients after ingestion of well-cooked fish, canned tuna (Caballero and Moneo 2004) or even during a deep-frozen seafood diet (Moneo *et al.* 2007). Furthermore, some Anisakis proteins are both heat stable and pepsin-resistant (Audicana *et al.* 1997; Caballero and Moneo 2004).

In order to give the correct diagnosis of infection and/or sensitization, several indicators have to be considered. The appearance of anisakiasis-related symptoms within 48 h after consumption of raw or undercooked seafood is a definite factor which requires further investigation. A performed radiography of the suspect's stomach and intestines could lead to the discovery of nematode larvae (Fig. 1.3). Blood eosinophilia can be indicative, but is not

always present (Maruyama *et al.* 1996). Regarding gastroallergic anisakiasis, skin prick testing (SPT) of *Anisakis*-specific IgE by enzyme-linked immunosorbent assay (ELISA) proved to be valuable in practice (Del Pozo *et al.* 1996; Garcia *et al.* 1997). Also, the patient's total IgE level in serum, determined by immunoblotting analysis, is typically elevated (Moreno Ancillo *et al.* 1997; Daschner *et al.* 1999; Magnaval *et al.* 2002). Several antigens of the excretory/secretory protein fraction have been elucidated for the *Anisakis simplex* complex (Nakata *et al.* 1990; Moneo *et al.* 2000; Perez-Perez *et al.* 2000; Kobayashi *et al.* 2007; Rodriguez-Mahillo *et al.* 2007). At least for some of them, a cross-reactivity to antigens of other arthropods has been demonstrated (Guarneri *et al.* 2007).

Besides the direct implications on public health, anisakid parasites can hamper economies by causing loss to the fishing industry due to decreased fitness of infected fish (see Woo 1995; Lymbery *et al.* 2002). Individuals with heavily infected muscular tissue have to be discarded.

In summary, the potentially high impact of anisakid nematodes on several fields confirms the need of comprehensive research for this family of parasites, their surveillance and, eventually, their control in affected populations.

1.4 Prevention of anisakiasis

Many countries with considerable marine fishing industry have taken preventive measures to reduce the risk for consumers of seafood, albeit with notable differences. EU regulations dictate that caught fish and squid have to be deep-frozen at -20 °C or colder for at least 24 h (CFP 2004). Furthermore, cooked seafood has to be heated above 60 °C for a minimum of 10 minutes, which is regarded to efficiently kill all parasites. According to US fisheries law, seafood destined for raw consumption has to be deep-frozen either at -35 °C for 15 h, or at -23 °C for a period of 7 days (FDA 2001). Heavily parasitized fish should be excluded from the market.

The Australian Fisheries Management Authority demands to keep fish and other seafood under permanent temperature control of 5 °C or below (AFMA 2006). Visual inspection of the abdominal cavity should be performed to locate parasites. Parasitized fish for raw consumption

should be frozen at -18 °C, whereas those destined for cooked consumption do not require this treatment.

Unfortunately, there is no control for the catches of recreational anglers in Australia and worldwide. The low awareness of seafood consumers of *Anisakis* and related species is also a matter of concern. In addition, the freezing process may change the flavor of fish and is therefore preferred to be consumed fresh.

1.5 Geographical distribution

Anisakid nematodes are globally distributed, but the different genera and species inhabit different oceanic regions. Since the emergence of molecular tools for the species identification of anisakid larvae (Boczon *et al.* 1989; Mattiucci *et al.* 1989; Sugane *et al.* 1989; Nadler and Hudspeth 1998; Zhu *et al.* 1998), the picture of their geographic range has changed notably. Formerly categorized taxa with broad spatial occurrence could be subdivided into sibling species with a more narrow distribution. For the *Anisakis simplex* complex, which are the most investigated anisakid species, following geographical distribution is confirmed (Mattiucci *et al.* 1997): *Anisakis simplex sensu stricto* is located in the northern hemisphere between 30°N latitude and the Polar Arctic Circle. Conversely, *A. pegreffii* has widely distribution in the southern hemisphere between 35°S and 55°S, and also throughout the Mediterranean Sea. *A. simplex C* has been found circumantarctically and in pacific Canada.

In a comparison study of anisakid nematodes between the arctic/sub-arctic and antarctic/sub-antarctic region (Mattiucci and Nascetti 2007), both a higher genetic diversity and intensity of host infection was found in the southern hemisphere populations, possibly due to a lower degree of habitat disturbance.

1.6 Occurrence in Australia

Compared with other regions in the world, currently available data of anisakids in Australian regions is still incomplete, especially on molecular investigation. Pioneer research was done by

Johnson and Mawson during the 1940's (Johnston 1937; Johnston and Mawson 1939; 1940; 1941c; 1941a; 1941b; 1942b; 1942c; 1942a; 1943b; 1943a; 1944; 1945b; 1945a; 1950; 1951a; 1951b; 1952). Their results, though, were often lacking sufficient information, and hence sometimes ambiguous or misleading. First comprehensive data came from Cannon (1977), who defined nine distinct larval types from marine fish in Queensland, namely *Anisakis* type I, *Terranova* types I and II, *Contracaecum* types I and II, *Hysterothylacium* (=*Thynnascaris*) types I, II, III and IV. Since then, the majority of authors studying anisakid nematode larvae in Australian waters, including the present study, refer to his morphologic descriptions (see in Hooper 1983; Lymbery *et al.* 2002; Doupe *et al.* 2003; Shamsi 2007).

Important work on anisakid nematodes in Australia – based on advanced tools for both morphology and molecular analysis – was conducted by S. Shamsi during her PhD project (2007). Most of her examined fish, marine mammal and aquatic bird specimens were from the coastal waters of Queensland and Victoria. Other authors focused on other regions around the Australian continent, including the north-west (Doupe *et al.* 2003), east (Hooper 1983), south-east and south (Sewell and Lester 1995), south-west (Lymbery *et al.* 2002) and New Zealand (Jones 1991; Sharples and Evans 1995; Wharton *et al.* 1999).

Their findings showed that there is a rich diversity of anisakids among numerous marine organisms in Australia. Prevalence rates of infection as high as 100 % have been found in several intermediate host species (Lymbery *et al.* 2002; Doupe *et al.* 2003). Nonetheless, further research is needed for more detailed insights of the host range and distribution from all occurring parasite species of the Anisakinae subfamily.

1.7 Identification of anisakid nematodes

***Anisakis*:** Recognized species include the three sibling species of the *Anisakis simplex* complex (*A. simplex s.s.*, *A. pegreffii* and *A. simplex C), A. typica*, *A. ziphidarum*, *A. schupakovi*, *A. physeteris*, *A. brevispiculata* and *A. paggiae* (D'Amelio *et al.* 2000; Mattiucci *et al.* 2005; 2007). Larvae of the latter three species belong to the *Anisakis* larval type II morphotype, which is morphologically distinct from *Anisakis* larval type I. *Anisakis* larval type I comprises the

remaining species except *A. schupakovi*, which is of unknown status. All these species have been confirmed at the genetic level using isozyme analysis and/or PCR-based approaches. Some other species have been described, but they do await unambiguous characterization, like *A. dussumierii* (Yamaguti 1941), *A. insignis* (Kreis 1945) and *A. alexandri* (Hsu and Hoeppli 1933).

***Contracaecum*:** Approximately 100 species have been discovered and classified to the *Contracaecum* genus, but the validity of some recorded taxa is arguable due to morphologic convergences, inadequate descriptions or lack of sufficient specimen numbers. Other species of similar morphology differ genetically and are therefore divided into two or more sibling species, e.g. *C. osculatum* (Nascetti *et al.* 1993; Orecchia *et al.* 1994), *C. ogmorhini* (Zhu *et al.* 2001a; Mattiucci *et al.* 2003) and *C. rudolphii* (Li *et al.* 2005; Zhu *et al.* 2007a).

***Hysterothylacium*:** Similar to *Contracaecum*, the taxonomy of *Hysterothylacium* spp. is not completely clear. New species that are reported frequently (e.g. Gopar-Merino *et al.* 2005; Li *et al.* 2007a, 2007b) add up to the already extended list of biological species, which is placed alongside *Contracaecum* to have the highest taxonomic diversity within the Anisakidae family. Especially molecular analysis for elucidation of the taxonomic status of its numerous described species is required.

1.7.1 Morphology

Because of the small size of the larval and adult stages of anisakid nematodes, their morphology is generally observed by light and scanning electron microscopy. Important features of adults include the spicules of the males, lips and papillae. As the present study solely deals with larval forms of anisakid nematodes, the morphologically relevant features for their classification are mentioned.

Body size: The length and width of a nematode worm are basic morphometric attributes which give an idea of the parasite's identity. However, it cannot be considered for specific or even generic identification.

Lips: Anisakid L3 have only weakly developed labiae, but additional features, like the presence or absence of a boring tooth, can help to distinguish between different genera. L4 of *Hysterothylacium* spp. and *Contracaecum* spp. exhibit well defined lips, similar to their adult

form. They are subdivided into one dorsal labium and two subventral labia, each segregated by a smaller interlabium. L4 of *Pseudoterranova* spp. and *Anisakis* spp. also have visible labia, but do not feature interlabia.

Digestive system: The mouth opening is leading into the cylindrical esophagus, sometimes ending in a ventriculus. Whereas *Contracaecum* spp., *Hysterothylacium* spp. and *Pseudoterranova* spp. have an appendix of the ventriculus, *Anisakis* spp. are missing this trait. Additionally, *Contracaecum* spp. and *Hysterothylacium* spp. have an intestinal caecum elongating anteriorly towards the esophagus. Hence, the presence or absence of a ventriculus, ventricular appendix and intestinal caecum, as well as the ratios between their sizes, is regarded as useful parameter for differentiating between genera and species of anisakid nematodes. The distance of the laterally ending anus to the posterior end is also a record of interest.

Excretory system: Depending on the genera of Anisakidae, the position of the excretion porus varies. For *Anisakis* spp. and *Contracaecum* spp., it is located between the base of its subventral lips. In contrast, *Hysterothylacium* spp. have its excretion pore extruding laterally near the nerve ring, which is close below the head.

1.7.2 Molecular tools

Currently, a number of molecular methods can be employed for species identification of ascaridoid nematodes (see Mattiucci and Nascetti 2008). Among them, MAE and PCR-linked methods showed the most promising approaches in terms of efficacy, performance and cost-effectiveness, and are therefore commonly used for diagnosis.

MAE – multilocus allozyme electrophoresis: MAE relies on the distinct properties of enzymes, including net charge, molecular size and 3D-structure, which determine the migration speed along an electrophoretic gradient. Initially employed on a larger scale for bacterial population genetics and systematic studies (Selander *et al.* 1986), the method was successfully established to discriminate isomorphic sibling species of anisakids, such as for *Anisakis simplex* (Orrechia *et al.* 1986; Abollo et al. 2001), *Contracaecum osculatum* (Nascetti *et al.* 1993; Orecchia *et al.* 1994) and *Pseudoterranova decipiens* (Paggi *et al.* 2001). Through the conducting at multiple genetically independent loci makes it a fast method with simple configuration and

moderate costs (Andrews and Chilton 1999), but main drawbacks are its higher usage of sample material than PCR- based approaches and the reduced genetic resolution (Monis *et al.* 2002).

PCR – polymerase chain reaction: This standard molecular technique enables minimal amounts of any desired double-stranded DNA fragment, up to 10 kilobases length, to be amplified in vitro by factor 12 and higher if the flanking sequences are known (Saiki *et al.* 1988). In the case of taxonomic differentiation of unidentified species, a template of high inter-specific but low intra-specific variance has to be chosen. For ascaridoid nematodes, the internal transcribed spacer sequences of nuclear ribosomal DNA (ITS-1 and ITS-2, respectively) provide useful genetic markers, and are well established (e.g. Zhu *et al.* 1998; 2000a; 2001b; Shih 2004; Nadler *et al.* 2005; Klimpel *et al.* 2007). A continually expanding list of ITS-1 and ITS-2 sequences from anisakids and other nematodes is publicly available at the GenBank™ database.

PCR-RFLP – PCR restriction fragment length polymorphism: A proven and tested procedure to process by PCR generated amplicons further is to digest them with a set of restriction endonucleases with subsequent separation and staining of the fragments on an electrophoretic agarose gel (Saiki *et al.* 1985). Due to resulting differences in length of the digested fragments, depending on the position of restriction sites, it is also applicable and used for species identification like anisakid nematodes (e.g. Sugane *et al.* 1989; Matsuura *et al.* 1992; D'Amelio *et al.* 1999; 2000; Umehara *et al.* 2007). However, sequence variations, which occur outside the cleavage sites of the used restriction enzymes, remain mainly undetected (Gasser and Zhu 1999).

DNA sequencing: DNA sequencing has become fast and inexpensive enough to be applied in many disciplines of molecular biology, including molecular taxonomy. By screening every nucleotide position, it is the most thorough way to analyze DNA sequences amplified by PCR. Modern systems operate automatically employing chain terminating dideoxynucleotides, fluorescent laser detection and capillary electrophoresis, and can be analyzed by bioinformatics software (Wilson *et al.* 1990). Nonetheless, it produces a substantial data load per specimen, making it a laborious and costly method when used for larger sample sizes (Gasser 1998).

SSCP – single strand conformation polymorphism: SSCP is a powerful genetic tool for taxonomic studies investigating genetic variation (Gasser and Chilton 2001). In principle, single-stranded DNA migrates through a non-denaturing gel, not only based on its molecular weight,

but also on its conformational structure, which depends on base composition. The arising band patterns on the gel give a visual representation of each sequence analyzed for quick and efficient comparison among samples (Gasser *et al.* 2006). Several studies have shown the high resolution power of even single nucleotide differences (e.g. Zhu and Gasser 1998; Gasser *et al.* 1999). An acceptable sequence length for analysis is 100 to 500 bp (Gasser *et al.* 2006).

