Molecular and immunological characterisation of proteins from *Anisakis pegreffii* and their immune stimulatory effect on the human health system

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy (PhD)

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis/project is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

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Published article from this research work

Larval anisakid nematodes in teleost fishes from Lizard Island, northern Great Barrier Reef, Australia

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Manuscripts under preparation for publication from this research work

1. Molecular Characterisation and Identificationof Ani p 3 (Tropomyosin), an allergen in *Anisakis pegreffii* ***(Chapters 3 and 5).***
2. Structural, immunological and functional properties of the *Anisakis pegreffii* allergen Ani p 4, a cysteine-protease inhibitor. ***(Chapter 6)***

Abbreviations

Terms	Means
A	alpha
Λ	Lambda DNA
3’	three prime
5’	five prime
°C	Degrees Celsius
A	Alanine
aa	amino acid
AGRF	Australian Genome Research Facility
A. simplex	*Anisakis simplex*
A. pegreffii	*Anisakis pegreffii*
AmpR	Ampicillin resistence
AP	Alkaline phosphatase
APS	Ammonium persulphate
ATP	Adenosine triphosphate
BLAST	Basic local alignment sequence tool
BSA	Bovine serum albumin
bp	base pairs
C	Cysteine
CPI	Cysteine Protease Inhibitor
cDNA	complimentary DNA
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTP	Deoxy ribonucleotidetriphosphate
EB	Elution buffer
EDTA	Ethylenediaminetetraacetic acid
e.g.	*Exempli gratia*, for example
et al	et alia, and other people
ES	excretory–secretory
G	Glycine
Gal	Galactose
h	hour(s)
H2O	Water
HCL	Hydrochloric acid
HRP	horseradish peroxidase
ITS-1/ITS-2	Internal transcribed spacer 1and 2
IPTG	Isopropyl-β-D-thiogalactosidase
kb	Kilobase
kDa	Kilodalton
L3	third stage larvae
L/mL/µL	Liter/Milliliter/microliter
LB	Luria Bertani broth
LBA	Luria Bertani agar
kg/g/mg/µg/ng/pg:	kilogram/gram/miligram/microgram/nanogram/picogram
M/mM/µM	Molar/Milimolar/ Micromolar
M	Methionine

min	minute(s)
mQ	Milli-Q
MW	Molecular weight
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NO	Nitric oxide
O.D.	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
pH	negative logarithm of hydrogen ion concentration
PBS	Phosphate buffered saline
rDNA	Ribosomal DNA
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Reverse transcription
RT	Room temperture
RTase	Reverse transcriptase
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
s	second(s)
ss/dsDNA	single-/double-stranded DNA
spp.	species (plural)
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
T	Threonine
Tm	Melting temperature
Tm	Tropomyosin
TEMED	NNN'N'-tetramethylethyleidiamine
Tris	Tris (hydroxymethyl) amino methane
Tris-HCL	Tris hydrochloride
Tyr	Tyrosine
U	units
UV	Ultraviolet light
V	Voltage
v/v	Volume for volume
WCL	Whole cell lysate
w/v	Weight for volume
X-gal	5-bromo-4-chloro-3-indoyl-b-D-galactose

Abstract

With the fast growth of seafood trading worldwide, the potential health risks of eating contaminated seafood have greatly increased. Biological effects of contaminated seafood are associated and caused by a variety of bacteria, viruses, and parasites; this various groups of pathogens results in an extensive diversity of clinical syndromes, each with its own epidemiological characteristics. According to a World Health Organization investigation, more than 1 billion people worldwide are infected with different species of the *Ascaris lumbricoides* worm parasite (282), which causes serious conditions ranging from mild to lethal. *Anisakis* parasites in marine fish have imposed a significant economic burden, reducing productivity and requiring elaborate and expensive control methods.

Nematodes of the family Anisakidae is a major group parasitise fish, mammals, birds and reptiles, with the larval stages of some species. Several species of *Anisakis* have been found to be parasitic in marine mammals such as whales and dolphins in their adult stages, and their larvae are found in a variety of fish species. Anisakid nematodes have complex life cycles that include invertebrate and vertebrate hosts at various developmental stages during their life. Most reports of anisakiasis infections are associated with eating raw or undercooked fish that contain larval *Anisakis*. Humans can also be accidental hosts for larval *Anisakis,* however they cannot progress their life cycles, but they can frequently cause hypersensitivity IgE-mediated reactions with or without several gastrointestinal manifestations ranging from urticaria to angioedema, called anisakiasis diseases. The development of an allergic reaction mediated by IgE may be against ES and somatic allergens of larval *Anisakis*.

Nuclear ribosomal DNA (rDNA) provides suitable genetic markers for the identification of larval *Anisakis*. Therefore, the sequences of internal transcribed spacers (ITS-1 and ITS-2)

are a powerful approach to identify and distinguish anisakid nematodes (at any developmental stage) for diagnostic or taxonomic purposes, for exploring the genetic composition of larval anisakid populations and for investigating their ecology. Although several allergens have been identified in *Anisakis simplex*, little information is available for other parasite species infecting fish. Therefore, this project aims to investigate the allergenicity of tropomyosin (Ani p 3) and a cysteine protease inhibitor (Ani p 4) from the sibling species, *Anisakis pegreffii*, using biochemical, genetic and immunological approaches

Larvae (L3) were harvested manually from tiger flathead fish and identified morphologically using a light microscope. For molecular characterisation and genetic diversity, total genomic DNA was extracted from individual samples and verified by species-specific PCR amplification using the sequences of the first and/or second internal transcribed spacers (ITS-1 and/or ITS-2) of ribosomal DNA (rDNA) as the species-specific genetic markers. For molecular characterisation of *Anisakis* allergens, RNA of *Anisakis pegreffii* was extracted, then cDNA synthesised and amplified using designed primers and used to amplify tropomyosin and cysteine protease inhibitor genes from *Anisakis pegreffii*.

Whole muscle proteins from *Anisakis* larvae were extracted directly with extraction solution and analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and confirmed by immunoblotting assays. Tropomyosin protein was detected using anti-tropomyosin and anti-crustacean polyclonal antibodies raised in rabbits while a cysteine protease inhibitor was detected using anti-cysteine protease inhibitor polyclonal antibodies raised in rabbit. The allergenicity of the nematode tropomyosin and cysteine protease inhibitor was evaluated using human serum of atopic patient with a shellfish allergy.

The result of SDS-PAGE and immunoblotting assays of *Anisakis pegreffii* extracts indicates the presence of tropomyosin and cysteine protease inhibitor proteins of 44 and 12.8 kDa molecular weight, respectively. These results are similar to the allergenic tropomyosin and cysteine protease inhibitor of *Anisakis simplex*. Also, strong reactivity with human serum designated these proteins as allergens in *Anisakis pegreffii* spp. The molecular sequencing was successful for tropomyosin and cysteine protease inhibitor genes from *Anisakis pegreffii,* and demonstrated that they are closely related to the *Anisakis simplex* proteins previously sequenced and submitted in the GenBank database. Our results indicated that, the cDNA sequence similarity searches using the BLAST program and phylogenetic analysis (Clustal W programme) revealed that tropomyosin of *A. pegreffii L3* has the highest homology to α tropomyosins isoform from *A. simplex L3* (98%) and *A. lumbricoides* (84%), respectively. In addition, cysteine-protease inhibitor of *A. pegreffii L3* has the highest identity to cysteine-protease inhibitor isoform from *A. simplex L3* (98%) using the BLAST program and 100% using phylogenetic analysis (Clustal W programme).

This analysis highlights that this nematode tropomyosin and cysteine protease inhibitor share high sequence identity with other parasitic nematodes, house dust and crustacean tropomyosin, respectively.

Chapter one: Introduction

Despite knowledge about the interaction between infectious disease and immunity being advanced, parasitic infection still represents a significant challenge to human population growth and animal life, and is responsible for significant morbidity and mortality worldwide. One category of parasitic worms, the helminthes, have been related to significant economic loss in livestock and food industries in the developing world (de Silva *et al*., 2003). Important helminths include *Schistosoma* genus, the cause of schistosomiasis (bilharzia), and the soil-transmitted helminths (STH), including roundworms (*Ascaris lumbricoides*), whipworms (*Trichuris trichuria*) and hookworms (*Ancylostoma duodenale* and *Necator americanus*), commonly known as intestinal worms. Recently, it has been estimated that *Ascaris lumbricoides* infects 1,221 million people, whereas *Trichuris trichuris* and hookworm infects 795 and 740 million people, respectively (de Silva *et al*., 2003). In addition, the greatest numbers of STH infections occur in sub-Saharan Africa, East Asia, China, India and South America.

It has been known that parasites cause prevalent diseases in humans and animals such as malaria, cysticercosis, toxoplasmosis, schistosomiasis, leishmaniasis and anisakiasis (White and Garcia, 1999, Hill et al., 2005, Brooker et al., 2006, Schwartz et al., 2006, Gardiner et al., 2005). These diseases cause symptoms from mild to lethal, such as nausea, tiredness, loss of appetite and abdominal pain. In addition, chronic and intense STH infections can contribute to malnutrition and iron-deficiency (anemia), and also can affect physical and mental growth in childhood (Drake *et al*., 2000, Hotez *et al*., 2005, Stephenson *et al*., 2000). Anisakiasis is parasitic zoonoses caused by the ingestion of live larvae anisakidae nematodes belonging to the genera *Anisakis*, *Pseudoterranova*, *Contracaecum* and *Hysterothylacium* found in the body cavities and muscular systems of fish and large crustaceans (Sakanari and Mckerrow, 1989 , Umehara et al., 2008). Humans accidentally ingest these larvae when eating raw or undercooked infected fish or crustaceans with the third larvae (L3). However, these cannot progress their life cycles in human, but they can cause gastrointestinal diseases known as anisakiasis and severe allergic reactions mediated by IgE antibody (Torres et al., 2004b, Sohn and Chai, 2005b, Davey, 1971). These symptoms also can appear after consumption of smoked, salted or dried salt fish.

Although a lot of progress has been made in parasite vaccine research in the last decade, there are few commercially available vaccines to animal parasites. Furthermore, there are no commercially available parasite vaccines for use in humans because parasites often elicit

unsuitable and ineffective immune responses in the host, or dampen the host immune system, thereby preventing a robust and effective immune response by the host (Cox, 1997). Moreover, parasites often display various immune avoidance strategies such as antigenic variation and molecular mimicry. In addition, they have complex life cycles and other biological characteristics such as their surface have multilayer structures and secrete proteases to prevent antibody opsonisation, and migrate in their hosts to avoid inflammation. All these mechanisms act as a predicament for developing effective vaccines (Good *et al*., 2004).

Rationale for this project

There is an urgent need to address the risks from eating raw or undercooked fish in Australia, because Anisakids have a global distribution among a wide variety of marine fish species. There is also a need for the development of diagnostic and vaccination therapies for allergic disease.

Research hypothesis

The combined analysis of all morphotypes and genotypes can be used to redefine anisakid populations, and also identify their taxonomy. In addition, the basis of the allergenicity of proteins has not yet been resolved. Thus, tropomyosin (Ani p 3) and cysteine-protease inhibitor (Ani p 4) allergens provide an excellent opportunity to study their protein structure and allergenicity.

Research aims

The main aim of this research study is to conduct a survey of commercially important fish species for anisakid infection in Australia and to use morphological, immunological and molecular approaches to identify the parasites and proteins inferred to be allergenic.

This main aim will be achieved by performing these following objectives:

1- To identify specimens of anisakidae from Australian fish hosts.

2- To classify different larval anisakid species from commercial marine fish.

3- To identify specimens from different species using morphological characterization.

4- To screen rDNA of larval anisakid species based on the sequences of the first and second internal transcribed spacers (ITS-1 and ITS-2).

5- **To screen rDNA in an order to distinct any nucleotide variation within the species, subspecies, or strains based on ITS-1 and ITS-2 sequence data.**

6- To detect and identify tropomyosin (Ani p 3) and a cysteine-protease inhibitor (Ani p 4) present in an extract of the third stage larvae of *A. pegreffii* as major allergens in Anisakidae.

7- To elucidate the primary structures of Ani p 3 and Ani p 4 from *A. pegreffii* using a cDNA cloning technique and generatge recombinant Ani p 3 and Ani p 4.

8- To identify the IgE-binding regions of Ani p 3 and Ani p 4 sequences and compare these homologous sequences in other allergenic Anisakidae species and non-allergenic species.

Chapter two: Literature review

2.1 Allergy

The term "allergy" was originally introduced by (Von Pirquet, 1906) who noticed that some of his patients exposed to foreign agents such as *Vaccinia virus* and *Corynebacterium diphtheriae* developed hypersensitive reactions, particularly fever, skin rash, arthropathy and lymph node swelling. These symptoms were termed as serum sickness. He also suggested that the word 'allergen' should be used to describe the effect of foreign agents on immune cell function after one or several exposures. Therefore, von Pirquet established the immunology and allergology basis in present science.

In the past, it was thought that types of hypersensitivity were caused by an inappropriate activation of the immune system. Therefore, they were classified as allergies. Four new types of hypersensitivity reactions (Types I, II, III and IV) were classified and described in 1963 based on the mechanisms involved and time taken for the reaction (Coombs and Gell, 1963). The Type I Reaction is considered most commonly associated with allergic reactions to foreign substances.

Prausnitz and Kustner, (1921) discovered a transferable tissue-sensitising factor in serum of sensitized subjects called "serum factor". Later on, serum factor was introduced as "atopic regain" and strange disease by Coca and Cooke, (1923). Serum factor was eventually recognized as a self-protein representing a new antibody subclass (immunoglobulin E or IgE) by (Johansson and Bennich, 1967, Ishizaka et al., 1967a). The discovery of modern allergology can be attributed towards the identification of immunoglobin E (Ig) E as the main mediator of allergic disease (Ishizaka et al., 1967b). In addition, allergy reactions to inhalation, ingestion, injection and contact allergens were defined medically as environmental agents that induce high levels of IgE antibodies, eosinophilia, and a dominance of T cells that secreted a type 2 profile of cytokines (Pritchard and R.A, 1997).

2.2 Food allergy

Food allergy (FA) has become a serious health issue that affects adults and children, and may be most prevalent during the first years of life (Bock, 1987). In general, approximately 4-6% of children and 1-3 % of the adult population experience food allergy (Gotua *et al*., 2008). In Australia 1 in 10 children have confirmed food allergy, the highest prevalence rate in the world (Allen-Health Nuts study, 2013).

Despite the fact that the most severe form of allergic reaction is potentially life threatening, a cure or effective treatment has not yet been discovered for allergic disease. The disease can only be managed by avoidance of allergen or food that cause allergenicity and/or treatment of symptoms. Medically, allergic food is defined as food that elicits an immunological response (hypersensitivity) to specific components of the food or ingredients within the food (typically proteins, but sometimes also chemical haptens) (Boyce *et al*., 2010).

Although an allergy could be triggered by virtually any food, it has been recently concluded that eight "major allergens" in foods are responsible for 90% of significant allergic reactions. These includes: milk, egg, peanut, tree nuts, wheat, soy, shellfish and fish (Sampson, 1999). Food allergy or allergic food hypersensitivity is induced by two major mechanisms (Table 1): IgE-mediated and/or non-IgE mediated. It is most practical to classify immunologic reactions to food into the three following groups: IgE mediated (type-I), non-IgE-mediated (cellular), and mixed disorders (Gülen, 2007). IgE-mediated food allergies are a representative of type I allergy that is associated with a wide variety of allergic reactions that may appear within a few minutes or hours after eating the allergen and can also potentially be mild to severe life-threatening in nature. Clinical manifestations of food allergy vary depending on the age of the subject. The allergen involved, and the amount of food consumed might lead to gastrointestinal tract symptoms (nausea, vomiting, diarrhea, abdominal pain, colic and blood in the stools); cutaneous symptoms (urticaria, eczema or atopic dermatitis, angioedema and pruritis); respiratory symptoms (wheezing, rhinitis and asthma) and other symptoms (laryngeal edema, anaphylactic shock and hypotension) (Ring *et al*., 2001).

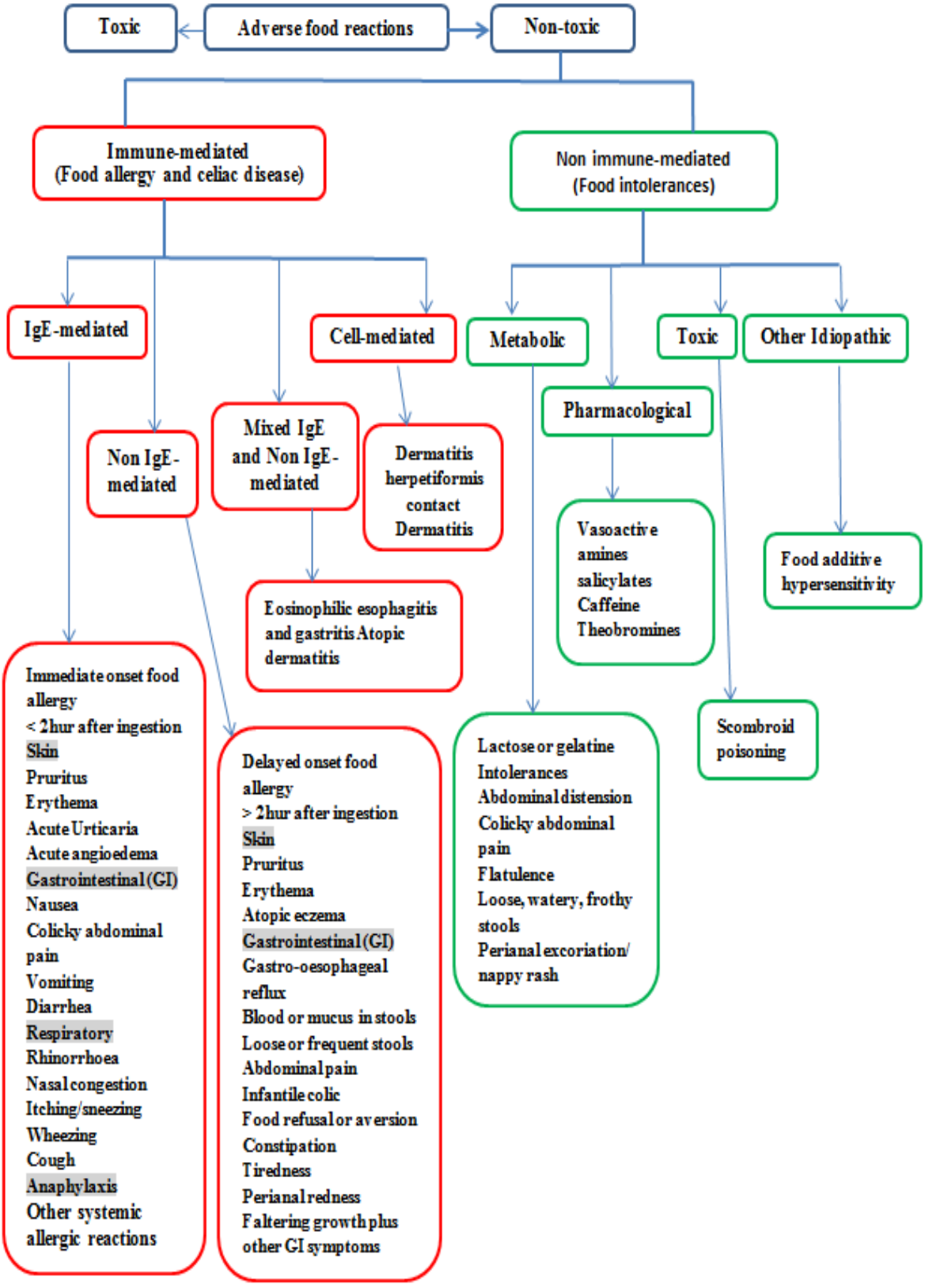


Figure 2.1 Adverse reaction to food, including IgE and non-IgE mediated reactions.

Source: https://www.jfhc.co.uk/living_with_food_allergy_20802.aspx

2.2.1 Changes in the Prevalence of Food Allergies

It has been observed that the prevalence of food allergies is increasing over the past several decades. For example; the parental report has shown that the prevalence of peanut allergy among children living on the Isle of Wight increased from 0.5% in 1989 to 1.0% in 1994-1996, and the prevalence of IgE antibodies increased from 1.1% to 3.3% (Grundy *et al*., 2002). In addition, the prevalence of peanut allergy among school going children in Montreal (assessed using an algorithm that included parental self-report, SPT, food-specific IgE, and food challenge) was 1.5% in 2000-2002 and increased to 1.63% in 2005-2007 (Kagan *et al*., 2003, Ben-Shoshan *et al*., 2009). A clinic in China reported an increased rate of food allergy as confirmed by a food challenge, from 3.5% in 1999 to 7.7% in 2009 (Hu *et al*., 2010). Self-reported survey data in the United States suggests an 18% increase in food or digestive allergies from 1997 to 2007 (Branum and SL, 2008, Branum and SL, 2009).