1.8 Conclusion

Anisakis and other species belonging to the Anisakidae family are common parasites of the world's marine ecosystems. Since mankind is greatly benefitting from its rich resources of fish and other sea animals, these parasites pose a significant risk to public health and fisheries welfare. As an initial step, it is imperative to address prevalence, geographic extent and host distribution of anisakid nematode populations, especially in locations with important fishing industry and markets. The accurate identification of species is crucial, and requires the use of latest technical advances, particularly for the larval stages, which cannot be identified to species based on morphology.

The main objectives of this study were: (1) to determine infection rates of larval anisakids in selected fish species of commercial value which are commonly caught around south-eastern Australia and (2) to characterize species within the family Anisakidae both by morphologic and molecular examination, and establish their distribution among infected fish hosts.

Chapter 2

Material and Methods

2. 1 Study objects

Extractions of intestinal parasites were performed in five different fish species caught from three different regions around the Southeastern coast of Australia (Tab. 2.1). A short introduction of the biology of each fish species is given in the following.

2.1.1 *Aldrichetta forsteri* (Valenciennes 1836) – Yellow-eye Mullet

Yellow-eye mullets (Fig. 2.1) are confined to southern Australia and New Zealand. Their habitat is demersal, and usually found at sandy bottoms near the coast but also in estuarine waters. They have a catadromous life cycle, with the juvenile stage living in freshwater and the mature stage in saltwater. Food intake is omnivorous, ranging from plant material to invertebrates and organic detritus. Within their occurrence in Australia (Victoria, northern Tasmania, South Australia), they are an important marine food item.

2.1.2 *Neoplatycephalus richardsoni* (Castelnau 1872) – Tiger Flathead

Tiger flatheads (Fig. 2.2) occur in subtropical waters in the southwest Pacific, and are a species endemic to Australia. They are demersal, piscivorous fish hunting mainly on smaller fish and crustaceans. Usually they are sold fresh on the domestic fish markets.

2.1.3 *Platycephalus bassensis* (Cuvier, 1829) – Sand Flathead

Sand flatheads (Fig. 2.3) are endemic to the Indo-Pacific of southern Australia. Normally found solitary or in loose aggregations, they are demersal ambush predators of fish and crustaceans. In Port Phillip Bay, 50-80 % of all catches of flathead species are *Platycephalus bassensis*. Catches from recreational anglers, though, outnumber commercial catches by factor 10.

Table 2.1. List of the examined fish species and basic information about their biology.

Fish species	Family	Habitat	Feeding type	Sample size	L_T [cm]
Aldrichetta forsteri – Yellow-eye mullet	Mugilidae	demersal	omnivorous	10	30.2
Neoplatycephalus richardsoni – Tiger flathead	Platycephalidae	demersal	piscivorous	5	33.2
Platycephalus bassensis – Sand flathead	Platycephalidae	demersal	piscivorous	5	27.6
Sardinops sagax – Pilchard	Clupeidae	pelagic	planktivorous	10	15.9
Seriola lalandi – King fish	Carangidae	bentho-pelagic	piscivorous	10	n/a

Figure 2.1. Lateral view of *Aldrichetta forsteri*.

Figure 2.2. Anterior and dorsal view of *Neoplatycephalus richardsoni*.

2.1.4 *Sardinops sagax* (Jenyns 1842) – Pilchard

Apart from the northern and southwestern Atlantic, pilchards (Fig. 2.4) are distributed ubiquitously in subtropical waters. The subspecies *Sardinops sagax neopilchardus* is confined to New Zealand, southern Australia and Tasmania, but there is no consensus about its validation as a subspecies. Generally, pilchards occupy a pelagic habitat within the sublittoral zone adjacent to the continental shelf. They are filter-feeders of phytoplankton and small crustaceans. Despite their relative small size, they form large schools of significant biomass, thus making them viable for the fishing industry. Commercial exploitation is various, ranging from fish meal processing to pet food, as bait fish, fresh sale, up to canning and oil production. Australian main fisheries lie in Western Australia and Victoria, where they are located east of Lakes Entrance, eastern Bass Strait and Port Phillip Bay.

2.1.5 *Seriola lalandi* (Valenciennes 1833) – Yellowtail Kingfish

These fish are prevalent in subtropical waters around the southern hemisphere and the north Pacific. They occur around the Australian continent, except on the northern coast. Yellowtail Kingfish (Fig. 2.5) can reach up to 2.5 m length and 70 kg weight, but caught fish are considerably smaller. Their habitat is bentho-pelagic, sometimes also estuarine. Diet consists mainly of fish, squid and crustaceans they hunt on. Due to their immense size they are popular targets for game fishing and also marketed as sashimi.

2. 2 Parasite collection

Most samples were collected from fishes of local fish markets or provided by a fish-food processing plant from Melbourne. Apart from *Seriola lalandi*, which viscera had been handed over directly, the abdominal cavity of the fish were opened ventrally.

Nematodes were collected from the internal organs, including digestive tract, gonads, liver, body cavity and kidney. They were washed extensively in PBS solution immediately after collection. A small section of the middle part (<1 mm) of the worm, which is dispensable for

Figure 2.3. Dorsal view of *Platycephalus bassensis*.

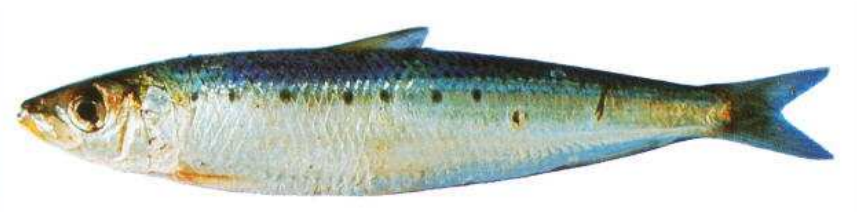

Figure 2.4. Lateral view of *Sardinops sagax* (picture from SeaFIC, New Zealand).

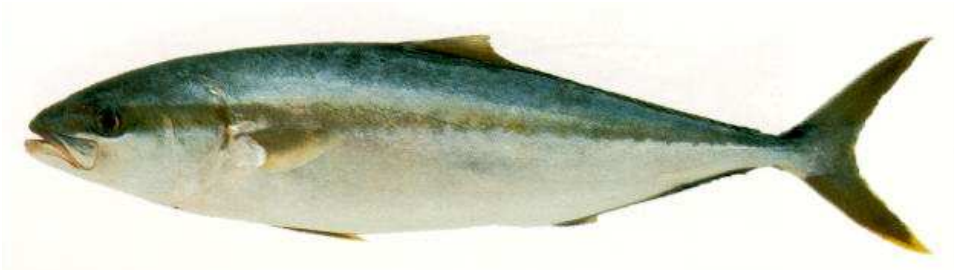

Figure 2.5. Lateral view of *Seriola lalandi* (picture from SeaFIC, New Zealand).

morphologic studies, was cut out with a clean scalpel, transferred into a tube and kept frozen at -80°C. The anterior and posterior ends were transferred to 70 % ethanol for preservation.

2.3 Morphological examination

Prior to watching the specimen under the light microscope, it was cleared in lactophenol to remove any stainings. Characters of systematic importance were measured by an eyepiece micrometer and sketch drawings made using a camera lucida. All measures are given as the arithmetic mean in millimeters unless stated otherwise, followed by the range in parentheses. These parameters include total body length, maximum body width, distance of the nerve ring to the anterior end, length of the esophagus, ventriculus, ventricular appendix, intestinal caecum, and distance of the anus to the posterior end. Special consideration was taken for the morphology of the lips, tail and the position of the excretion porus. Larval stages of anisakid nematodes were classified among different "morphotypes" (Cannon 1977).

2.4 Genomic DNA extraction

Genomic DNA from the frozen mid sections were isolated according to a standard sodium dodecyl sulphate (SDS)-Proteinase K method (Gasser *et al.* 1993). Briefly, samples get transferred into individual 1.5 ml tubes, each containing 500 µl of extraction solution (containing 350 µg/ml Proteinase K in DNA extraction buffer). At 37°C, they were incubated for 18 h. Purification was performed using Wizard^{TM} DNA Clean-Up minicolumns (Promega) according to the manufacturer's protocol. Control DNA samples from the host muscle tissue were also prepared. All purified samples were stored at -20°C until further use.

2.5 PCR – polymerase chain reaction

The second internal transcribed spacer of nuclear ribosomal DNA (ITS-2) was used in the present study, as it provides species-specific markers for anisakid nematodes (Zhu 1998; D'Amelio 1999). The following primer set – SS2: 5'-TTGCAGACACATTGAGCACT-3' (forward) and NC2: 5'-TTAGTTTCTTTTCCTCCGCT-3' (reverse) was used for amplification by the PCR. 3 µl of gDNA (~20 ng) were added to 47 µl PCR master mix buffer (overlaid with paraffin oil) containing 250 µM of each dNTP, 3.5 mM $MgCl_2$, each 100 pmol of forward and reverse primer, and 0.5 U *Taq* polymerase (Promega). The solution was kept on a freeze block (-20°C) and subsequently placed in a 480 thermal cycler (Perkin Elmer). The following cycling conditions were applied: initial denaturation at 94°C for 5 minutes, then 35 cycles of 94°C denaturation temperature for 30 seconds, 53°C annealing temperature for 30 seconds and 72°C elongation temperature for 30 seconds, followed by 72°C for 5 minutes.

In order to check for the quality of the amplified template, 5 µl of the PCR products were separated on 1.5 % agarose gel at 100 V in TBE buffer (Biorad) for 1 h and then stained for 30 minutes in an aqueous ethidium bromide solution (c = 5 µg/ml). Images of the gels under UV illumination were taken. ΦX174-*Hae III* (Promega) was used as molecular weight marker. Mock treated DNA sample of the host tissue and negative controls (dH_2O) were also prepared.

Due to a failure to produce utilizable sequencing data for the majority of submitted samples, PCR products were carried over to the Institute of Tropical Medicine in Tübingen, Germany, and reprocessed for sequencing. Following steps for the PCR diverge from the original approach. On ice, commercially available KAPA BloodDirect™ 2x Readymix (Peqlab), containing per reaction 2 U BloodDirect™ DNA polymerase, 1x BloodDirect™ buffer and dNTPs, were mixed to SS2 and NC2 primer (each 20 pmol), 3 µl DNA sample and dH_2O (50 µl reaction volume). The Omn-E thermal cycler (Hybaid) was used for amplification. For quality control of the product, 1.8 % agarose gel with ethidium bromide (c = 0.06 µg/ml) in 1x TAE buffer was used. 5 µl of each amplicon was mixed with 7 µl of 10% bromophenol loading dye solution, loaded and run at a voltage of 90 V for 30 to 60 minutes.

2.6 SSCP – Single strand conformation polymorphism

For rapid and efficient identification on the species level, single strand conformation polymorphism (SSCP) was used for the screening of ITS-2 amplicons for sequence variations among anisakid nematode individuals. This mutation scanning technique enables the differentiation of single stranded DNA fragments on a non-denaturing gel both by molecular weight and by secondary structure (Gasser and Chilton 2001). The non-isotopic SSCP method was adapted from Gasser *et al.* (2006) with the following modifications: 3 µl of the amplicons were diluted in 7 µl dH_2O and mixed with 10 µl DNA sequence stop solution (Elchrom Scientific); after the samples were denatured at 96°C for 15 minutes and immediately snap frozen on a freeze block (-20°C), 12 µl of each product were loaded on a SSCP gel (Elchrom Scientific) in TAE buffer and run at 7.4°C for 16 h at a current of 74 V; staining agent was SYBR gold (Invitrogen), 20 µl diluted in 100 ml dH_2O, incubated for 30 minutes with subsequent destain in dH_2O for 30 minutes. Images of the SSCP gels were taken from an UV scanner.

2.7 DNA sequencing

In order to determine the species identity of specimens, representative samples with different SSCP patterns were chosen for sequencing of the DNA template. PCR products were purified using Wizard™ PCR Preps mini columns (Promega) according to instructions of the manufacturer, and eluted in 35 µl of dH_2O. DNA concentration was determined photometrically, and sufficient amounts were submitted for automated sequencing using the BigDye™ v3.1 system. In Australia, sequencing was carried out both with forward and reverse primers (SS2 and NC2, respectively). A similar procedure was used in the laboratories of the Institute for Tropical Medicine, Tübingen, Germany, with following deviances. The PCR Purification Kit (Quiagen) was used for the amplicons' purification following the manufacturer's instructions, and eluted in 30 µl dH_2O. The sequencing reaction was carried out with the BigDye™ v.1.1 system using 40 ng of DNA template in a volume of 20 µl per reaction. The samples were purified over Centri-sep™-columns according to the manufacturer's protocol and subsequently

stored at -20°C. A 3100 DNA Capillar Sequencer (Applied Biosystems) was used for the evaluation of the sequencing products. The International Union of Pure and Applied Chemistry (IUPAC) code was used (Tab. 2.2).

2.8 Molecular analysis

ITS-2 sequences were aligned manually and compared with database entries of other nematode sequences with the aid of the algorithm BLAST (NCBI, US). The GenBank™ database was used for this purpose. The algorithm ClustalW served for sequence alignments. Pair-wise comparisons of sequence differences (D) were determined using the formula D = 1-(M/L), where M is the number of alignment positions at which two sequences have a base in common and L is the total number of alignment positions over which the two sequences are compared (Chilton *et al.* 1995).

Table 2.2. International Union of Pure and Applied Chemistry (IUPAC) codes.

Code	Base
A	adenine
B	not A
C	cytosine
D	not C
G	guanine
H	not G
K	G or T
M	A or C
N	any base
R	A or G
S	C or G
T	thymine
V	not T
W	A or T
Y	C or T
-	gap

Chapter 3

Results

3.1 Molecular analysis

In total, 133 samples of anisakid parasite specimens, plus three host DNA samples from different fish species, were subjected to molecular investigation by the PCR. Among them, 121 showed a single band with an estimated length of 450 bp (Fig. 3.1), whereas twelve samples failed to produce a band on the gel. No amplification of host DNA was observed.