US studies used administrative data from hospital discharges, self-reporting, and food-specific IgE to estimate changes in food allergy prevalence over time and estimated that 3.3% of US children had food allergies in 1997 vs 3.9% in 2007 (Branum and SL, 2009). A random-calling telephone survey across the United States in 1997, 2002 and 2008 suggested the rates of allergy in children increased significantly for tree nuts (0.2%, 0.5%, and 1.1%) and peanut (0.4%, 0.8%, and 1.4%) (Sicherer *et al*., 2010). In the Australian Capital Territory, the estimated minimum incidence of peanut allergy in children born in 2001 and 2004 was 0.73% and 1.15%, respectively (Mullins, 2007). In addition, in the period between 1995 and 2007, the percentage of the population sensitised to peanut was 5% and based on clinical criteria, 4.3% were diagnosed with peanut allergy (Mullins *et al*., 2009). Prevalence rates of admissions for food-induced anaphylaxis in Australia increased by 350% from 1994 to 2005 (Liew *et al*., 2009). Rates of increase were greater for children between 0- to 4-years compared with other age groups. The increase was most distinguished for peanut-induced anaphylaxis, with more modest increases observed for cow's milk–induced and egg-induced anaphylaxis (Liew *et al*., 2009). Similar data have been reported in the United Kingdom between 1990–2004 (Sicherer *et al*., 2010).

2.3 Mechanism of IgE mediated-allergic reaction

The discovery of the IgE antibody was made in the 1960s by two independent research groups; one in Denver, Colorado, US, led by Ishizaka *et al.* (1966), and the other in Uppsala, Sweden, by Wide *et al*. (1967). This detection played a significant role in understanding the mechanisms of allergy and also is considered to be the starting point in the diagnosis and treatment of allergic disease.

The initial phase in the production of IgE-mediated allergy is termed sensitization. The mechanism of this reaction starts when an allergen enters the body through the epithelial barrier of the skin, airway or gut and is then taken up by high-affinity receptor (FcεRI) that is expressed on mast cells and basophils and low affinity receptor (FcεRII), which is present on macrophages, monocyte, lymphocytes and platelets called antigen-presenting cells (APCs). After that, APCs direct the naïve CD4+ antigen-specific T cells to develop into Th1, Th2 or Th17 effector cells or regulatory T cells (Treg). In individuals, signals from the APCs will cause differentiation to Th2 cells and production of IL-4 and IL-13. Therefore, Th2 cells and interleukins will drive B cell class-switching to IgE production and secretion of allergen specific IgE. The secreted allergen-specific IgE will subsequently bind to mast cells in the tissue. Upon the second encounter with the same allergen, the mast cells will be activated and release potent inflammatory mediators, such as histamine and proteases.

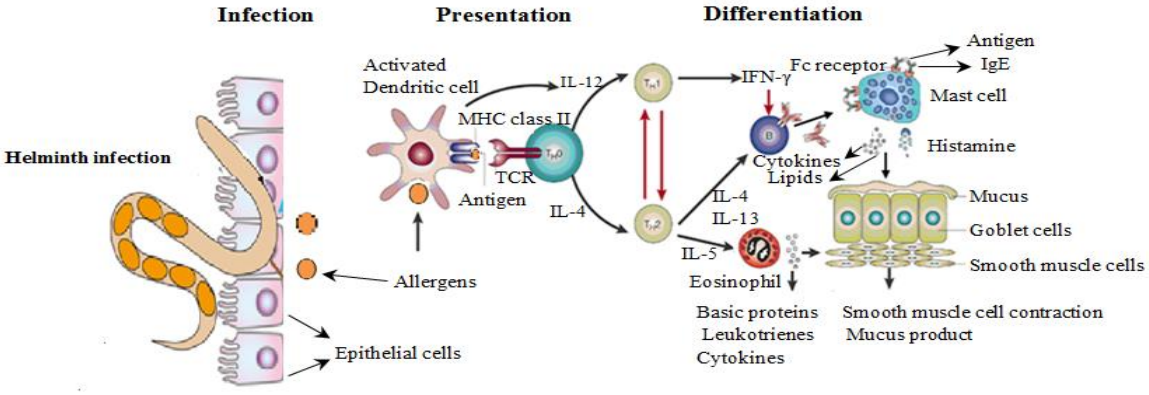


Figure 2.2 Mehanism of allergic reactions

Source: Modified and adapted from Nature Reviews/ Immunology (http://www.nature.com/nri/journal/v1/n1/full/nri1001-069a.html)

2.4 Allergens

Allergens can be glycoproteins, lipoproteins, or proteins conjugated with chemical or drug haptens that are present in well-defined (usually biological) sources. Generally, these allergens can be categorized as follows: water-soluble glycoproteins, relatively low molecular weight (10-70 kDa in size), resistant to heat and digestion, stable to acid and proteases, and high solubility in body fluids. More than 200 allergens of clinical importance have been identified among the weeds, grasses, trees, animal danders, molds, house dust mites, parasites, insect venoms, occupational allergens, drugs and foods (Leung *et al*., 2003).

2.4.1 Allergen History

During 1940–1950s, different "classical" biochemical separation methods were used to purify pollen and house-dust allergens such as phenol extraction, salt precipitation, and electrophoretic techniques. In the 1960's, the first allergen was purified from ragweed using ion exchange and gel filtration media, and named "antigen E" (King, 1962). After a few years, (Johnson and Marsh, 1965, Johnson and Marsh, 1966) Johnson and Marsh (1965, 1966) isolated an important allergen from rye grass pollen (*Lolium perenne*), and used the name "Rye 1" to indicate that allergen. It was considered to be the first allergen that was purified from this species.

Later on, many allergens were purified from ragweed (Ra3, Ra4, Ra5, and Ra6), rye grass (Rye 2 and Rye 3), cat (Cat-I) and codfish (M), and used for immunological and molecular genetic studies (Marsh et al., 1975, Lowenstein et al., 1981, Ohman et al., 1974, Elsayed and Aas, 1970, Elsayed and Bennich, 1975). Marsh (1975) published a first seminal book chapter called Allergens and the genetics of allergy. It was the first explicit definition of a "major" purified allergen that induced immediate skin test responses in >90% of allergic individuals in contrast to a "minor" allergen, to which <20% of patients gave skin test responses. In addition, several mechanisms between allergen and immune system were described in this chapter such as; molecular properties of allergens, the factors that influenced allergenicity, basic immune response to allergens, and immunogenetic studies of IgE responses to purified pollen allergens.

2.4.2 Allergen Nomenclature

After the first recognition of allergic proteins and cloning in the late 1980s, hundreds of allergens have been identified and their nucleotide sequences determined and added to the

database. To discriminate and avoid complication of allergens names, King *et al.* (1995) and Larsen and Lowenstein (1996) have developed a standardized way to describe the methods for designation (nomenclature) of the characterized allergens as follows; the allergen is named by the first three letters of the genus, a space, the first letter of the species, a space, and an Arabic number, all in normal type (not italics). For example, Lol p 1 refers to the first pollen allergen identified from *Lolium perenne* (rye grass), Ani s 3 refers to the third Anisakidae allergen identified from *Anisakis simplex,* and Cyn d l refers to the homologous pollen allergen from *Cynodon dactylon* (Bermuda grass). In other cases where the first letters of the genus and species of two different species are the same, an adjustment must be made by adding a letter to either the genus or the species. Allergens that share similar biochemical properties, biological function, and have >67% identity of amino acid sequence are assigned to the same group. Additionally, the use of the prefixes "r," "s," and "n" to designate recombinant, synthetic, and natural allergens is optional. There are some criteria to be considered when evaluating the allergens and their allergenicity. For example; only allergens with greater than 5% IgE reactivity are included in the nomenclature. Also, allergens are designated as major if more than 50% of patients tested have serum IgE that reacts to the allergen.

2.4.3 Allergen Structure (Isoallergens and isoforms)

Isoallergens are broadly defined as multiple molecular forms of the same allergen that share extensive IgE cross-reactivity. Isoallergens are also defined as allergens from a single species with 67% or greater amino acid sequence identity. For example, tropomyosin allergen, birch pollen allergen and Bet v 1 have more than 40 sequences representing 31 isoallergens, showing 73% to 98% sequence identity (Chapman, 2003). The Bet v 1 isoallergens are distinguished by additional numbers: Bet v 1.01 through Bet v 1.31. Similarly, 4 isoallergens of ragweed allergen, Amb a 1, are listed as Amb a 1.01, Amb a 1.02, Amb a 1.03, and Amb a 1.04 (Smith *et al*., 2001, Piboonpocanun *et al*., 2006). Isoforms are defined as polymorphic variants of the same allergen that show > 90% amino acid sequence identity (Chapman *et al*., 2007). Isoforms are distinguished in the nomenclature by 2 additional numbers. For example, birch pollen has 42 isoforms of Bet v 1 and are listed as Bet v 1.0101, Bet v 1.0102, Bet v 1.0103 and similarly, dust mite has 23 isoforms of Der p 1and are listed as Der p 1.0101, Der p 1.0102, Der p 1.0103, and 13 isoforms of Der p 2 are listed as Der p 2.0101, Der p 2.0102, Der p 2.0103 (Chapman *et al*.,

2007, Smith *et al*., 2001, Piboonpocanun *et al*., 2006). As isoforms differ in only a few amino acid substitutions, analysis of immunoreactivity to isoforms can be useful in defining antibody binding sites and T cell epitopes on allergens (Piboonpocanun *et al*., 2006).

2.4.4 Types of allergens

2.4.4.1 Proteases

Proteases have been currently classified into six broad groups such as cysteine proteases, serine proteases, aspartate proteases, threonine proteases, metalloproteases and glutamic acid proteases (Barrett A.J Rawlings N.D Woessner, 2003, Hedstrom, 2002). Protease activated receptors have been identified on a variety of cell types and mediates protease response activity (Alshurafa *et al*., 2004). The cysteine protease of dust mite, Der p1, the aspartic protease of cockroach, Bla g 2 and the serine protease of *Aspergillus fumigatus* are all major allergenic molecules responsible for the increase in asthma and atopic conditions worldwide (Donnelly *et al*., 2006).