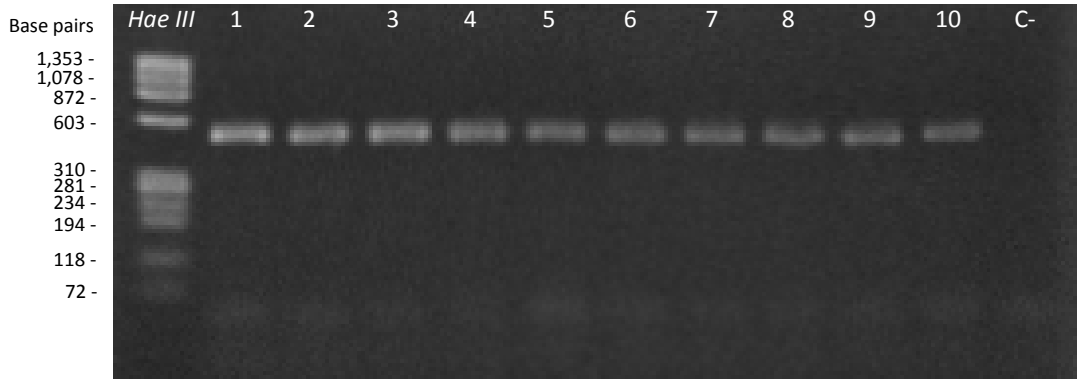

Figure 3.1. Image of PCR products of anisakid nematode samples in an 1.5 % agarose gel under UV light. *Hae III*: DNA ladder; 1 - 10: amplified DNA samples; C-: dH_2O negative control.

3.2 Anisakids in fish

In 60% of all examined fish individuals (n=40), nematodes of the Anisakidae family could be found within the abdominal cavity. A summary of the infection rate of anisakids for each fish species is given in Table 3.1. The average intensity of infected fish is 19.75 worms per specimen, with a range of 1 to 112 anisakids per specimen. Figure 3.2 depicts the anisakid parasite burden for each fish species.

Table 3.1. Percentage of anisakid infection for selected fish species from south Australian waters.

Fish species	n	Infection rate
Aldrichetta forsteri	10	100%
Neoplatycephalus richardsoni	5	90%
Platycephalus bassensis	5	20%
Sardinops sagax	10	90%
Seriola lalandi	10	0%

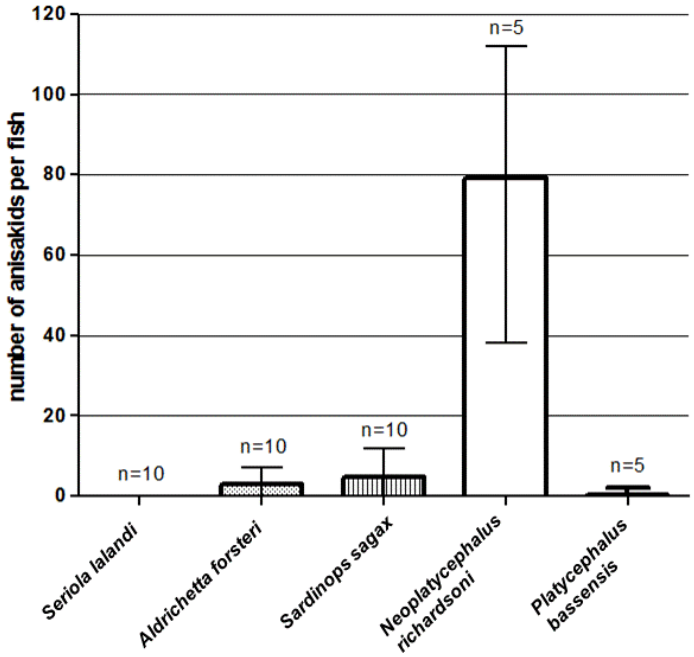

Figure 3.2. Mean intensity of infection with anisakids (Nematoda: Anisakidae) of selected fish species from south Australian waters. Error bar shows range.

3.3 *Anisakis*

3.3.1 *Anisakis* larval type I of Cannon, 1977

Material examined: Third stage larvae (n=52) usually found encysted in a coiled, spring-like state on the walls of intestines, stomach, gonads and rarely in the liver. All specimens were obtained from tiger flathead *Neoplatycephalus richardsoni* (n=4).

Morphology (Fig. 3.3): Body length 21.74 (14.99-27.12), width 0.48 (0.39-0.59). Poorly defined labia, one dorsal and two subventral. Boring tooth present. Excretion pore below tooth. Distance nerve ring – anterior end 0.31 (0.08-0.37). Muscular esophagus, 2.16 (1.67-2.66) long, ends in ventriculus, 0.84 (0.36-1.30) long. Ventriculus joins obliquely with intestines. Distance anus – posterior end 0.12 (0.08-0.15). Three anal glands encircling rectum. Tail short and rounded, ending with distinct mucron.

Genetic analysis: All collected *Anisakis* larval type I morphotypes were subjected to molecular analyses using ITS-2 primers. Two samples failed to produce an amplified DNA fragment. The remaining cohort showed following SSCP pattern: A-1 (n=41) and A-2 (n=8) (Fig. 3.4).

The length of the ITS-2 sequence was 308 bp for A-1 and A-2, respectively. Alignment of the ITS-2 sequences of A-1 and A-2 revealed they were identical with database sequences of both *Anisakis pegreffii* and *A. simplex s s.* (Fig. 3.5 and 3.6).

3.4 *Contracaecum*

3.4.1 *Contracaecum* larval type I

Material examined: These third-stage larvae (n=26) were found exclusively inside the liver of yellow eye mullet *Aldrichetta forsteri* (n=9).

Morphology (Fig. 3.7): Body length 22.71 (14.99-28.11) long, width broad, 1.08 (0.92-1.18) wide. Lips weakly developed. Tooth present, with excretion pore below. Distance nerve ring –

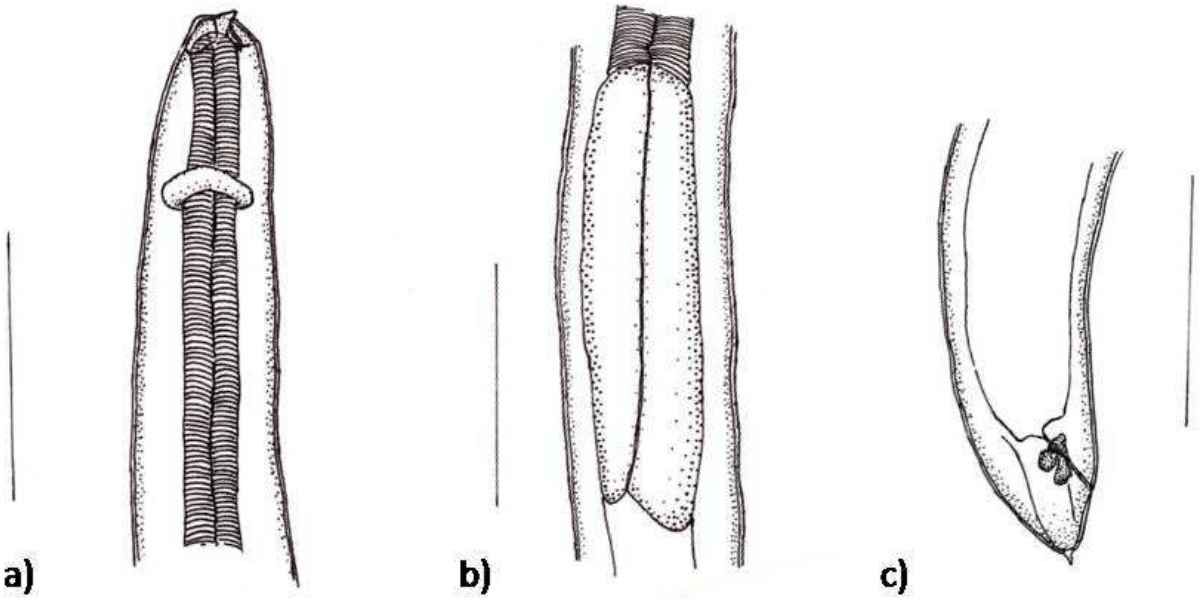

Figure 3.3. *Anisakis* larval type I of Cannon, 1977 from *Neoplatycephalus richardsoni*, third stage larvae: (a) anterior end showing boring tooth, excretion porus and nerve ring; (b) ventriculus; (c) posterior end showing anal glands and mucron. Scale bar = 500 µm.

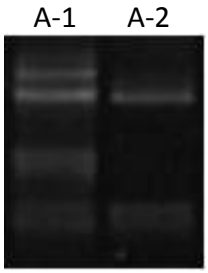

Figure 3.4. SSCP patterns of *Anisakis* larval type I morphotype.

TTACGGTGAACTGTCTTCACGGTTTTTCTGGACTGTGAAGCATTCGGCAAGCAATTGCTGTTGTGTTGTTG
GTGATTCTATCATGGACAATATGACGAGCGGTTCCTTGCTTAGTGATGACAAAAGAAGACGTCAACACCGA
ATCTACTATACTACTAATACTAGTATATAGGTGAGGTGCTTTTGGTGGTCACAAAAGTGACAAGTATGCCA
TTTCATAGGGGCAACAACCAGCATACGTGATAAGTTGGCTGGTTGATGAAACGGCAACGGAATGACGGACG
TCTATGTGATCAAAAATGATACTA

Figure 3.5. ITS-2 region sequence of third-stage larva from tiger flathead *Neoplatycephalus richardsoni* (A-1/A-2) which matched database sequences representing *Anisakis pegreffii* and *A. simplex s s*. (see in Abe *et al.* 2005; Zhang *et al.* 2007; Zhu *et al.* 2007b).

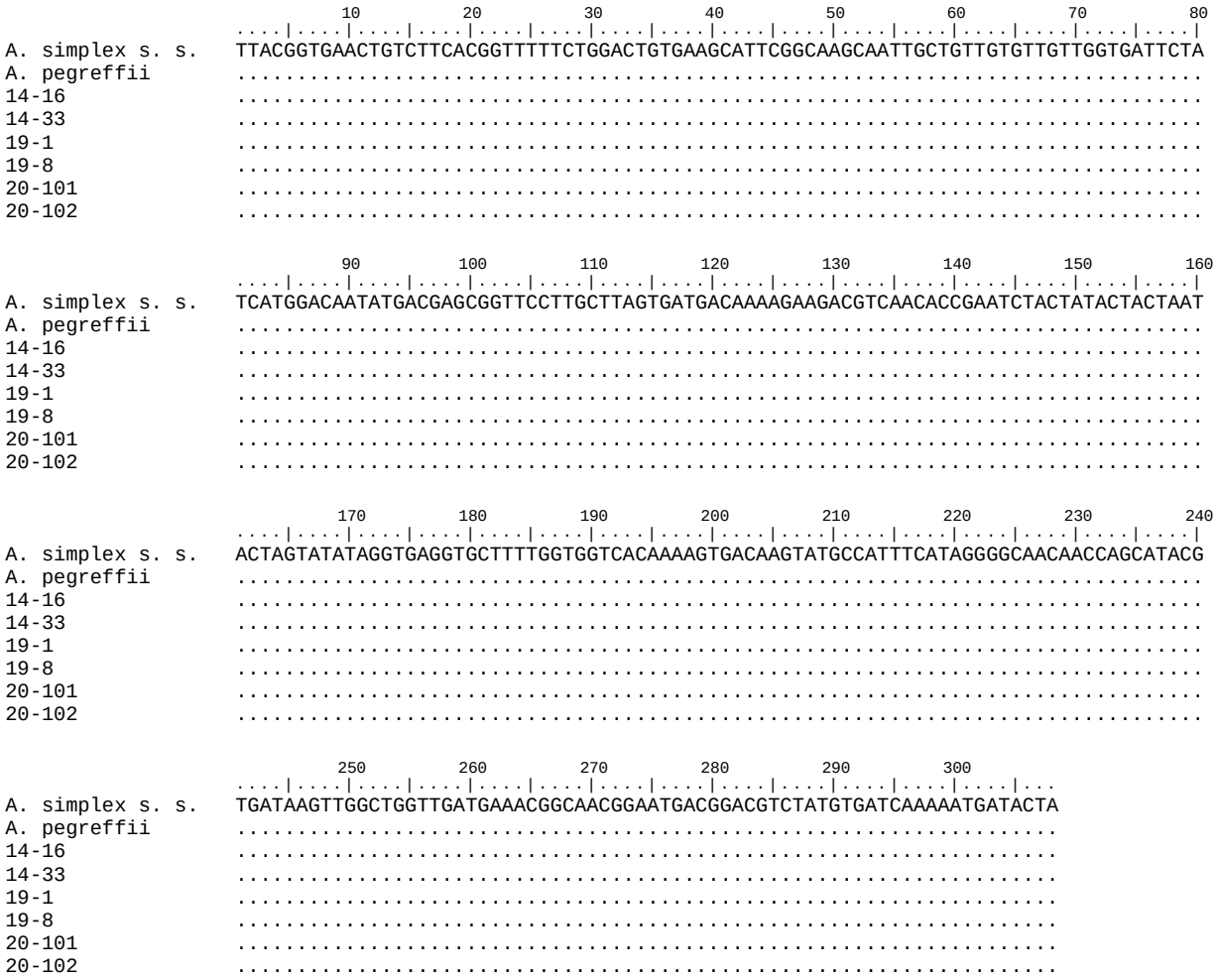

Figure 3.6. Alignment sequences of the ITS-2 region of *Anisakis* larval type I to database sequences of selected *Anisakis* spp. (Accession numbers from top to bottom: AF411202 and EU624343). Samples are labeled according to host and parasite number. Polymorphic sites were designated using IUPAC codes.