Several types of protease-activated receptor (PAR) are identified and expressed on epithelial cells of pancreas, kidney, colon, liver, small intestine, and airways (Bohm *et al*., 1996, D'Andrea *et al*., 1998, Cocks *et al*., 1999), such as PAR1, PAR2, PAR3 and PAR4 (Dery *et al*., 1998, Nystedt *et al*., 1994). The subtype PAR2 has been identified as a receptor for endogenous serine proteases such as respiratory epithelial trypsin, mast cell tryptase, coagulation cascade and exogenous serine proteases from parasites, insects, mites, molds and pollens (Reed, 2007, Schmidlin *et al*., 2001). It has been demonstrated that PAR2 receptors play a significant role during inflammatory diseases in both airways and intestine, and participate in the control of ion transport in these tissues, and are activated by serine proteases (Kunzelmann *et al*., 2002). Exogenous serine proteases from mite, fungal, and parasite origins have been involved in allergic airway disease, where they stimulate the component of innate and adaptive immune system such as attraction and activation of neutrophils and eosinophils, degranulation of mast cells and releasing of inflammatory mediators such as histamine, proteoglycans, and cytokines, increase response of afferent neurons, smooth muscle contraction, angiogenesis, fibrosis, and production of immunoglobulin E (IgE). Therefore, both endogenous and exogenous serine proteases can be considered allergic organisms and associated with allergenicity (Reed, 2007).

The prime house dust mite allergen Der p 1 allergen (cysteine protease), Der p 3, Der p 6 and Der p 9 allergens (serine proteases) induce release of proinflammatory cytokines from bronchial epithelial cells, mast cells and basophils. This increases the bronchial permeability by disrupting epithelial tight junctions. Therefore, the study of the biochemical properties and physiological function of protease allergens might be an important factor for investigation of allergenicity. In addition, proteolytic enzymes act directly on cells of the human immune system by cleaving cell-surface proteins of CD23 in activated B cells, CD25, CD40, and in T cells. Therefore, it enhances B-cell IgE synthesis by promoting differentiation into plasma cells (Chapman *et al*., 2007, Furmonaviciene *et al*., 2007, Wan *et al*., 1999).

Per a 10, a serine protease allergen extracted from *Periplaneta americana* (cockroach) could induce significant histamine release ($P < 0.05$) in blood of sensitized patients. The secretion of interleukin-4 (IL-4) ($P < 0.05$) and IL-5 ($P < 0.05$) were also significant. In addition, more than 80% of sensitized patients show IgE reactivity to Per a 10 by skin tests and immunoblot (Sudha *et al*., 2008). Ani s 1, Ani s 4 and Ani s 6 are serine protease from *Anisakis*, which gains entry into their host's tissues, and feed on them. Their action leads to tissue damage and the activation of the innate immune system cells such as; dendritic cells, mast cells, basophils and macrophages. In addition, these proteases induce Th2-driven inflammatory responses in the airways by disrupting the epithelial cell junctions and increasing bronchial permeability (Donnelly *et al*., 2006).

2.5 Allergens in the Different Seafood Groups

2.5.1 Seafood Allergens

Recently, the demand of seafood (fish and shellfish) has increased worldwide; subsequently many reports of adverse reactions associated with allergens in seafoods have been globally recorded, highlighting the need for more precise diagnosis and treatment of this illness and discovering reasons for the persistence of this allergy. Despite years of intensive data studies, the reasons why some people are allergic to seafood are still vague. Generally, the prevalence of seafood allergy for occupational protein contact dermatitis ranges from 3% to 11% and for occupational asthma from 7% to 36% (Jeebhay *et al*., 2001). The manifestations of seafood allergy vary widely, but it tends to be more severe than most other allergic food. The major routes of sensitization to allergic proteins of seafood are

through ingestion, handling and inhalation of vapours allergens during cooking or processing.

It has been demonstrated that over 50% of the allergic reactions associated with patterns of IgE seen among 105 sensitised individuals were to different crustacean species, followed by 30% to molluscs and 20% to fish in South Africa (Zinn et al., 1997, Lopata and Jeebhay, 2001). More than half of the individuals reacted to one seafood group, 36% to two seafood groups and surprisingly only 11% to all three seafood groups. Almost 50% of the mollusc-sensitive group reacted to abalone, which subsequently resulted in the identification and characterisation of a novel allergen (Lopata *et al*., 1997). Importantly, new allergens and significant potential cross-reacting allergens have been identified within the fish family and between shellfish (crustaceans and molluscs), arachnids, insects and nematodes (Nakano *et al*., 2008, DeWitt *et al*., 2004, Ayuso *et al*., 2008, Jeong *et al*., 2004a, Santos *et al*., 1999a). Therefore, there is a possibility that patients reacting to fish infested with the parasite Anisakis might also react to molluscs due to cross-reactivity. Interesting and new findings of immunological and particularly clinical IgE allergic cross-reactivity between individual families (Table 2.2) give valuable information to improve the diagnosis and management of this potential sensitization and life-threatening allergy and therefore is essential for future immunotherapy (Lopata *et al*., 2010).

Table 2.1 Identification of amino acid sequence between allergenic tropomyosins from different crustacea species and also compared to allergenic and non-allergenic tropomyosins from other invertebrates and vertebrates organisms.

Species	Cross reactivity of tropomyosin	Identity (%)	References
Crustacea	Eup s 1(krill) to Eup p 1 (krill)	98	Nakano *et al*. 2008
	Eup s 1(krill) to Pen a 1 (shrimp)	89	Nakano *et al*. 2008
	Eup s 1(krill) to Met e 1 (shrimp)	89	Nakano *et al*. 2008
	Eup s 1(krill) to Hom a1 (lobster)	90	Nakano *et al*. 2008
	Eup s 1(krill) to Cha f 1 (crab)	89	Nakano *et al*. 2008
	Pen a 1 (shrimp) to Cha f 1 (Crab)	92	DeWitt *et al*. 2004
	Pen a 1 (shrimp) to Hom a1 (lobster)	99	DeWitt *et al*. 2004
	Pen a 1 (shrimp) to (Locust)	82	DeWitt *et al*. 2004
	Pen a 1 (shrimp) to Onc v (Nematode)	71	DeWitt *et al*. 2004
	Pen a 1 (shrimp) to Sch m (Trematode)	60	Ayuso *et al*. 2002
	Pen a 1 (shrimp) to Dro m (Fruit fly)	78	Ayuso *et al*. 2002

Pen a 1 (shrimp) to Der p 10 (House dust mite)	81	Ayuso *et al*. 2002
Pen a 1 (shrimp) to Der f 10 (House dust mite)	81	Ayuso *et al*. 2002
Pen a 1 (shrimp) to Pa (cockroach)	82	Santos *et al*. 1999
Pen a 1 (shrimp) to Bg (cockroach)	84	DeWitt *et al*. 2004
Pen a 1 (shrimp) to Per a7 (cockroach)	83	DeWitt *et al*. 2004
Pen a 1 (shrimp) to Pf (cockroach)	84	Jeong *et al*. 2004
Met e 1 (shrimp) to Pf (cockroach)	81	Jeong *et al*. 2004
Met e 1 (shrimp) to Pf (cockroach)	80	Jeong *et al*. 2004
Pen a 1 (shrimp) to (Human)	57	DeWitt *et al*. 2004
Pen a 1 (shrimp) to Gal g (chicken)	58	Ayuso *et al*. 2002
Pen a 1 (shrimp) to Ory c (rabbit)	56	Ayuso *et al*. 2002
Hom a1 (lobster) to Cha f 1 (Crab)	92	DeWitt *et al*. 2004
Hom a1 (lobster) to (Locust)	82	DeWitt *et al*. 2004
Hom a1 (lobster) to (Nematode)	71	DeWitt *et al*. 2004
Hom a1 (lobster) to (Trematode)	60	DeWitt *et al*. 2004
Hom a1 (lobster) to (Fruit fly)	79	DeWitt *et al*. 2004
Hom a1 (lobster) to Der p 10 (House dust mite)	81	DeWitt *et al*. 2004
Hom a1 (lobster) to Bg (cockroach)	83	DeWitt *et al*. 2004
Hom a1 (lobster) to (Human)	57	DeWitt *et al*. 2004
Cha f 1 (Crab) to (Locust)	84	DeWitt *et al*. 2004
Cha f 1 (Crab) to (Nematode)	73	DeWitt *et al*. 2004
Cha f 1 (Crab) to (Trematode)	58	DeWitt *et al*. 2004
Cha f 1 (Crab) to (Fruit fly)	81	DeWitt *et al*. 2004
Cha f 1 (Crab) to Der p 10 (House dust mite)	84	DeWitt *et al*. 2004
Cha f 1 (Crab) to Bg (cockroach)	84	DeWitt *et al*. 2004
Cha f 1 (Crab) to (Human)	60	DeWitt *et al*. 2004

Table 2.2 Identification of amino acid sequence between allergenic tropomyosins from different molluska species and also compared to allergenic and non-allergenic tropomyosins from other invertebrates and vertebrates organisms.

Species	Cross reactivity of tropomyosin	Identity (%)	References
Molluska	Scallop to (Blue mussel)	68	DeWitt *et al*. 2004
	Scallop to (Nematode)	60	DeWitt *et al*. 2004
	Scallop to (Trematode)	64	DeWitt *et al*. 2004
	Scallop to (Human)	55	DeWitt *et al*. 2004
	Scallop to Bg (cockroach)	60	DeWitt *et al*. 2004
	Scallop to (Fruit fly)	60	DeWitt *et al*. 2004
	Scallop to Der p 10 (House dust mite)	62	DeWitt *et al*. 2004
	Blue mussel to Bg (cockroach)	57	DeWitt *et al*. 2004

Table 2.3 Identification of amino acid sequence between allergenic tropomyosins from different crustacea and molluska species.

Species	Cross reactivity of tropomyosin	Identity (%)	References
Crustacea and molluska	Eup s 1(krill) to Tod p 1 (squid)	84	Nakano *et al*. 2008
	Cha f 1 (Crab) to (Blue mussel)	56	DeWitt *et al*. 2004
	Cha f 1 (Crab) to (Scallop)	62	DeWitt *et al*. 2004
	Hom a1 (lobster) to (Blue mussel)	57	DeWitt *et al*. 2004
	Pen a 1 (shrimp) to (Scallop)	62	DeWitt *et al*. 2004
	Hom a1 (lobster) to (Scallop)	62	DeWitt *et al*. 2004
	Pen a 1 (shrimp) to (Blue mussel)	57	DeWitt *et al*. 2004

Table 2.4 Comparison of amino acid sequence between allergenic and non-allergenic tropomyosins from other invertebrates and vertebrates organisms.

Species	Cross reactivity of tropomyosin	Identity (%)	References
Other organisms	Pf (cockroach) to Pa (cockroach)	100	Jeong *et al*. 2004
	Pf (cockroach) to Bg (cockroach)	98.2	Jeong *et al*. 2004
	Bg (cockroach) to (Locust)	90	DeWitt *et al*. 2004
	Bg (cockroach) to *C. elegan*	69	DeWitt *et al*. 2004
	Bg (cockroach) to (*S. haematobium*)	56	DeWitt *et al*. 2004
	Bg (cockroach) to (Fruit fly)	86	DeWitt *et al*. 2004
	Bg (cockroach) to Der p 10 (House dust mite)	81	DeWitt *et al*. 2004
	Bg (cockroach) to (Human)	51	DeWitt *et al*. 2004
	Pa (cockroach) to Dro m (Fruit fly)	83	Santos *et al*. 1999
	Pa (cockroach) to Der p 10 (Fruit fly)	81	Santos *et al*. 1999
	Pa (cockroach) to Der f 10 (Fruit fly)	81	Santos *et al*. 1999

2.5.2 Studies of Fish Allergens

Fish is one of the most important sources of food for human nutrition and health. The rising supply and request for varieties of fish worldwide is associated with exposure to fish allergens in various forms that can cause allergic disease and asthma (Sharp and Lopata, 2013). Although there are more than 32,400 different species of fish described (Froese, 2012), most studies on fish allergens have focused on the bony fish group including; cod, hake, whiting, carp, salmon, trout, herring, sardine, catfish, perches, mackerel, and tuna (Sharp and Lopata, 2013). The major fish allergen shown to display IgE cross-reactivity

among fish species is parvalbumin and several other allergens, which are unknown (Sharp and Lopata, 2013).

The first allergen characterized and sequenced in the Atlantic cod is Gad c 1 (originally designated Allergen M). It contains 113 amino acids and a glucose molecule, with a molecular weight of about 12 kDa (Elsayed and Aas, 1971, Elsayed and Bennich, 1975, O'Neil et al., 1993). This allergen was isolated, characterized and identified as a family of parvalbumins, a group of calcium-binding proteins (Lopata and Potter, 2000). Parvalbumin is present in high quantities in lower invertebrates and in low quantities in high vertebrates including humans (Permyakov *et al*., 2006). It plays a vital role in basic vertebrate calcium buffering in the relaxation process of muscles (Hamada *et al*., 2003).

The allergenicity of purified parvalbumin from Atlantic salmon (Salmo salar (Sal s 1) has been confirmed using various molecular cloning techniques (Lindstrom *et al*., 1996b). Furthermore, in 2002, Das Dores *et al*., determined the DNA sequence encoding parvalbumin allergen (Gad m 1) in the Atlantic cod. This recombinant allergen of a molecular weight of 11.5 kDa had 62.3% identity with Gad c 1 and 75% with Sal s 1. In addition, parvalbumin has also been characterized as the major allergen in horse mackerel (Hamada *et al*., 2003) and carp (Bugajska-Schretter *et al*., 1999). It has been demonstrated that sera from allergic subjects displayed IgE reactivity to parvalbumin from pilchard (*Sardinops sagax*), anchovy (*E. encrasicolus*), hake (*M. merluccius*), snoek (*Thyrsites atun*) and yellowtail (*Seriola lalandi)*, respectively (Beale *et al*., 2009). Parvalbumins of 12 kDa can show one of two distinct isoform lineages; α and β. Fish muscle often contain both α and β parvalbumin, although the majority of allergenic parvalbumins reported belong to the β lineage ((Lindstrom *et al*., 1996a). Furthermore, there are seven different β parvalbumin isoforms expressed in most fish muscle, which are subsequently named β1, β2, to β7, respectively (Brownridge *et al*., 2009).

2.5.3 Studies of Crustacean Allergens

Approximately 50,000 living crustacean species including shrimps, crabs, crayfish, rock lobster, prawn, barnacles and more families are found worldwide, and most varieties are consumed either raw or cooked (Lopata *et al*., 2010). Major allergens responsible for ingestion-related allergic reactions due to crustaceans are tropomyosins with molecular weight between 34 and 39 kDa (Lopata *et al*., 2010). Molecular comparison of tropomyosin

from many different crustacean species reveals very high identities of up to 98% (Jeebhay and Lopata, 2012). The first two allergens isolated from raw and cooked shrimp are termed as antigen I and antigen II, respectively (Hoffman *et al*., 1981). From the allergenicity and homology comparison, it was observed that the heat stable antigen II binds to IgE antibodies in the sera of all 11 shrimp allergic subjects tested. Subsequently, another study confirmed that antigen II is the major allergen isolated from shrimp *Penaeus indicus* and recognized as Pen i 1 tropomyosin (Shanti *et al*., 1993).

The first report of the cloning of the major shrimp allergen from *Metapenaeus ensis* (Met e 1) was by (Leung *et al*., 1994), and identified by screening a cDNA library of shrimp muscle with sera from patients with a shellfish allergy. The cDNA of Met e 1 consists of 281 codons, coding for a 34-kDa protein (Leung *et al*., 1994). In the same year, another shrimp allergen was also identified from the shrimp *Penaeus aztecus* and named (Pen a 1) (Daul *et al*., 1994). The identity of Pen a 1 as a tropomyosin has subsequently been confirmed by molecular cloning and nucleotide sequence analysis (Reese *et al*., 1997). In addition, the percentage of amino acid sequence identity was also compared between allergens. For instance; the percentage of amino acid sequence identity from tropomyosin allergens from shrimp *Penaeus indicus* (Pen i 1), shrimp *Penaeus aztecus* (Pen a I) and shrimp *Metapenaeus ensis* (Met e I) compared to the muscle protein of the fruit fly *Drosophila melanogaster* was found to be high at 86%, 87% and 87%, respectively. Therefore, the phylogenic relationship between these two arthropods is well established (Daul *et al*., 1994, Leung *et al*., 1994).

Similarity between shrimp tropomyosin allergen Met e I, Pen a I, Sa-II, and antigen II has shown that they are similar or identical allergens, suggesting that the major shrimp allergen is tropomyosin isoforms among various shrimp species (Leung et al., 1994). Furthermore, the identity of tropomyosin as an allergen has subsequently been confirmed in other crustaceans. Additionally, (Leung *et al*., 1998b) identified the allergen Pan s 1 from the spiny lobster *Panulirus stimpsoni*, Hom a 1 from the American lobster *Homarus americanus* (Mykles *et al*., 1998), and Cha f 1 (Leung *et al*., 1998a) from the crab *Charybdis feriatus*. All those allergens have similar molecular weight (34 kDa) and their deduced amino acid sequences are highly similar to tropomyosin.

The major heat-stable allergic protein that has been identified in the muscle of crustacean species and is responsible for allergic reactions due to consumption of infected crustaceans

is tropomyosin (Lopata *et al*., 2001). This molecule belongs to a family of actin filament-binding proteins with highly conserved structure. It has to be found with multiple isoforms in both muscle and non-muscle cells of all species of vertebrates and invertebrates. Tropomyosin has a native structure consisting of two parallel alpha–helical tropomyosin molecules that are wound around each other forming a coiled–coil dimer in the muscle (Reese *et al*., 1997, Lopata *et al*., 2001).

Although, tropomyosin is the primary allergic protein in muscle tissue among crustacean species (Lopata *et al*., 2001), there have been reports on the presence of multiple allergens in crustacean species (Shiomi *et al*., 2008, Ayuso *et al.*, 2008, Lopata *et al*., 2001). For example, using two-dimensional immunoblotting methods and the response of IgE antibody binding of patients sera to allergic proteins, allergen from shrimp *Penaeus monodon* called (Pen m 2) has been identified and compared with known protein sequences and revealed extensive similarity with arginine kinase (Yu *et al*., 2003). This protein has shown 60% similarity in sequence to arginine kinase of crustacean *Penaeus japonicas* (Pen i 1) with a molecular weight of 39.9 kDa. In addition, Pen m 2 exhibited arginine kinase activity and reacts with IgE serum from shrimp-sensitive patients suggesting that arginine kinase is a common allergen among crustaceans. It has also been demonstrated using mass spectrometric analysis that the high levels of arginine kinase in shrimp (*Pandalus borealis*) suggest it is easily aerosolized (Abdel Rahman *et al*., 2013). Arginine kinase has also been classified as a significant allergen in the moth (*Plodia interpunctella*) (Binder *et al*., 2001).

Interestingly, crustaceans show various degrees of sequence identity with tropomyosins of other arthropods (Lopata *et al*., 2010, Auerswald and Lopata, 2005) such as cockroaches (Lopata *et al*., 2005b), fruit fly (Reese *et al*., 1997), house-dust mites and locusts (Lopata *et al*., 2005a), Anisakis nematode (Guarneri *et al*., 2007), suggesting that the documented cross-reactivity between tropomyosins from different allergen sources can result in immune cellular activation and subsequently asthmatic responses. On the other hand these immunological findings will improve diagnosis and control of this potentially life-threatening allergy and is important for future immunotherapy (Lopata *et al*., 2010).

2.5.4 Studies of Mollusk Allergens

Molluscs comprises over 100,000 different species and have been subgrouped into the following classes: gastropods (snails, whelks, slugs, abalone and limpets), bivalves

(mussels, scallop, oysters and clams), and cephalopods (the pearl boat Nautilus, sepias, squids and octopuses, and the many fossil ammonites and belemnites) (Haszprunar and Wanninger, 2012). Even though the amino acid sequence identity of shrimp crustaceans tropomyosin with mussels and abalone tropomyosin molluscs is lower with 57% and 61%, respectively (Jeebhay and Lopata, 2012), the allergic tropomyosin protein from molluscs can cause hypersensitive reactions upon ingestion, as well as occupational reactions in sensitized workers (Tomaszunas *et al*., 1988, Glass *et al*., 1998). Therefore, a number of reports demonstrate that tropomyosin is a major allergic muscle protein identified in molluscan shellfish such as snail (Asturias *et al*., 2002), abalone (Miyazawa *et al*., 1996, Lopata *et al*., 1997), limpet (Morikawa et al., 1990), cuttlefish (Shibasaki *et al*., 1989), turban shell Turbo (Ishikawa *et al*., 1998a), squid (Miyazawa et al., 1996), *Helix aspersa* (Asturias et al., 2002), *Octopus vulgaris* (Ishikawa *et al*., 2001) and *Penaeus monodon* (Rahman *et al*., 2010).