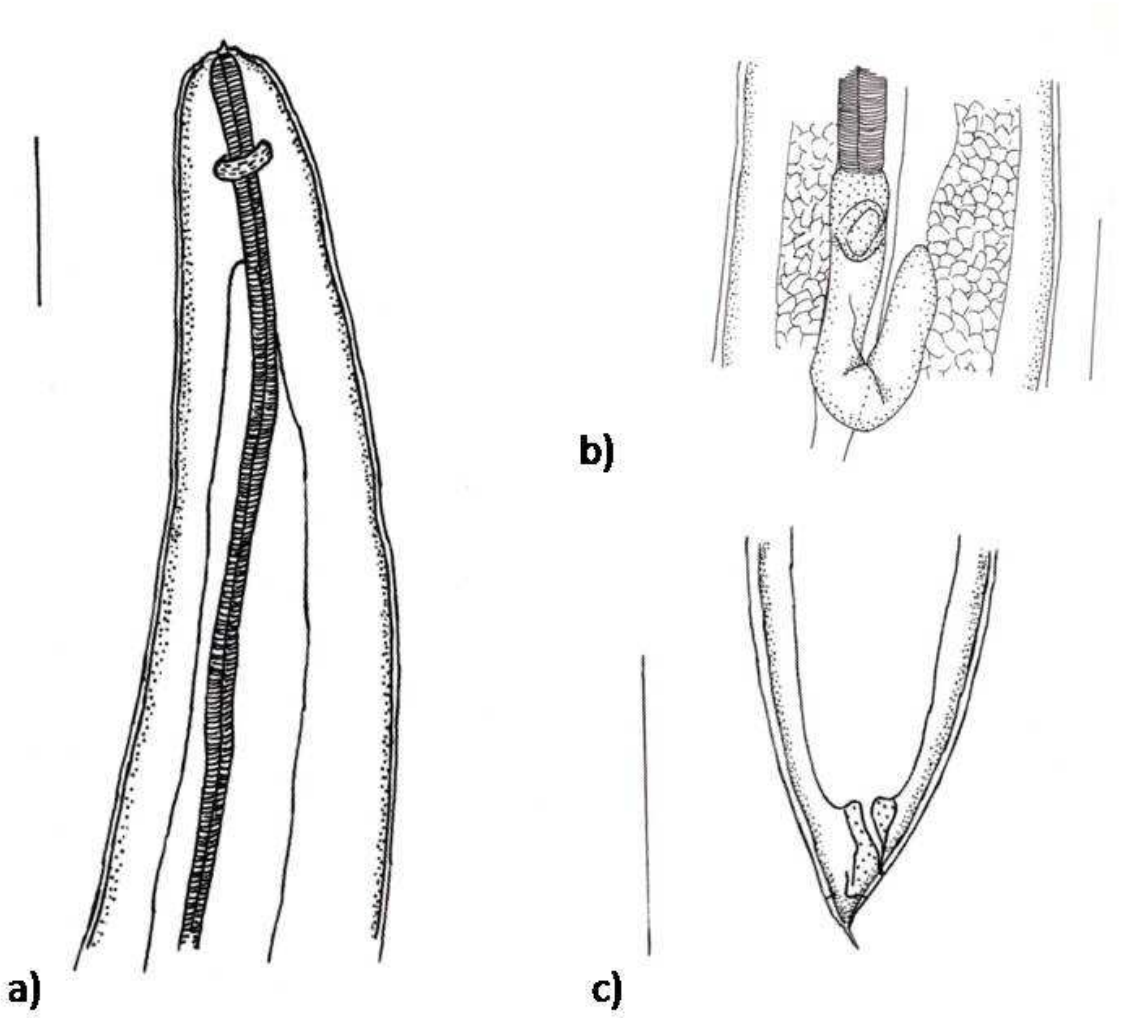

Figure 3.7. *Contracaecum* larval type I from yellow-eye mullet *Aldrichetta forsteri*: (a) anterior end showing boring tooth, nerve ring and intestinal caecum; (b) ventriculus with ventricular appendix; (c) posterior end. Scale bar = 500 µm.

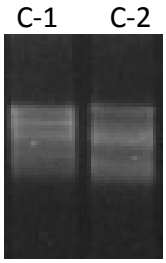

Figure 3.8. SSCP patterns of *Contracaecum* larval type I morphotype.

ATATTCAACACAATCCGCTGAAGCGGTGATTTCGGTGAGCAATGTCTCTTCCTTTTGAGCATTCCTCATCT
AGTAAAGAAAGTACCGCATAGTTAGTCGGTAAGGTGCGGTTAAGGCCATCAATATGTTGTCATTGCTCAAT
GCGGCTTTCAGTGTATGTTAAGAGTGGCTGAAACCGACGAGTGGCTAGAAATGCACAACATATCGAACAAT
GATGGTACTATTTGTGTT

Figure 3.9. ITS-2 region sequence of third stage larva from yellow-eye mullet *Aldrichetta forsteri* (C-1) which matched database sequences from *Contracaecum multipapillatum D* (see in Shamsi *et al.* 2008).

ATATTCAACACAATCCGCTGAAGCGGTGATTTCGGTGAGCAATGTCTCTTCCTTTTGAGCATTCCTCATCT
AGTAAAGAAAGTACCGCATAGTTAGTCGGTAAGGTGCGGTTAAGGCCATCAATATGTTGTCATTGCTCAAT
GCGGCTTTCAGTGTATGTTAAGAGTGGCTGAAACCGATGAGTGGCTAGAAATGCACAACATATCGAACAAT
GATGGTACTATTTGTGTT

Figure 3.10. ITS-2 region sequence of third stage larva from yellow-eye mullet *Aldrichetta forsteri* (C-2) which matched database sequences from *Contracaecum multipapillatum D* (see in Shamsi *et al.* 2008), apart from one nucleotide difference.

anterior end 0.36 (0.29-0.45). Long, slim esophagus, 3.97 (2.61-5.04). Ventriculus short, 0.22 (0.14-0.31) long. Ventricular appendix 1.16 (0.39-1.51) long, sometimes ending anteriorly, about one third the length of intestinal caecum, 3.35 (1.95-4.42) long. Distance anus – posterior end 0.18 (0.09-0.26). Tail conical with sharply pointed end, no spine.

Genetic analysis: Ten specimens from 9 individual hosts were subjected to molecular characterization using ITS-2 primers. There were two different SSCP pattern distinguishable: C-1 (n=9) and C-2 (n=1) (Fig. 3.8).

The ITS-2 sequence of C-1 and C-2 were 231 bp long. Alignment of the ITS-2 sequence of C-1 was homologous to database sequences of *Contracaecum multipapillatum* D (Fig. 3.9). Alignment of the ITS-2 sequence of C-2 was identical with database sequences of *C. multipapillatum* D with the exception of one base substitute at sequence position 180 (Fig. 3.10).

3.4.2 *Contracaecum* larval type III of Cannon, 1977

Material examined: Third-stage larvae (n=26) were found in the liver and pancreas – in one case in the intestines – of tiger flathead *Neoplatycephalus richardsoni* (n=2), and a single specimen extracted from the liver of yellow-eye mullet *Aldrichetta forsteri* (n=1).

Morphology (Fig. 3.11): This morphotype had similar features with *Contracaecum* sp. (larval type II) described by Cannon. Cuticula annulated in ripple-like pattern. Body length and width 3.28 (1.49-4.77) and 0.23 (0.13-0.33), respectively. Lips inconspicuous. Tooth present, with excretion pore below. Distance nerve ring – anterior end 0.11 (0.05-0.25). Muscular esophagus 0.55 (0.35-0.73) long. Ventriculus short, 0.07 (0.03-0.20) long, and adjacent ventricular appendix 0.37 (0.11-0.61) long. Intestinal caecum 0.33 (0.13-0.50) long, equates length of ventricular appendix. Distance anus – posterior end 0.10 (0.08-0.13). Three anal glands around rectum. Conical shape of tail with rounded tip; phasmids not distinguished, though paired, drop shaped structure visible in tail.

Genetic analysis: Seven specimens, collected from all hosts (n=3) were successfully amplified using ITS-2 primer and subjected to SSCP electrophoresis. This analysis resulted in two

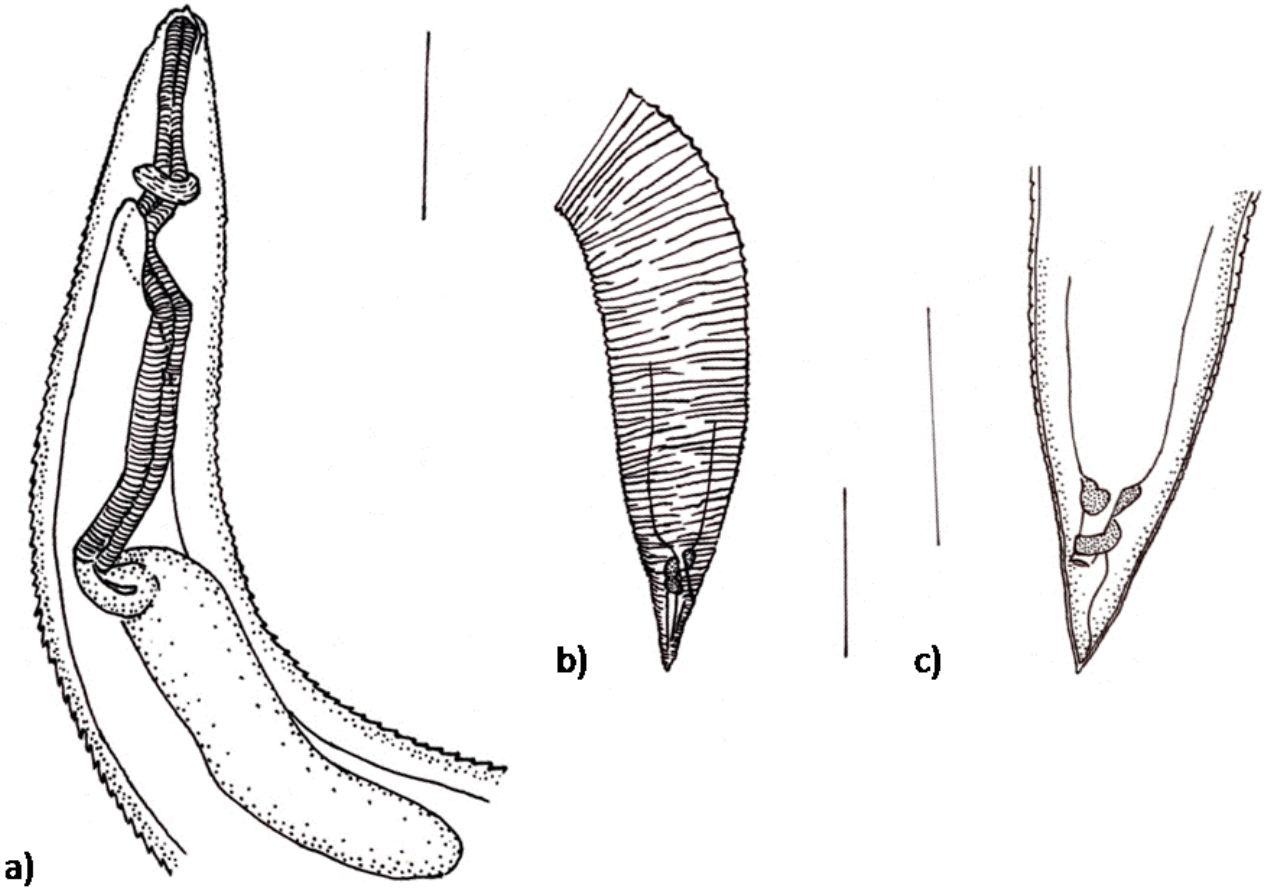

Figure 3.11. *Contracaecum* larval type III of Cannon, 1977 from *Neoplatycephalus richardsoni*, third stage larvae: (a) anterior part showing nerve ring, intestinal caecum and ventricular organ; (b) posterior part, surface structure; (c) posterior end showing anal glands. Scale bar = 250 µm.

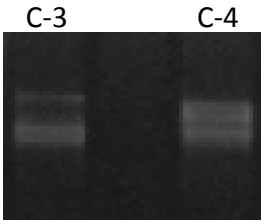

Figure 3.12. SSCP patterns of *Contracaecum* larval type III morphotype.

```
                10         20         30         40         50         60         70         80
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C-3.1   ATATTCAGAGTGATCAGCCACANCGTCNTCTTAACGGAATGGGCTCGAAGCATG----TGCGGGCATTCAGACAGCGCAA
C-3.2   ...N....N..TT.NC...-...N.-...NN..N...G-AN.TGT...C...TTTGCNNGACA..NC-----..NNA-.N
C-3.3   -......ATACT...C..G-T-.G.---.TCA...C.CT--..TGTA.-N.C----GCGA.CTAT...-G....-TNT.T

                90         100        110        120        130        140        150        160
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C-3.1   GGTGCCCGAC--AGGGCGACKCAATGTCTCTTNNNAACGG-YAAGCT-ATTACTCAGTCAMTACA-AGKTAAGCAGCAGA
C-3.2   ......ATTTCGG.N..-NN-T.TA....NN.........G.....NA..AN.NN..N-TNN..NCGN..N.....N..N
C-3.3   -N..T..AG.TTG.A..TG...CT.A...GG.......A.G..-...T...GTAA.CCT-T.T.TCT..A.G.TGC-TT.

                170        180        190        200        210        220        230        240
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C-3.1   TAATGGCTATAGYGTGTG--ATATGGTCATCATATGATACGGCTMTG-M-GCACGYCAGTATCAA-TTGGCTGTCMTATA
C-3.2   AGNN.NG.NGNAN.N...AT.NT.NNNAGCA--N.N....N..NNN.-.A..........C.N..G......T.NTATA.
C-3.3   A.CC...C-CTAA.C...-A.AT..A.TG.A-G.C.-.C.CT.G..-A.G..-.C.T.A.....TG...A...C.-A.C.

                250        260        270        280        290        300        310
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
C-3.1   ATGGTTGACNGGTGGCTAAGCCTTTGGA-AATCGAAGGAACTACTGACGASTAGGTGTGCTATCTAGCAGCT
C-3.2   N..........G...N...N.NNN.N.-N...N.....N........CG..T..N.-..NNC.N.A..T..
C-3.3   .G..C......A..TT...C...CGAC.CT...AT.C..CTGCGAC..TGNCTTA.A-CTA..ACG.T.T..
```

Figure 3.13. Alignment sequences of the ITS-2 region of third stage larva from tiger flathead *Neoplatycephalus richardsoni*. All sequences arose from one specimen of *Contracaecum* larval type III with the SSCP pattern C-3. Polymorphic sites were designated using IUPAC codes.