Tropomyosin has subsequently been confined into three groups of mollusks; oyster (Cra g 1), abalone (Hal m 1), and squid (Tod p 1) (Taylor, 2008). (Lopata *et al*., 1997) confirmed two major allergens with molecular weights of 38 kDa and 49 kDa, respectively from abalone (*Haliotis midae*), which responds to IgE from patient serum. The 38-kDa protein was believed to be tropomyosin while another allergen (49-kDa) named Hal m 1, remains to be identified. (Ishikawa *et al*., 1998a) purified a 35-kDa allergen (Tur c 1) in the turban shell *Turbo cornutus* using various molecular methods. The amino acid composition and partial amino acid sequences of Tur c 1 imply that it is a tropomyosin protein. (Asturias *et al*., 2002) isolated and cloned tropomyosin from *Helix aspersa* (brown garden snail) and found that the tropomyosin (Hel as 1) was a 36-kDa protein and shared 84% amino acid sequence identity with abalone (*Haliotis diversicolor*), 70% with mussel (*Mytilus edulis*), and 72% with scallop (*Chlamysnobilis*) tropomyosins.

Mollusc and crustacean allergens possibly share common antigen epitopes. Therefore, they are potentially significant because of their cross-reactivity of tropomyosin. For example, using molecular methods, it has been demonstrated that Cra g 1 and 2 allergens from *Crassostrea gigas* are isoforms of tropomyosins. Interestingly, the amino acid compositions of Cra g 1 and Cra g 2 are similar to shrimp tropomyosins (Ishikawa et al., 1998b, Ishikawa *et al*., 1997). In another study, (Leung and Chu, 2001) have successfully identified two allergens from oyster called (Cra g 1.01, Cra g 1.02 and Cra g 1.03). The features of Cra g

1.01 and Cra g 1.02 allergens are similar to isoforms of Cra g 1 and Cra g 2 and are biochemically purified from the Pacific oyster (Ishikawa *et al*., 1997). In contract, Cra g 1.03 was confirmed as tropomyosin using recombinant DNA technology. (Patwary *et al*., 1999) isolated and characterized cDNA clones that encode tropomyosin from the adductor muscle of sea scallop (*Placopecten magellanicus*). The amino acid sequence is about 70% identical to tropomyosins from other mollusks, and this protein had a molecular weight of approximately 30 kDa.

(Chu *et al*., 2000) amplified tropomyosin cDNA from abalone (*Haliotis diversicolor*), scallop (*Chlamys nobilis*), and mussel (*Perna viridis*), using reverse transcription polymerase chain reaction (RT-PCR). The cDNAs were cloned and expressed, and the IgE reactivity of the recombinant proteins was demonstrated. (Miyazawa *et al.*, 1996) isolated a 38-kD heat-stable allergen (Tod p 1) from the squid *Todarodes pacificus*. The sequences of Tod p 1 exhibited significantly high identity to the snail tropomyosin. Tropomyosin has been identified as a major allergic protein in muscle of *Octopus vulgaris* (Oct v 1) (Ishikawa *et al*., 2001).

2.5.5 Studies of parasite Anisakis Allergens

Allergic reactions to commercially important seafood are not always caused by allergens derived from seafood (fish and shellfish) species. Therefore, these allergic symptoms may be produced by seafood-borne parasite after repeated ingestion of raw or undercooked food or by coming in contact with parasitized seafood (Lopata and Lehrer, 2009). For example, seafood contaminated with the parasite *Anisakis* can cause severe allergic reactions (Audicana and Kennedy, 2008). The twelve allergens from *A. simplex* that have been currently characterized include somatic allergens and excretory–secretory (ES) allergens (Table 2.4). These allergens have been classified into major and minor allergens depending on the percentage of recognition of the allergen protein by IgE in infected patients sera, while some are considered as pan-allergens. Pan-allergens are considered to be highly conserved proteins, which explains crossreactive antibodies recognising different food sources (Daschner *et al*., 2012). Most of them are present in ES products, when they are secreted during expulsion and surgery to remove them or in cases when the larva penetrates mucous membrane of the host tissue (Audicana and Kennedy, 2008). Ani s 1, 4, 5, 6, 7, 8 and 9 are all heat-stable ES products, although some of them such as Ani s 9 have reportedly been abundant in crude extracts, with their biological function yet to be

determined (Caballero *et al*., 2008, Rodriguez-Perez *et al.*, 2008, Kobayashi *et al*., 2007a, Kobayashi *et al*., 2007b). Similar findings have been made with somatic allergens such as Ani s 2, 3, 10, 11 and 11-li. The functions of Ani s 10, 11 and 11-li are still to be determined.

Table 2.5 Characterised allergens of Anisakis simplex

Allergen	MW (kDa)	Compartment	Function	IgE reactivity (%)	Major allergen	Pan-allergen
Ani s 1	24	ESP	Kunitz-type trypsin inhibitor	85	Yes	
Ani s 2	97	Somatic	Paramyosin	88	Yes	Yes
Ani s 3	41	Somatic	Tropomyosin	4	Yes	Yes
Ani s 4	9	ESP	Cystatin	27		
Ani s5	15	ESP	SXP/RAL proteins	25–49		
Ani s 6	7	ESP	Serpin	18		
Ani s 7	139	ESP	Glycoprotein	83–100	Yes	
Ani s 8	15	ESP	SXP/RAL protein	25		
Ani s 9	14	ESP	SXP/RAL protein	13		
Ani s 10	22	Somatic?	?	39		
Ani s 11	55	Somatic?	?	47		
Ani s 11-li	?	Somatic?	?	?		
Ani s 12	?	?	?	57	Yes	
Ani s 13	37.6	ESP	Haemoglobin	64.3–80.9	Yes	

ESP, excretory–secretory products; ?, denotes unknown function, source or molecular weight (Nieuwenhuizen, N.E., Lopata, A.L. 2013; González-Fernández *et al*., 2015)

2.6 Tropomyosin

Tropomyosin (TPM) constitutes a family of a highly conserved protein in a chain that lies along the actin microfilament system and covers the binding sites and together performs a broad range of cellular functions from regulating cell structure to cell motility of muscle and non-muscle tissue and cytokinesis (Schevzov *et al*., 2005). TPM has been found in α-helical coiled-coil form, head-to-tail dimers wrapped around actin microfilaments. In mammals, TPMs are derived from four highly conserved genes known as the αTm fast, βTm, γTm (Tm5nm), and δTm genes, while, in non-muscle cells, over 40 TPM isoforms are derived using alternative splicing (Lees-Miller *et al*., 1991).The TPM structure is based on a repeated pattern of seven amino acids with hydrophobic residues at the first and fourth positions. TPM has been found in the animal kingdom from yeast to humans and displays a complexity of isoforms. This isoform diversity is produced by a combination of multiple

TPM encoding genes, of which some contain alternative promoters and some exhibit alternative splicing of primary RNA transcripts. Based on the presence and apparent molecular weight, TPM isoforms from animals are differentially expressed in different cell types and developmental stages and divided into high molecular weight (HMW) isoforms with approximately 284 amino acid residues (44,000 kDa), and low molecular weight (LMW) isoforms, with approximately 247 amino acid residues (33,000 kDa) (Pittenger *et al*., 1994).

It has been demonstrated that tropomyosin is the major food allergen of shellfish and parasites in fish. Additionally, it is considered as a pan allergen due to its wide distribution in several taxonomical allergenic sources. Functionally, allergic tropomyosin has shared several highly conserved IgE-binding B-cell epitopes that induce the immune system and it has high cross-reactivity with invertebrate tropomyosins of different species. In regard to natural infection with parasitic worms, they constantly challenge their hosts by producing large numbers of somatic and excretory/secretory antigens and thereby induce strong allergic response to these antigens. Tropomyosin is a major somatic allergen and has been identified from zoonotic parasites and is present in third-stage larvae, fourth-stage larvae and adult worms (O'Donnell *et al*., 1989).

2.6.1 Tropomyosin as Allergens in *A. simplex* and other Invertebrate Groups

Tropomyosin has been classified as a major parasite allergic protein from *Ascaris suum/lumbricoides* (Asc 1 3) (Acevedo and Caraballo, 2011) and *Anisakis simplex* (Ani s 3) (Guarneri *et al*., 2007) with MW 40 and 41kDa, respectively. It is a heat stable somatic allergen found from dead larvae in food or after degradation inside the host tissue, and is an important source of cross-reactivity between *Anisakis* and other invertebrates (Sereda et al., 2008) (Table 2.4). Tropomyosin is an alpha helical protein that forms a coiled-coil secondary structure of two parallel helices. The resistance of tropomyosin to freezing and heat treatment during processing techniques can be attributed to stability of this structure.

Anisakis tropomyosin belongs to a family of phylogenetically conserved structural proteins. The amino acid sequence has regions of high percentage of identity among and between different invertebrate and vertebrate tropomyosins species (Asturias *et al*., 2000, DeWitt *et al*., 2004, Jenkins *et al*., 1998). Therefore, these finding have suggested that immunological cross-reactivity may be clinically relevant (Sereda *et al*., 2008).