ATATTCAATACTATCCGCACAATGCTTCAGACGGTTCGTGTGAAGCGTGCGGTGCATTCGACAAGCAGTGT
CCCTTTGGGGCGCTCCTTGTCTGGTTTGAACGGCAAATTATTGCAAAGATTTACTCGGTAAGCAGCAATAA
TGGCCGTAAGTGTGAGATTGATTGTGTACGTCCCTCGATGCGGCCCCCAGTATTTGTTGACTGCCTCTGGT
GGTGACTGGGGGTTAAGTATCGGATTATCGAAAGAATGTGACATGTCTTATACGGTTATGTGCT

Figure 3.14. ITS-2 region sequence of third stage larva from yellow-eye mullet *Aldrichetta forsteri* (C-4) which matched database sequences from *Contracaecum ogmorhini s.s.* (see in Zhu *et al.* 2001; Nadler *et al.* 2005; Shamsi 2007), apart from two bases difference.

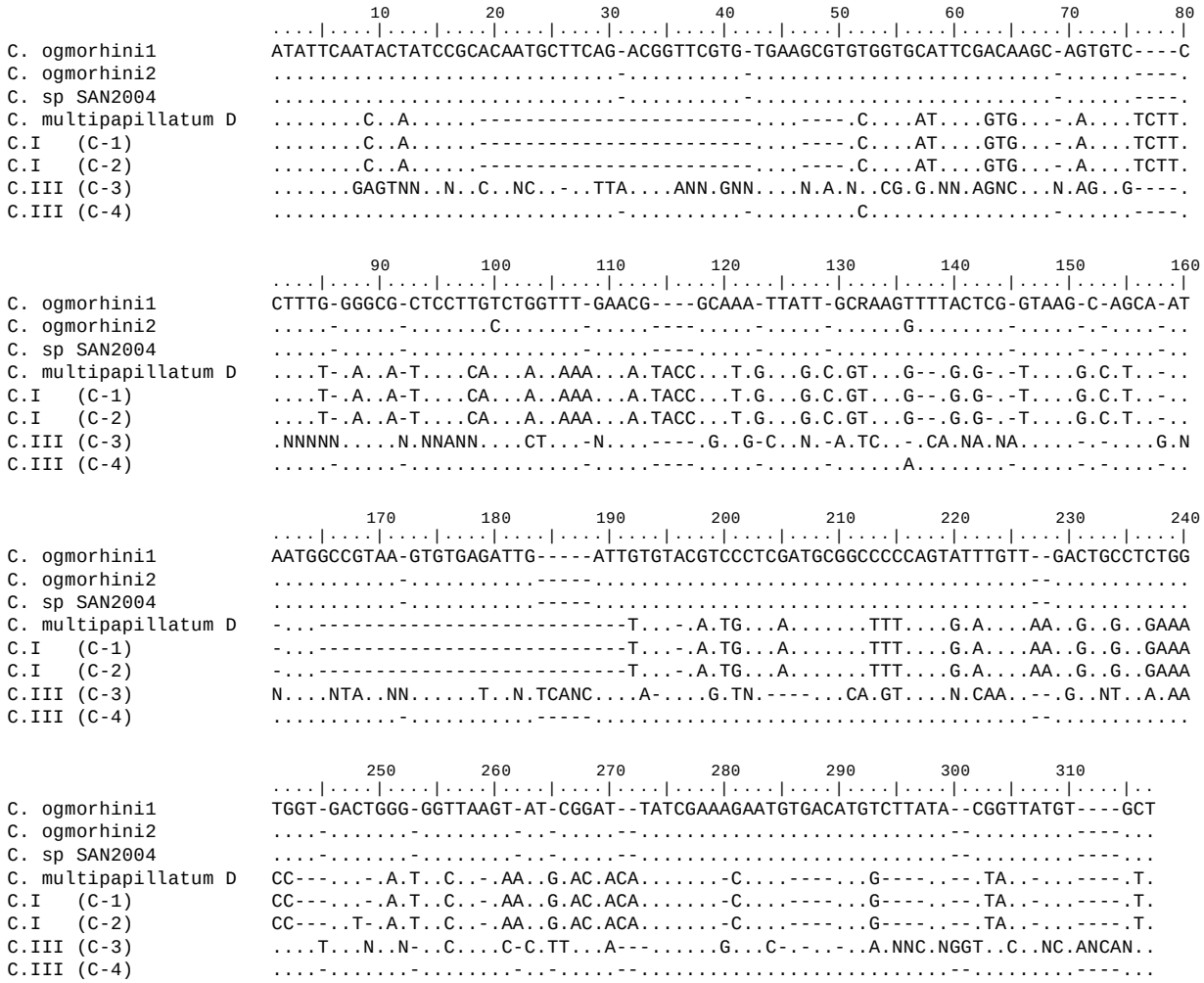

Figure 3.15. Alignment sequences of the ITS-2 region of *Contracaecum* larval types with different SSCP patterns – indicated in brackets – to database sequences of selected *Contracaecum* spp. (Accession numbers from top to bottom: AJ291471, AJ291472, AY821753 and AM940060). Polymorphic sites were designated using IUPAC codes.

SSCP patterns, namely C-3 (n=6) from *Neoplatycephalus richardsoni* and C-4 (n=1) from *Aldrichetta forsteri* (Fig. 3.12).

The ITS-2 sequence of C-4 was 277 bp long. Alignment of the ITS-2 sequence of C-4 matched the GenBank™ database sequences of *Contracaecum ogmorhini s.s.,* apart from two bases difference at position 50 and 120 (Fig. 3.14). The ITS-2 sequence of C-3 could not be determined unequivocally. However, through alignment of three sequences from the same DNA sample (Fig. 3.13), one putative sequence of C-3 was constructed. When the putative ITS-2 sequence from C-3 was compared with the sequence from C-4, it exhibited 55.18 % homology to it. An overview of all *Contracaecum* alignments is given in Figure 3.15.

3.5 *Hysterothylacium*

3.5.1 *Hysterothylacium* larval type IV of Cannon, 1977

Material examined: All fourth-stage larvae (n=63) extracted from the intestines, pyloric caeca, liver and pancreas of tiger flathead *Neoplatycephalus richardsoni* (n=4), except for one specimen obtained from the intestines of yellow-eye mullet *Aldrichetta forsteri* (n=1).

Morphology (Fig. 3.16): Variable body length of 7.26 (2.49-19.25), width 0.23 (0.11-0.44). Lips well developed, with small interlabia. Nerve ring 0.18 (0.06-0.33) from anterior end. Excretion pore near nerve ring. Muscular esophagus 0.72 (0.45-1.16). Ventriculus 0.13 (0.03-1.05). Ventricular appendix 0.39 (0.06-1.74). Caecum 0.31 (0.10-1.05). Gonads visible. Anus 0.14 (0.06-0.35) from posterior end. Tail short, with cluster of spines resembling a crown at posterior end.

Genetic analysis: Seven specimens from *N. richardsoni* (n=4) were subjected to molecular characterization using ITS-2 primers. No differences of the SSCP pattern were detected.

The ITS-2 sequence was 345 bp long. Alignment of the ITS-2 sequence (Fig. 3.17) with database sequences showed no match, but 87.57 % identity with 6.67 % gaps from sequences of *Hysterothylacium bidentatum*, as well as 85.96 – 86.44 % identity with 5.06 to 4.52 % gaps, respectively, from sequences of both *H. aduncum* and *H. auctum* (Fig. 3.20).

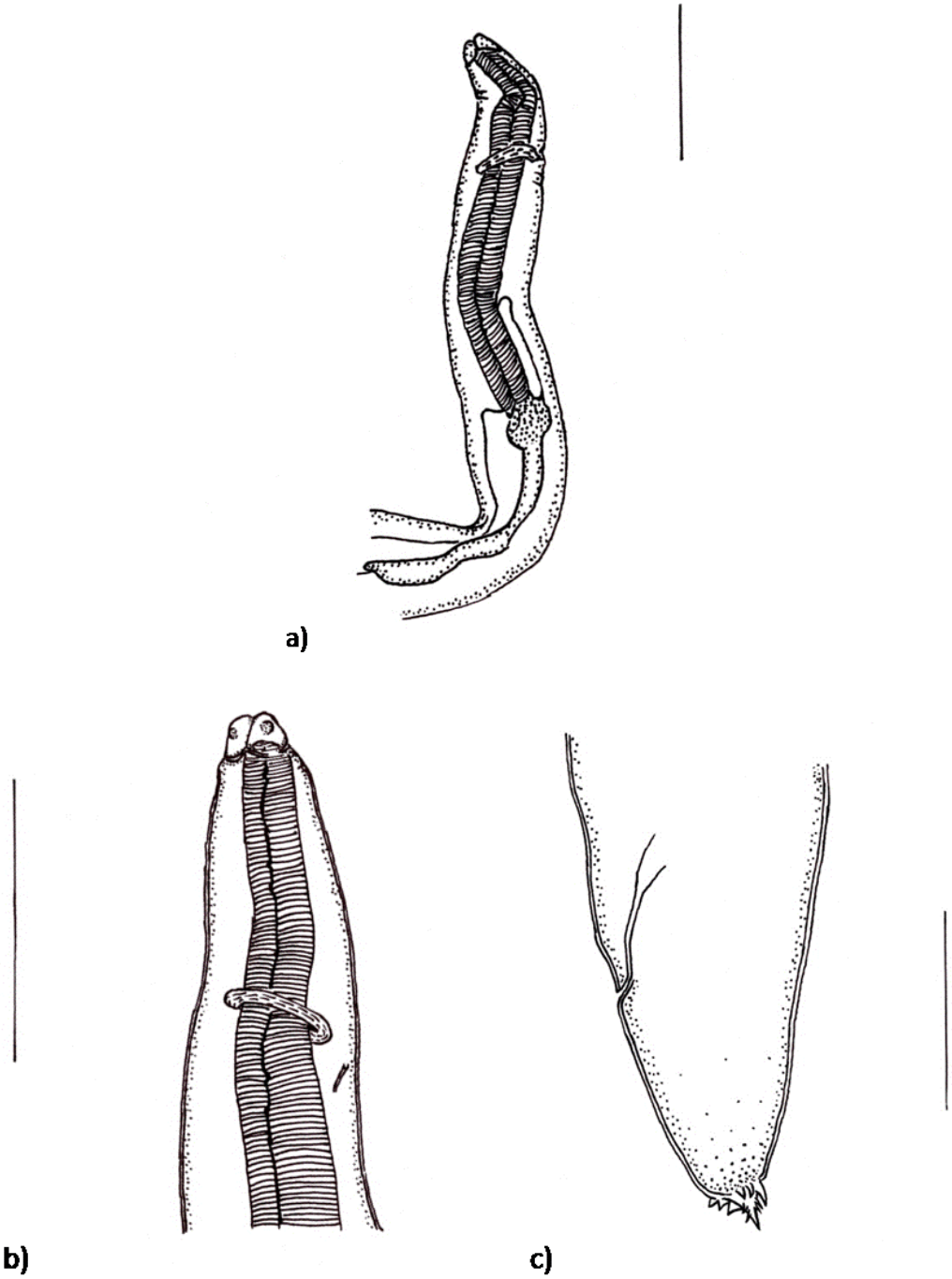

Figure 3.16. *Hysterothylacium* larval type IV of Cannon, 1977 from *Aldrichetta forsteri* (a) and *Neoplatycephalus richardsoni* (b and c), fourth stage larvae: (a) anterior end showing lips, nerve ring, intestinal caecum and ventricular organ (scale bar = 250 µm); (b) anterior end showing lips, interlabia, nerve ring and excretion porus (scale bar = 250 µm); (c) posterior end (scale bar = 100 µm).

TTTGAATATCAAACATCCGCGTGTTTTTGCGAACATGCGGTGTATTCGGTGAGCTATGGTGGTGTTGTTGC
TGCTGCTGTGTGTATGTTGAGGCCTCTCGTTTCCACGGGTCGGTCACCTCATATGCAGGGCGGCCGCCGCG
CCGGCATACCTTGCTTAGGCTGTGTGCCATATATCGCTCGTAATCATTAGCTCCATGCGAGGCGATGATGG
CCGTCAAGTGTCGCTCTCTTGTCCATAGATACGGCTCCGAGCACGTGTTTGCCAATCGGCTATCGGTTTGG
AGTGCCCGTATCGATCGCTGGAAATGGAAGAGGGGGTGATGCGAGGTGGCTGTCACTTTGT

Figure 3.17. ITS-2 region sequence of fourth stage larva from *Neoplatycephalus richardsoni,* which showed similarities with database sequences from *Hysterothylacium aduncum, H. auctum* and *H. bidentatum* (see in Zhu *et al.* 1998; Nadler *et al.* 2000; Umehara *et al.* 2008).

3.5.2 *Hysterothylacium* larval type VIII

Material examined: Third stage larvae (n=90) extracted from the intestines, pyloric caeca, liver, pancreas and the body cavity of pilchard *Sardinops sagax* (n=9) and tiger flathead *Neoplatycephalus richardsoni* (n=3).

Morphology (Fig. 3.18): Body measures variable, length 5.97 (2.37-12.83), width 0.24 (0.11-0.51). Labia inconspicuous with tooth present. Nerve ring 0.22 (0.06-0.46) past anterior end. Excretion pore near nerve ring. Esophagus slender, 0.91 (0.40-1.45) long, followed by short ventriculus, 0.08 (0.02-0.17) long. Ventricular appendix 0.30 (0.07-0.78) long. Intestinal caecum's length highly variable, 0.37 (0.12-0.98) long. Distance anus – posterior end 0.14 (0.07-0.20). Tail conical, with single terminal spine at round tip.

Genetic analysis: A subdivision of the total sample size (n=27) from both host species (*N. richardsoni*: n=3; *S. sagax*: n=7) were used for further analysis *via* molecular methods. No variations of the SSCP pattern were observed.

The ITS-2 sequence was 348 bp long. Alignment of the ITS-2 sequence (Fig. 3.19) revealed it was 96.57 – 97.13 % identical (12 and 10 bases deviance, respectively) with database sequences of both *Hysterothylacium aduncum* and *H. auctum* (Fig. 3.20).

3.6 Morphologically unidentified members of the Anisakidae family

Material examined: A substantial proportion of all morphologically examined anisakids (n=67) were not distinguishable, or only with great uncertainty. Furthermore, anisakid parasites of *Neoplatycephalus richardsoni* (n=1) and *Platycephalus bassensis* (n=5) were not analyzed by light microscopy due to time limitations (Tab. 3.2). Therefore, a subset of these unidentified samples were subjected to molecular-based investigation.

Genetic analysis: A total of 17 specimen, one from *Aldrichetta forsteri* (n=1), twelve from *N. richardsoni* (n=4), two from *P. bassensis* (n=1), and two from *Sardinops sagax* (n=2) were taken for molecular analysis. Among them, 15 amplicons of ITS-2 could be generated and further used

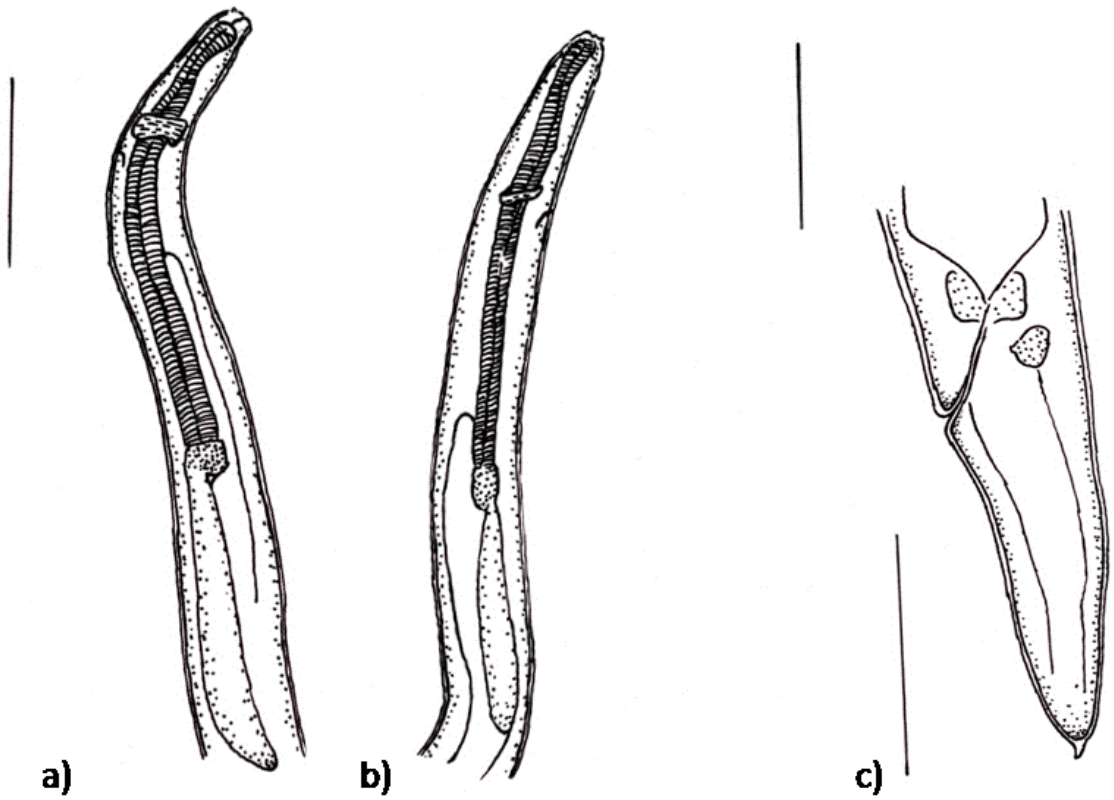

Figure 3.18. *Hysterothylacium* larval type VIII from *Sardinops sagax*, third stage larvae: (a) and (b) anterior part showing nerve ring, excretion porus, intestinal caecum and ventricular organ (scale bar = 250 µm); (c) posterior end showing anal glands (scale bar = 100 µm).

TTTGACTATCAAACATCCGTGTGTTTTCCAACTCATGGTGTATTCGGCGAGCTATGGTGGTGTTGTTGGCA
ATGCTGTGAGTGTGTGTTGCGGCCTCTCTTAAGGAGAGGTCACTTCATGTGCTTGAGGCAATGGCCGACGC
GCCGACATACCTTGCTAAGGCTTTGTGCCATATATCGCTCGTAATCATTTGCTCCATGCGAGGCGATGATG
GCCGTCAAGTGTCGCTCTCTTAACCATTGATACGGCTCCGAGCACGTGTTTGCCAATTGGCTATCGGTTTG
GGGTGCCCGTATCAGAATTGTTGGAAGTGGAAGAGAGGGTGATGCTAGGTTGCTGTCACTATGT

Figure 3.19. ITS-2 region sequence of third stage larva from pilchard *Sardinops sagax*, which showed high similarity with database sequences from *Hysterothylacium aduncum* and *H. auctum* (see in Zhu *et al.* 1998; Nadler *et al.* 2000; Umehara *et al.* 2008).

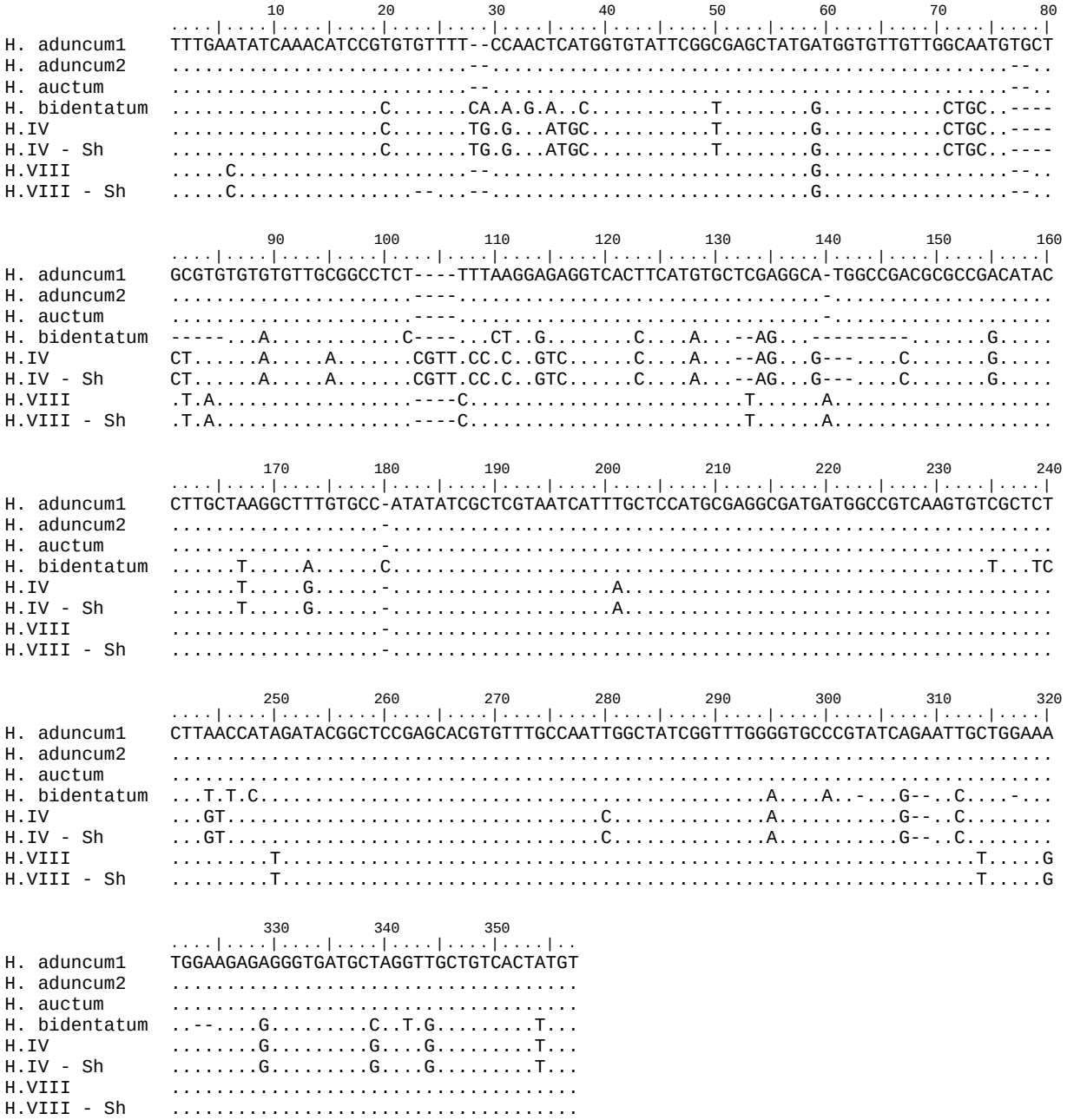

Figure 3.20. Alignment sequences of the ITS-2 region of *Hysterothylacium* larval types to database sequences of selected *Hysterothylacium* spp. (Accession numbers from top to bottom: AB277826, AJ225069, AF115571 and AY603539). H.IV – Sh and H.VIII – Sh indicate *H.* larval type IV and VIII, respectively, from Shamsi (2007). Polymorphic sites were designated using IUPAC codes.

for SSCP pattern comparison. Four different patterns were evident: H-4 (n=9) from *N. richardsoni* and *A. forsteri*, H-8 (n=3) from *N. richardsoni* and *S. sagax*, A-1 (n=1) from *N. richardsoni* and X-2 (n=2) from *P. bassensis* (Fig. 3.21).

SSCP patterns of H-4 and H-8 matched the SSCP profiles of *Hysterothylacium* larval type IV and *H.* larval type VIII from the present study, respectively. The ITS-2 sequence was 308 bp long for A-1 and 287 bp long for X-1, respectively. Alignment of the ITS-2 sequence of A-1 revealed it was identical with database sequences of both *Anisakis simplex s.s.* and *A. pegreffii* (Fig. 3.6). Alignment of the ITS-2 sequence of X-1 (Fig. 3.22) had no corresponding entry in the database. However, it featured similarities (76.60 – 77.60 % identity, including 11.26 – 10.29 % gaps, respectively) with ITS-2 sequences from *Contracaecum muraenesoxi* n. sp. (Fig. 3.23).

Table 3.2. Total number and percentage of unidentified anisakids of each examined fish species.

Fish species	Total number of anisakids	Unidentified	Percentage
Aldrichetta forsteri	29	1	3.5%
Neoplatycephalus richardsoni	396	210	53.0%
Platycephalus bassensis	2	2	100.0%
Sardinops sagax	47	2	4.3%
Seriola lalandi	0	0	0%

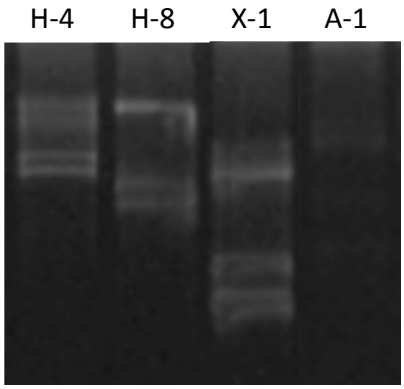

Figure 3.21. SSCP analysis of unknown anisakid morphotypes showing four different patterns.

TTATTGAAAGCAATCCGCGTTGGTCAGCTTCGCGCTGGTAATCGAAGCGTCGACCATGTGGTGTATTCGGC
GAGCTATGGTTGAGTTTGTTGCACAACCGTACCTTGCTAAGGCTAAGCGAAATTTCTCGCAGTCATTTGCT
CCGTGCGAGGCGATGATGGCCGTCAAGTGTTGCTCCCTGATGCGGCTCCGAGCATGTGTTGCTTGTCGTGG
GTGATATAAGGTTTGGGGCTGGCATCGACAGCTGGTATAATGGGAGTAATGCGAGTGCGTGGCTATCACTA
TGT

Figure 3.22. ITS-2 region sequence of third stage larva from sand flathead *Platycephalus bassensis* (X-1). This sequence showed similarity with the database sequence from *Contracaecum muraenesoxi* (see in Xu *et al.* 1999).