Table 2.6 Amino acid sequence identity among allergenic and non-allergenic tropomyosins from different organisms

Species	Cross reactivity of tropomyosin	Identity (%)	References
Anisakis simplex	*Ani s 3 (A.simplex)* to Hel as 1(snail)	66	Asturias *et al*. 2000
Nematoda	*Ani s 3 (A.simplex)* to (trichostrongylus)	93	Asturias *et al*. 2000
	Ani s 3 (A.simplex) to (caenorhabditis)	92	Asturias *et al*. 2000
	Ani s 3 (A.simplex) to (onchocerca)	91	Asturias *et al*. 2000
	Ani s 3 (A.simplex) to Onchocerca volvulus	69-98	Asturias *et al*. 2000
	Ani s 3 (A.simplex) to (shrimp)	70	Asturias *et al*. 2000
	Ani s 3 (A.simplex) to (snail)	63-56	Asturias *et al*. 2000
	Ani s 3 (A.simplex) to (insect)	69	Asturias *et al*. 2000
	Ani s 3 (A.simplex) to (human)	58	Asturias *et al*. 2000
	Ani s 3 (A.simplex) to (chicken)	58	Asturias *et al*. 2000
	Ani s 3 (A.simplex) to (mouse)	58	Asturias *et al*. 2000
Ascaris lumbricoides	*A. lumbricoides* to Ani s 3 (Anisakis simplex)	98	Santos *et al*. 2008
Nematoda	*A. lumbricoides* to Per a7 (cockroach)	69	Santos *et al*. 2008
	A. lumbricoides to Dro m (Blattella germanica)	74	Santos *et al*. 2008
Caenorhabditis	*C. elegans* to Pen a 1 (shrimp)	71	DeWitt *et al*. 2004
elegans	*C. elegans* to Hom a1 (lobster)	71	DeWitt *et al*. 2004
Nematoda	*C. elegans* to Cha f 1 (Crab)	73	DeWitt *et al*. 2004
	C. elegans to Bg (cockroach)	69	DeWitt *et al*. 2004
	C. elegans to Locust	71	DeWitt *et al*. 2004
	C. elegans to Der p 10 (House dust mite)	72	DeWitt *et al*. 2004
	C. elegans to (Fruit fly)	69	DeWitt *et al*. 2004
Trichostrongylus	*T. colubriformis* to D.melanogaster	58	Frenkel *et al*. 1989
colubriformis	*T. colubriformis* to rabbit	58	Frenkel *et al*. 1989
Nematoda			
Onchocerca volvulus	*O. volvulus to T. colubriformis*	91	Jenkins *et al*. 1998
Nematoda	*O. volvulus* to Caenorhabditis elegans	91	Jenkins *et al*. 1998
	O. volvulus to D.melanogaster	66	Jenkins *et al*. 1998
	O. volvulus to human skeletal α-TM	57	Jenkins *et al*. 1998
Schistosoma	*S haematobium* to Caenorhabditis elegans	57	DeWitt *et al*. 2004
haematobium	*S haematobium* to Pen a 1 (shrimp)	60	DeWitt *et al*. 2004
Trematode	*S haematobium* to Hom a1 (lobster)	60	DeWitt *et al*. 2004
	S haematobium to Cha f 1 (Crab)	58	DeWitt *et al*. 2004
	S haematobium to (Scallop)	64	DeWitt *et al*. 2004
	S haematobium to Bg (cockroach)	56	DeWitt *et al*. 2004
	S haematobium to Locust	57	DeWitt *et al*. 2004
	S haematobium to Der p 10 (mite)	59	DeWitt *et al*. 2004

2.7 Cysteine proteinase inhibitors (CPIs)

According to their properties, protease inhibitors (PIs) have been primarily classified into serine, cysteine, aspartic and metallo protease inhibitors (Abbenante and Fairlie, 2005). Cysteine protease inhibitors (CPI) or cystatin are widely distributed across the animal and plant kingdoms (Gregory and Maizels, 2007), and based upon their possession of conserved functional motifs they can be classified into three major families, stefins (family 1), cystatins (family 2) and kininogens (family 3) (Abrahamson et al., 2003a). In general, stefins are predominantly intracellular cystatins A and B, and contain about 100 amino acid residues (11 kDa) that possess no disulphide bonds or glycosylation sites, while cystatins are generally found in extracellular and/or transcellular cystatins (cystatins: C, D, E, F, S, SA, and SN). Cystatins are widely distributed as secretory proteins of about 100 amino acid residues, 13-15 kDa. In addition, they typically possess an N-terminal signal peptide and 2 conserved disulphide bonds. Kininogens are mostly found in the extracellular fluid and typically glycosylated with larger MW from 50 to 120 kDa (Abrahamson, 1994).

The numerous parasite species, helminths (nematodes, cestodes and trematodes) follow extremely diverse and complicated routes to invade the host tissues and thereby survive and/or complete part of their life cycle in the host (Dzik, 2006). The infective stage of the helminths release a variety of proteinases and their inhibitors help them to penetrate the defensive barriers and avoid the attack of the immune system of the host and are in addition involved in various endogenous physiological and pathological processes such as; oogenesis, moulting during larval development and migration through host tissues (Dzik, 2006, Lustigman *et al*., 1996). Based on the percentage of identity, there are significant levels of similarity between cystatins from several species of nematode as well as with human cystatin. Thus, cysteine protease inhibitors (CPI) or cystatins are involved in inhibiting the parasites own proteinases and proteinases of the host tissue for invasion and contributes to extracellular protein digestion. In addition, cystatins are equimolar reversible complexes acting as tight-binding inhibitors of cysteine proteinases (Nicklin and Barret, 1984) to promote parasite development and migration within the host tissue and might cause pathological disease in the host (Dzik, 2006).

The first cysteine proteinase inhibitor or “cystatin" isolated and characterized from parasite origin was the “onchocystatin” of the human filarial nematode *Onchocerca volvulus*

(Lustigman *et al*., 1991, Lustigman *et al*., 1992). It was found in the eggshell of microfilariae and cuticle of L3, L4, male and female adult of filaria *Onchocerca volvulus*. The function of this protein was demonstrated in the way it was used to regulate physiological processes of parasite proteinases during the moulting from the larval stage L3 to L4 and the development of microfilariae in the uterus of the nematode (Lustigman *et al*., 1991, Lustigman *et al*., 1992). Hashmi *et al*. (2006) characterized a sterile cpi-2a (ok1256) from *Caenorhabditis elegans* and showed that CPI-2a has an essential regulatory role during oogenesis and fertilization. This result was in accordance with cystatins of other parasitic nematodes. These reports support the function of homologous cystatins from various parasitic nematodes in endogenous physiological roles.

Investigation of the features of recombinant onchocystatin (rOv17, Ov‑CPI‑2) cystatins from filaria *Onchocerca volvulus* (Schönemeyer *et al*., 2001), Av‑cystatin (Av17, from *Acanthocheilonema viteae*) and Bm‑CPI‑2 (from *Brugia malayi*), recombinant nippocystatin (rNbCys), recombinant *H. contortus* cystatin of gastrointestinal nematodes (Dainichi et al., 2001, Newlands et al., 2001) showed that all recombinant cystatins inhibit the cysteine proteinases cathepsin B, L and S that are involved in the proteolytic processing of polypeptides and suppressed specific immune responses (Schönemeyer *et al*., 2001). In parasitic trematodes, recombinant CsSteFin-1, a cysteine proteinase inhibitor from *Clonorchis sinensis*, effectively inhibited various cysteine protease including human cathepsin B, human cathepsin L, papain and CsCFs (Kang *et al*., 2011). In addition, a multi‑domain cystatin of *Fasciola hepatica* efficiently inhibits parasite cathepsin L1 activity, which is important for penetrating the host intestine and might modulate host immune responses (Khaznadji *et al*., 2005).

In parasitic cestodes, a protein of 11 kDa on SDS-PAGE was purified and characterized as cysteine protease inhibitor from spargana. Since parasitic cestodes were able to inhibit papain and 27 kDa cysteine protease of spargana, they were thought to regulate endogenous cysteine proteases of the parasite, rather than being interactive with cysteine proteases from their hosts (Chung and Yang, 2008). Parasite cysteine proteases play critical roles in penetration, modulating host immunity, and migration into the host tissue (Sloane and Honn, 1984, Hartmann and Lucius, 2003). At the same time the proteolytic activity of cysteine proteases is inhibited by cysteine protease inhibitors which may play both roles of

regulating cysteine proteases and protection of cysteine proteases from the host (Pandey, 2013). Ani s 4 as a cysteine protease inhibitor was identified from *A. simplex* and defined as a heat-resistant 9 kDa allergen that causes allergy in humans. It was also shown to inhibit the papain family of cysteine proteases (Rodriguez-Mahillo *et al*., 2007).

2.7.1 Cysteine-protease inhibitors as Allergens in *A. simplex* and other Invertebrate Groups

Ani s 4 (MW: 9 kDa) has been identified as a significant (ES) allergen from live and dead larvae (L3) of *Anisakis simplex*. It belongs to the cystatin family of cysteine protease inhibitors and is resistant to freezing, heat and pepsin digestion. It is recognised by only 27–30% of patients but seems to be particularly important in provoking anaphylaxis in allergic consumers (Moneo *et al*., 2005). Although, Ani s 4 is classified as a minor allergen (less than 50%), it's an important allergic protein due to its clinical relevance as it is related with allergic reactions after eating well-cooked or canned fish (Rodriguez-Perez *et al*., 2008).

2.8 Allergen cross-reactions

Cross-reactions occur where the antibodies are provoked against a specific allergen or allergens from one biological source, and at the same time can recognise other allergen or allergens from other sources (Garcia and Lizaso, 2011). Thus, the allergic reaction is sensitised to those different allergen sources with the same antibody. In accordance with this, cross-reactivity reveals deep phylogenetic relationships between two or more allergens depending on the degree of similarity of linear amino acid sequence data in the primary sequence of the proteins (Aalberse, 2007). Assessment of cross-reactivity between two allergens depends on three following parameters: 1) the fraction of antigenic epitopes with high affinity to target IgE that are cross-reactive; 2) the fraction of IgE with high affinity to target antigen that is cross-reactive; and 3) the relative affinity of the interaction between IgE and the two allergens or more (Aalberse, 2007). The type of reaction due to antibody cross-reactivity is characterized as Type I hypersensitivity reactions as it is principally by antigen/allergen-specific IgE antibody.

With the aim of comparing between cross-reactions in individuals with Type I hypersensitivity disease, there are fundamentally four potentially harmful immune reactions due to allergen cross-reactivity. These are: (i) cross-reactions can occur between closely related *Anisakis* genus nematode parasitic in fish, *A. pegreffii, A. simplex sensu stricto (s.s.), A. simplex C, A. typica, A. ziphidarum* and *Anisakis* sp. (ii) cross-reactions can occur

between closely related nematode parasites of fish, specifically between those of the genera *Anisakis*, *Pseudoterranova*, *Phocascaris*, and *Contracaecum* within the Anisakidae. (iii) Cross-reactions between fish-derived parasites and others in the Order Ascaridida, for other members of which humans can be the definitive (*Ascaris lumbricoides*) or accidental (e.g. *Ascaris suum*, *Baylisascaris* spp., *Toxocara* spp., *Toxascaris* spp.) hosts. (iv) cross-reactions between Anisakid nematodes in fishery product and food allergens from edible crustaceans (e.g. prawns, shrimps) or environmental (e.g. house dust mites, cockroaches). Cross-reactivity between *A. simplex* and numerous invertebrate species has been experimentally shown by different methods; however, the clinical importance of such cross-reactivities is poorly understood and requires further analysis.