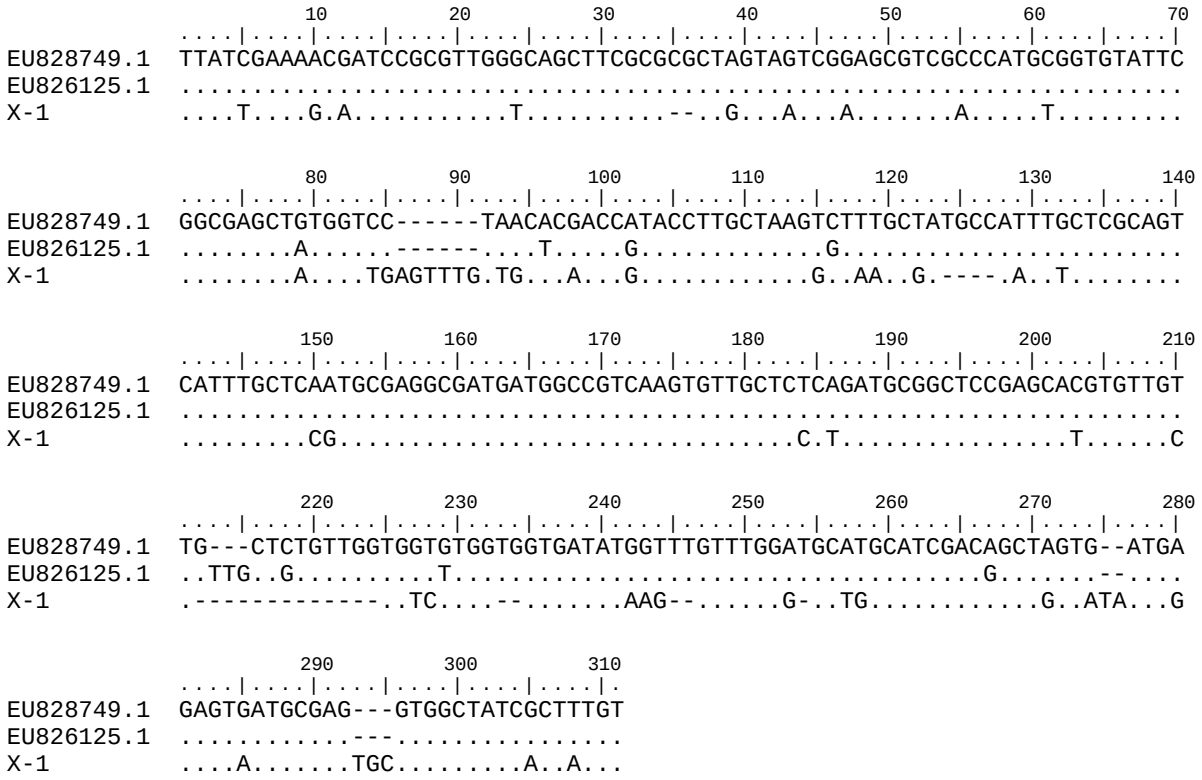

Figure 3.23. Alignment of ITS-2 region sequence of third stage larva from *Platycephalus bassensis* (X-1) with database sequences from *Contracaecum muraenesoxi* n. sp. (Accession number EU828749.1 and EU826125.1). Polymorphic sites were designated using IUPAC codes**.**

Chapter 4

Discussion

The present epidemiological survey of marine fish parasites of the Anisakidae family revealed five larval morphotypes representing three different genera of anisakid nematodes. Among them, nine different SSCP patterns were defined, resulting in seven different genotypes of the ITS-2 region in their rDNA. Figure 4.1 illustrates the spatial distribution of fish observed in this study, whereas Fig. 4.2 depicts the proportion of each genera of Anisakidae which are prevalent in infected fish. Classified into different anisakid genera, we can conclude follows:

Neoplatycephalus richardsoni harbored infections with *Anisakis pegreffii* and/or *A. simplex s.s. Contracaecum multipapillatum D* and *C. ogmorhini s.s.* were found in *Aldrichetta forsteri*, whereas a larval morphotype of *Contracaecum* type III with a different ITS-2 sequence from *C. ogmorhini s.s.* infecting *N. richardsoni*. Furthermore, an anisakid nematode related to *C. muraenesoxi* n. sp. could be shown in *Platycephalus bassensis.* A species of the *Hysterothylacium* genus with unregistered ITS-2 signature was found in *N. richardsoni* and in *A. forsteri*, another species with high genetic similarity to both *Hysterothylacium aduncum* and *H. auctum* in *Sardinops sagax* and *N. richardsoni*. Interestingly, no intestinal nematodes were found in *Seriola lalandi*.

***Anisakis*:** *A. simplex s.l.,* consisting of at least three valid sibling species, exhibits a comparatively high similarity in its internal transcribed spacer regions. For example, the ITS-2 region from *A. simplex C* differs from the corresponding sequences of *A. pegreffii* and *A. simplex s.s.* only at two base positions (0.65 % difference). Based on the ITS-2 sequences, *A. pegreffii* and *A. simplex s.s.* cannot be distinguished from one another. Thus, additional molecular markers used for species identification of anisakids, such as the ITS-1 region of nuclear rDNA or the cytochrome *c* oxidase subunit 1 (*cox*-1) locus of mitochondrial DNA, should be used to provide unequivocal discrimination of the species.

Interestingly, the two SSCP patterns A-1 and A-2 of morphologically classified *Anisakis* larval type I had the same sequence. Although this objects the conjecture that DNA-sequences of a

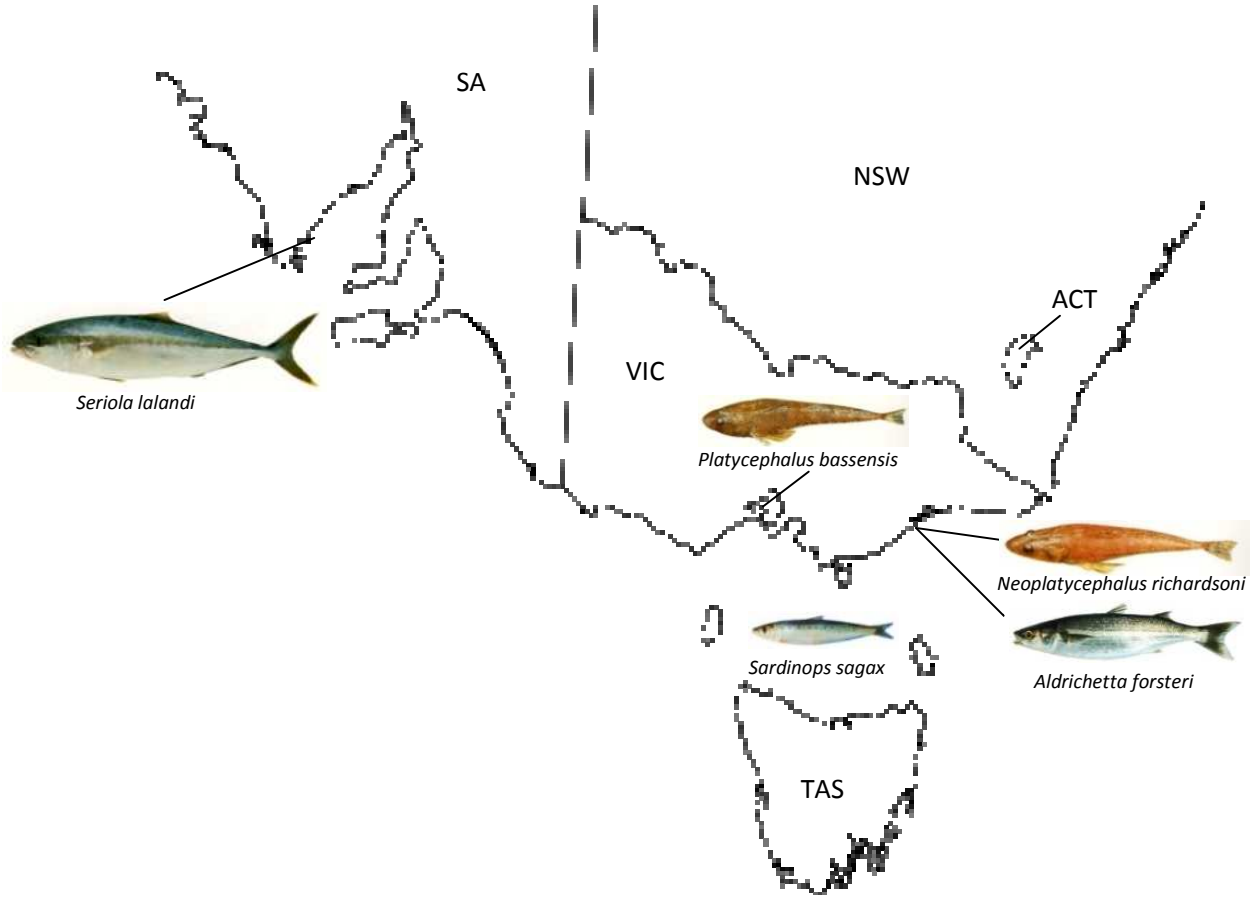

Figure 4.1. Geographical location along the southeast Australian continent of caught and examined fish from this study. ACT: Australian Capital Territory; NSW: New South Wales; SA: South Australia; TAS: Tasmania; VIC: Victoria.

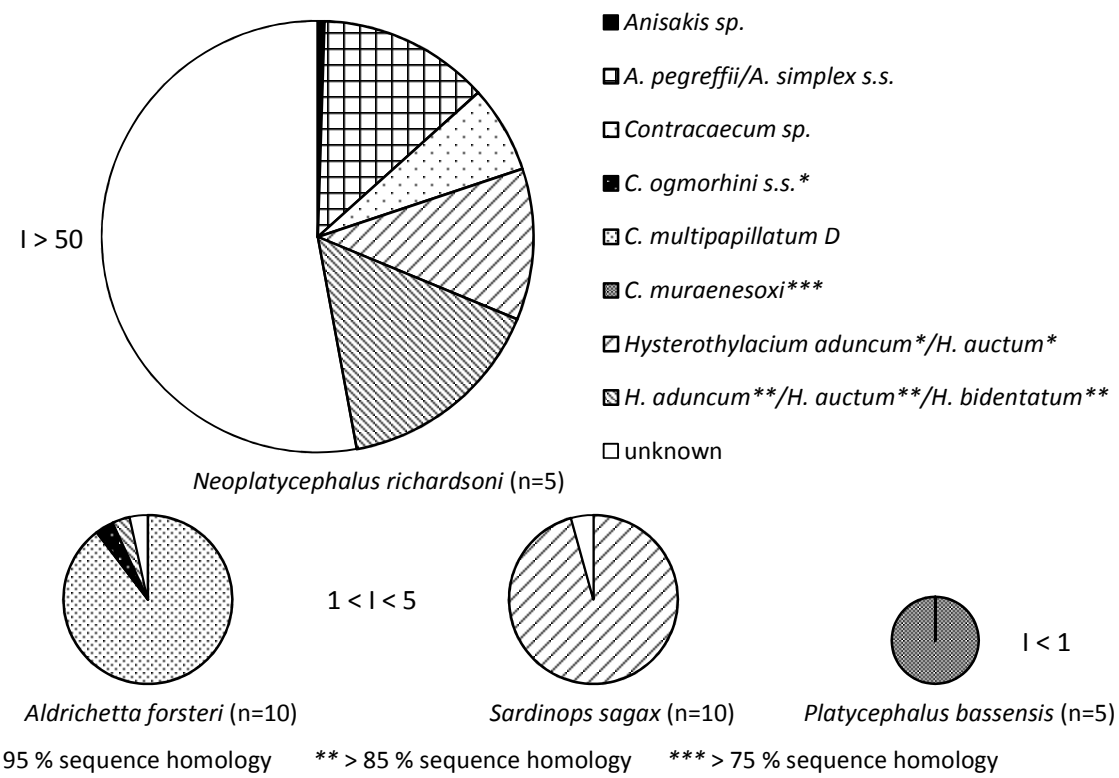

Figure 4.2. Species composition of anisakid nematodes in four different host species from south Australia. The pie size represents mean intensity of infection (I).

determined base composition produces one single band pattern by SSCP, differences in the proportion of polymorphic sites which go undetected *via* DNA sequencing can effect conformations and, therefore, the number of bands displayed in the gel. By direct comparison of the SSCP patterns of A-1 and A-2 (Fig. 3.4) it was evident that A-2 features the same conformational states as A-1, but lacks other conformers that A-1 has. According to this observation, we speculate a higher number of polymorphic sites in A-1 than in A-2.

During her investigations, Shamsi (2007) found similar morphometric parameters for third stage larvae of *A. pegreffii* (n=63) and *A. simplex s.s.* (n=11) from southern Australia, which are also in the same range than the parameters of *Anisakis* L3 of the present study (n=52). Only the ventriculus was specified significantly larger in *A. simplex s.s.* (1.24 mm, 0.80-1.84 mm range) than in *A. pegreffii (*0.78 mm, 0.46-1.14 mm range). The size of the ventriculus of *Anisakis* larval type I from this study (0.84 mm, 0.36-1.30 mm range) matches more closely to *A. pegreffii* from Shamsi's study. However, it was not considered as species determinant, also due to the low sample number of *A. simplex s.s*.

Whether the present species is *A. simplex s.s.*, *A. pegreffii* or a combination of them, clinical significance is given for both. Whereas *A. simplex s.s.* is the main etiological agent of anisakiasis in Japan (Umehara *et al.* 2007), infections with *A. pegreffii* are confirmed in the Mediterranean (D'Amelio *et al.* 1999). Most studies on allergenicity focused on *A. simplex s.l.*, but studies with *A. pegreffii* showed similar outcomes (Niewenhuizen *et al.* 2002). *A. pegreffii* is the common anisakid species of the southern hemisphere (Mattiucci *et al.* 2007), but its geographic distribution overlaps with *A. simplex s.s*. In Victoria and South Australia, Shamsi has discovered L3 of all three members of the *Anisakis simplex* complex (2007). Moreover, it is believed that *A. pegreffii* is more likely encountered in pelagic hosts, conversely *A. simplex s.s.* in bentho-pelagic hosts (Mattiucci *et al.* 1997).

***Contracaecum*:** The morphological and genetic findings of *Contracaecum* larval type I to be *C. multipapillatum D* von Drasche, 1882 are consistent with results from Shamsi (2007). This species, as well as *C. multipapillatum E*, occur southwards from the equator (Shamsi *et al.* 2008), in contrast to the sibling species *C. multipapillatum A*, *B* and *C*, which are restricted on the northern hemisphere (Nadler *et al.* 2000; D'Amelio *et al.* 2007). The detected sequence

variation of *C. multipapillatum D* in the ITS-2 region was 0 – 0.43 %. Aquatic birds like pelicans and egrets are final hosts of this species (Huizinga 1967; Sepulveda *et al.* 1999; Kinsella *et al.* 2004), and mullet species are known to act as intermediate hosts (Valles-Rios *et al.* 2000, Shamsi 2007). A risk of infection for terrestrial mammals could be shown by experimental inoculation of larvae to domestic cats leading to the transformation of the parasite to its adult stage (Vidal-Martinez *et al.* 1994).

For *Contracaecum* larval type III, two genotypes from different hosts have been identified, suggesting they are presumably two distinct species with analog larval morphology. However, despite several runs, the quality of one genotype sequence remained impaired, possibly due to gradual degradation of the DNA sample. Thus, the identity of the species can only be narrowed down to genus level. The other genotype could be classified to *C. ogmorhini s.s.* Johnston & Mawson, 1941, with 0.72 – 1.08 % sequence variation in the ITS-2 from Genbank™ database entries. In comparison, morphologic and molecular data of larvae by Shamsi (2007) identified *C. rudolphii D* Hartwich, 1964 in the flathead species *Platycephalus laevigatus* from Victoria. Although the sequence variation of 2.17 % between samples of the present and Shamsi's study is relatively low, this high inter-specific parity in the ITS region is known for several complexes of the *Contracaeum* genus. *C. ogmorhini s.s.* distributes along the southern hemisphere (Fagerholm and Gibson 1987; Fagerholm 1990), whereas the sibling species *C. margolisi* is spread in British Columbia (Zhu *et al.* 2001a; Mattiucci *et al.* 2003).

A novel species have been discovered in the sand flathead *Platycephalus bassensis*, based on ITS-2 data. It belongs to the *Contracaecum* genus and shows genetic similarity with another newly found species from the Taiwan Strait: *C. muraenesoxi* Luo-Damin & Fang-Wenzhe, 1999. However, the sequence variation of 22.40 – 23.40 % is too high to bring them into direct relationship.

***Hysterothylacium*:** As illustrated in figure 3.19, there is a high degree of ITS-2 sequence similarity between *Hysterothylacium* larval types of the present study and of Shamsi's work (2007). *Hysterothylacium* larval type IV is identical with Shamsi's *Hysterothylacium* larval type IV genotype B, and *Hysterothylacium* larval type VIII equals *Hysterothylacium* larval type VIII from Shamsi, except for a deletion of nucleotide position 23 and 24 (0.58 % sequence variation).

Moreover, morphologic parameters match respectively, but morphometric differences exist. We suggest the increased variability in body size and lengths of caecum, ventriculus and ventricular appendix from the present study due to a higher sample number than in the survey by Shamsi (n = 63 vs. 11 and n = 90 vs. 11 for *H.* larval type IV and VIII, respectively). Despite the verification on existing records from the same area, the identities of the species are not resolved. However, with merely 2.87 – 3.43 % sequence variation in the ITS-2 region, *H.* larval type VIII may represent an allopatric population of *H. aduncum* or *H. auctum*, respectively, which could possibly carry its own status as a valid species. The Genbank™ database reveals no difference in the ITS-2 region between *H. aduncum* and *H. auctum*. In fact, *H. auctum* is regarded as sibling species of *H. aduncum* (Hartwich 1975), but their taxonomy is still unresolved (Koie 1993). *H. auctum* Rudolphi, 1802 is a parasite of eelpout and other fish from the Baltic Sea (Fagerholm 1987; Szostakowska *et al.* 2001). According to published literature, only one adult specimen was described outside that zone, namely from the North Pacific near Japan (Moravec and Nagasawa 2000). The geographic extent of *H. aduncum* Rudolphi, 1802 includes the North Atlantic, where it is very common and abundant, the Baltic Sea, Black Sea, Yellow Sea and north Pacific (e.g. Hartwich 1975; Moravec *et al.* 1985; Palsson 1986; Telli and Doran 1997; Klimpel and Ruckert 2005; Zhang *et al.* 2007). Furthermore, occurrences on cultured salmon farms in Chile have been reported (Gonzalez and Carvajal 1995). Few cases of human anisakiasis were traced back to this species (Yagi *et al.* 1996).

For the ITS-2 sequence of *Hysterothylacium* larval type IV, Genbank™ entries of *H. bidentatum* Linstow, 1899 matched with highest accordance (12.43 % sequence variation), followed by entries of *H. aduncum* and *H. auctum* with 13.65 – 14.04 % sequence variation. As proposed by Shamsi (2007), who found eight distinct genotypes among larvae of the *Hysterothylacium* genus, the present findings support the presence of currently unknown *Hysterothylacium* species in Australian waters.

One of the striking findings of this study was the high infection rate of tiger flatheads *Neoplatycephalus richardsoni* regarding prevalence, parasite load and species diversity of anisakid nematodes. This is even more surprising as the closely related sand flathead *Platycephalus bassensis*, which shares similar biology and prey preference, showed only a low

infection rate of a species not occurring in *N. richardsoni*. The differences of anisakid infestations between the flathead species may be reasoned by variances in locality, diet or host preference of the parasite. A seasonal variation of the abundance of anisakid larvae in its intermediate host is also possible, as observations have shown for *A. simplex* in Norwegian waters (Stromnes and Andersen 2000). Another remarkable discovery was the absence of any anisakids in king fish *Seriola lalandi*. The obtained specimens came from an aquaculture farm in South Australia. In contrast, infections of wild conspecifics from southern Australia with larval stages of *Anisakis* and *Hysterothylacium* spp. are known (Shamsi 2007). The aquaculture management utilizes anti-parasitic treatment, such as hydrogen peroxide, to combat gill and skin flukes, however no specific treatment against intestinal helminths that are performed broadly (personal communication). As they are reported cases of *Anisakis* infections in cultured marine fish (Yoshinaga *et al.* 2006), care should be exercised whether aquaculture in general is a safe haven for fish against anisakids. Further investigations may help to clarify this matter in the future.

An additional objective of this survey was to evaluate the usefulness of both morphologic examination and molecular analysis, and whether reliance on a single approach brings equally adequate results compared to a combined approach. On the one hand, microscopic characterization minimizes costs and is simple to operate, but is very time consuming and also prone to subjective interpretation (Thompson 1982). Furthermore, this approach holds limitations when looked at larval stages of anisakids (Oshima 1972). On the other hand, SSCP and ensuing DNA sequencing of its emerging patterns comes with higher costs, but is precise and much faster in return, making it efficient for large-scale surveying (Gasser 1998).

For newly discovered species, information on morphology has to be compared with available molecular data. Since larval forms of anisakid nematodes do not allow unambiguous identification at species level (Bagrov 1982; Fagerholm 1988), retrieval of adult specimens, preferably male, which possess more stringent characteristic traits (Hartwich 1975; Fagerholm 1991), would alleviate its validation as a species. Genetic leveling of marker regions between validated species of anisakids, given they are species-specific, enables immediate identification of the species. Like previous studies, the present study shows that the ITS-2 region of nuclear

rDNA is an appropriate marker, but support through the use of at least one additional genetic marker, for example the ITS-1 region, would strengthen the significance for species identification of anisakid nematodes (e.g. Zhu *et al.* 2000a, b; Hu *et al.* 2001). As in Australia, there are certainly multiple species of the Anisakinae subfamily still awaiting comprehensive description, features of morphology, ideally in combination with genetic or proteomic information, are pivotal for their recognition and classification (Andrews and Chilton 1999; Shamsi *et al.* 2008). For epidemiological surveys, which focus on analysis of already well researched species and require large sample sizes, molecular investigation alone may be satisfactory to meet this task.

This study confirmed that *Anisakis* and related species are distributed on a global scale. Infections of anisakids occur frequently in marine fish from Victoria, and reach the fish markets often alive in freshly sold goods (personal observation). The tiger flathead, a locally popular food fish, is particularly likely to harbor infective L3, including socio-economically relevant *Anisakis* spp. Furthermore, this study contributes to additional insights into the species diversity of anisakid nematodes in south-eastern Australia, as well as genetic heterogeneity, host preferences and geographic range.

The increased demand for raw or lightly cooked seafood in Western countries, including Australia, is likely to increases the impact of fish-borne diseases like anisakiasis (Takabe *et al.* 1998; Chai *et al.* 2005). In particular, the severity of gastroallergic anisakiasis, which is potentially lethal, requires precaution and intervention. It will be important to establish a capable management plan for the control and prevention of this disease. In the Netherlands, for example, the number of anisakiasis cases has plummeted to almost zero after preventive regulations for the fishery industries were implemented (Bouree *et al.* 1995). Besides direct measures, information campaigns for the Australian business sector, institutions and consumers of fish could raise awareness; the continuing monitoring of both fish and human populations should ensure the success of such measures.

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Appendix A

Reagents

Phosphate Buffer Saline (PBS)	20mM $NaPO_4$
	150mM NaCl
	pH 7.4
Lactophenol	20% (v/v) lactic acid
	20% (v/v) phenol
	20% (v/v) glycerin
	20% (v/v) dH_2O
DNA extraction buffer	20mM Tris-HCl, pH 8.0
	100mM EDTA
	1% SDS
TBE buffer (Biorad)	65mM Tris-HCl
	27mM Boric acid
	1mM EDTA
	pH 9.0
TAE buffer	40mM Tris-acetate
	1mM EDTA
	pH 8.5

Appendix B

Complete list of collected intestinal parasites

Besides anisakid nematodes, also other parasitic helminths have been collected from the visceral cavity of fish. The following table and figures present the data of infection rates and intensity for each examined fish species.

Table A. Percentage of intestinal helminth infections for selected fish species from south Australian waters.

		Infection rate [%]		
Fish species	n	Cestoda	Trematoda	Acanthocephalia
Aldrichetta forsteri – yellow-eye mullet	10	-	-	90
Neoplatycephalus richardsoni – tiger flathead	5	-	40	-
Platycephalus bassensis – sand flathead	5	100	20	-
Sardinops sagax – pilchard	10	-	10	-
Seriola lalandi – king fish	10	30	-	-

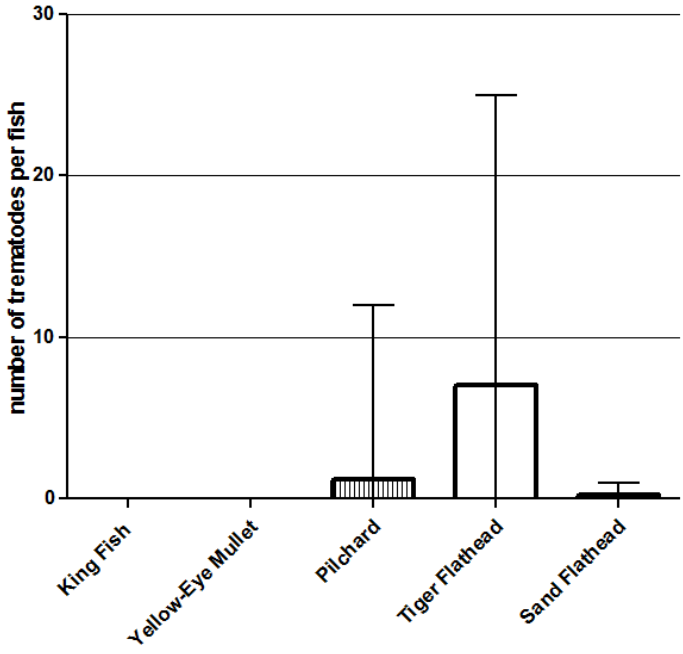

Figure A. Mean intensity of infection with intestinal trematodes of selected fish species from south Australian waters. Error bar shows range.

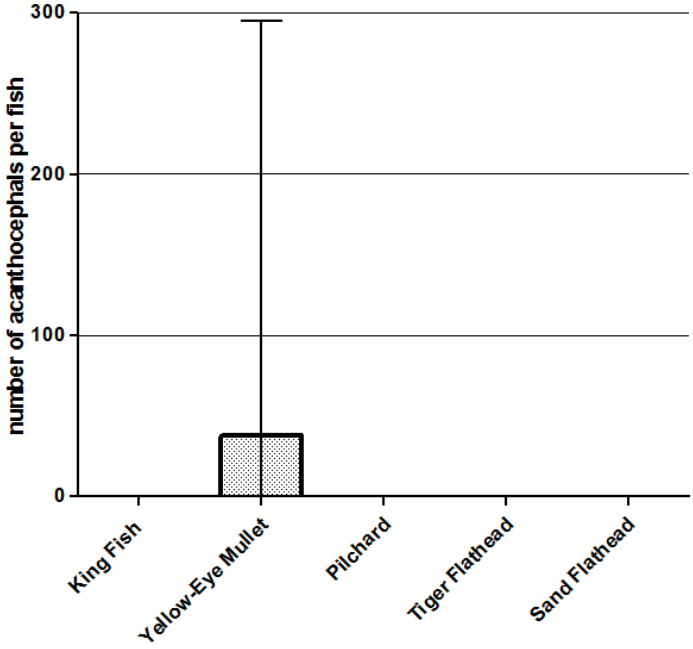

Figure B. Mean intensity of infection with acanthocephals of selected fish species from south Australian waters. Error bar shows range.

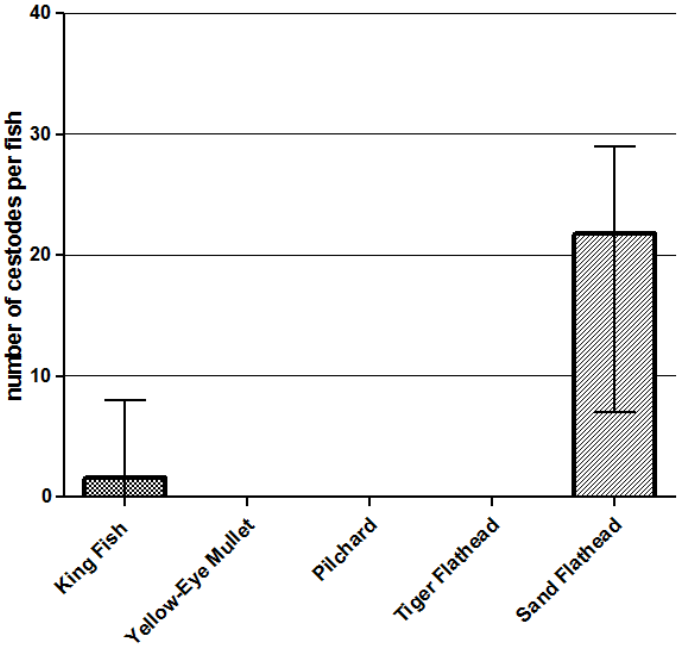

Figure C. Mean intensity of infection with cestodes of selected fish species from south Australian waters. Error bar shows range.