2.8.1 Antigenic and allergenic cross-reactions between *A. simplex (s.s.)* and other *Anisakis* nematodes

Anisakiasis is a gastrointestinal disease caused by infection with anisakid nematodes, mostly *Anisakis* type I larvae and *Pseudoterranova decipiens* (Ishikura *et al*., 1998, Ishikura et al., 1988). *Anisakis* type I larvae have been categorized into five species by allozyme and genetic analyses: (i) *A. simplex s.s*, (ii) *A. pegreffii*, (iii) *A. simplex C*, (iv) *A. typica*, and (v) *A. ziphidarum* (Mattiucci *et al*., 1997, Mattiucci *et al*., 2002, Nascetti *et al*., 1986). Importantly, the translated amino acid sequences of mtDNA cox2 gene showed 100% similarities among the three sibling species of *A. simplex* complex (*A. simplex s.s*, *A. pegreffii* and *A. simplex* C), while using nucleotide sequences showed 99.0% and 98.2% similarities with the previously reported sequences of *A. simplex* ssp and *A. pegreffii*, respectively (Suzuki *et al*., 2010). Although the *A. simplex s.s* and *A. pegreffii* have been detected in many fish species in Japan, the *A. simplex* ssp larvae have more pathogenetic potential than *A. pegreffii* (Suzuki *et al*., 2010, Arizono *et al*., 2012), whereas, in Italy, *A. pegreffii* is more a pathogenetic species than *A. simplex s.s* (Romero *et al*., 2013).

2.8.2 Antigenic and allergenic cross-reactions between *A. simplex (s.s.)* and anisakid nematodes

Three nematodes genera including *Anisakis, Pseudoterranova, and Contracaecum spp* are most relevant for public health. Therefore, the ingestion of raw or uncooked fish, molluscs or crustraceans infected with the third stage Larvae (L3) of these genera is responsible for Anisakidosis (Ruitenberg *et al*., 1979). Nevertheless, the cross-reactions between *A. simplex* and other anisakids at the clinical or experimental levels have been shown to share IgE binding components. However, the cause-and-effect relationship between allergen

exposure and symptoms is unclear. According to molecular features of cross-reactivity among acarida and environmental allergens, there are different levels of similarity between them as mentioned in Table (2.4). For instance; Arruda *et al.* (2005) and Santos *et al.* (2008) showed the cross reactivity between two groups of nematodes, *A. simplex* and *Caenorhabditis elegans*. It was observed that the percentage of similarity between them was around 69-98 %. Jenkins *et al*. (1998) reported that the identity at the amino acid level sequence between nematode groups *O. volvulus* to *T. colubriformis* and *O. volvulus* to *Caenorhabditis elegans* were roughly 91 %,. In addition, the patient serum response to Ani s 7 (nAni s 7) showed that it is cross-reactive with by *Pseudoterranova* allergen (Anadon *et al*., 2009).

2.8.3 Antigenic and allergenic cross-reactions between *A. simplex (s.s.)* and other groups of nematodes

Resulting from the experimental results and clinical data of sera from anisakidosis, ascariosis and toxocariasis patients by ELISA and western-blot techniques, there is strong evidence that there is extensive antigenic cross-reactivity among *Anisakis, Ascaris* and *Toxocara* species. Therefore, identification of these molecules may provide a basis for designing novel diagnostic and therapeutic strategies for controlling allergic immune responses. The existence of taxonomically related epitopes of proteins between nematodes species has been suggested by (Sakanari *et al*., 1988). In fact, extensive similarity between both somatic and excretory-secretory antigens of *A. simplex* and other ascaridoid nematodes mainly *A. suum*, *A. lumbricoides* and *T. canis* has been reported (Kennedy *et al*., 1988). High antigenic cross-reactivity between *A. simplex* and five other nematodes (ascaridoids *Ascaris suum*, *Toxocara canis*, *Hysterothylacium aduncum*, nonascaridoids *Trichinella spiralis* and *Trichuris muris*) has also indicated, confirming that there is extensive antigenic similarity within this group of nematode parasites (Iglesias *et al*., 1996). Furthermore, it has been shown that cross-reactivity among somatic and excretory-secretory antigens of larval three (L3) was high among anisakid (*Anisakis simplex*, *Hysterothylacium aduncum* and *H. fabri*), ascarids (*Ascaris lumbricoides* and *A. suum*) and toxocara (*Toxocara canis*). Lillywhite *et al*. (1991) showed the presence of cross reactivity amongst *T. trichiura* and *A. lumbricoides* and *T. canis* when IgG, IgE, and IgM antibodies were measured. (Rodero *et al*., 2005) showed the presence of immunological cross reactivity amongst *T. canis*, A. *lumbricoides* and *A. simplex* antigens.

2.8.4 Antigenic and allergenic cross-reactions between *A. simplex (s.s.)* and non-nematode environmental, food allergens or transmissible agents

Recently, several clinical trials have demonstrated cross-reactions between acarida nematodes and environmental allergens including other parasites, crustaceans, mollusc, insects, mites, mouse, chicken and human (Frenkel *et al*., 1989, Jenkins *et al*., 1998, Asturias *et al*., 2002, DeWitt *et al*., 2004, Santos *et al*., 2008). These reactions might be due to crude or complex antigen mixtures containing carbohydrates, which have antigenic determinants that are widespread in nature (Johansson *et al*., 2001, Pascual *et al*., 1997). According to molecular features of cross-reactivity among acarida and environmental allergens, there are different levels of similarity between them as mentioned in Table (1). For example; DeWitt *et al*. (2004) showed cross reactivity between nematode (*Caenorhabditis elegans*) and trematode (*Schistosoma haematobium*) and 57% similarity was observed between them. Asturias *et al*. (2000) revealed the existence of cross reactivity among anisakid (*Anisakis simplex*), shrimp, snail, insect, human, chicken and mouse. Use of the BLAST program revealed that *Anisakis simplex* tropomyosin has higher identity to tropomyosins from other invertebrates, crustaceans, insects and mollusks, than tropomyosins from vertebrates (human, chicken and mouse). Asturias *et al*. (2000) and Santos *et al*. (2008) also identified tropomyosin as a cross-reactive allergen and it has high sequence identity to tropomyosin of other invertebrates. Pascual *et al*. (1997a) and Pascual *et al*. (1997b) showed serum samples collected from 60 children with specific IgE to *A. simplex* responded to *Blatella germanica* (German cockroach) and *Chironomus* spp (red mosquito larvae). In addition, immunoblot of *Anisakis* inhibited with *Chironomus* and *B. germanica* yielded a partial blot inhibition on bands below 41 KDa.

2.9 The Life Cycle of *Anisakis species*

Anisakis spp. belong to the subfamily Anisakinae, family Anisakidae, superfamily Ascaroidea, suborder Ascaridina, order Ascarida, subclass Secernentrea and Class Nematoda (Smith and Wootten, 1978). The *Ascaris* are common parasites of the human gastrointestinal tract (Blaxter *et al*., 1998, Nielsen, 1998). As a genus, *Anisakis* is found world-wide, but *Anisakis* species are differentially distributed geographically and comprise of different species (Mattiucci and Nascetti, 2006, Paggi *et al*., 2001, Mattiucci *et al*., 1997b). Based on nuclear data sets from allozymes and mtDNA cox2 sequence analysis,

Anisakis include two main clades (Mattiucci *et al*., 2008a, Mattiucci *et al*., 2005, Mattiucci and Nascetti, 2006a, Valentini *et al*., 2006b).

The first clade includes the species of the *A. simplex* complex [*A. simplex s.s*, *A. pegreffii* and *A. simplex* C], *A. typica*, *A. ziphidarum* and *Anisakis* sp. The second includes the species *A. physeteris*, *A. brevispiculata* and *A. paggiae* (Mattiucci *et al*., 2005, Valentini *et al*., 2006a). The other gerera belonging to the subfamily Anisakinae, are collectively known as anisakids and are termed Pseudoterranova, Contracaecum and Hysterothylacium. All these nematodes appear to have similar life-cycles (Fig. 1.4), although their host species vary (Ishikura *et al*., 1993, Audicana *et al*., 2002b, Mattiucci *et al*., 1997).

It has been known that the primary/definitive hosts of anisakids are aquatic mammals, piscivorous birds, aquatic reptiles, or fish (Anderson, 1992). Adult *Anisakis* spp are found in the alimentary tract (particularly the stomach), mainly in sea mammals (cetaceans) such as whales, dolphins and porpoises, while adult *Pseudoterranova* spp. utilize pinnipeds (seals, sea lions, and walrus) as primary/definitive hosts (Anderson, 1992). Eggs are passed into the sea via the faeces of adult cetaceans. The first moult (L1 to L2) takes place inside the egg, releasing free-swimming larvae that are ingested by tiny crustaceans such as krill (e.g. Euphasia, Tysanoessa), the first intermediate hosts (Audicana *et al*., 2002a, Smith and Wootten, 1978, Koie *et al*., 1995). The crustaceans in turn are consumed by second intermediate hosts, which are a variety of fish species and large crustaceans such as squid or cephalopods. Inside these hosts, the larvae moult into third-stage larvae (L3) and become encapsulated on the surfaces of organs or muscles. Larger fish may become infected by eating smaller fish and large crustaceans containing L3 larvae, which enter the intestine and infect the tissues, where they may increase with the age of the fish (Anderson, 1992).

Humans become accidental hosts by eating raw or undercooked infested fish or seafood with L3 that moult into forth-stage larvae, but do not progress into adults in human; other accidental hosts include bears, otters and cats (Torres *et al*., 2004, Sohn and Chai, 2005, Davey, 1971). All types of L3-infected seafood can cause gastrointestinal diseases known as anisakiasis and severe allergic reactions mediated by IgE antibody. In the natural cycle, the third-stage larvae in fish are ingested by cetaceans and moult into forth-stage larvae and then into adults. They cluster inside the stomachs of the cetaceans, where the female adult worms are fertilized and lay eggs, completing the cycle. *Pseudoterranova* spp. is more

likely to moult into fourth-stage larvae than *Anisakis* spp. In a rare case, an adult male worm of *Pseudoterranova* spp was found in a patient (Kliks, 1983a).

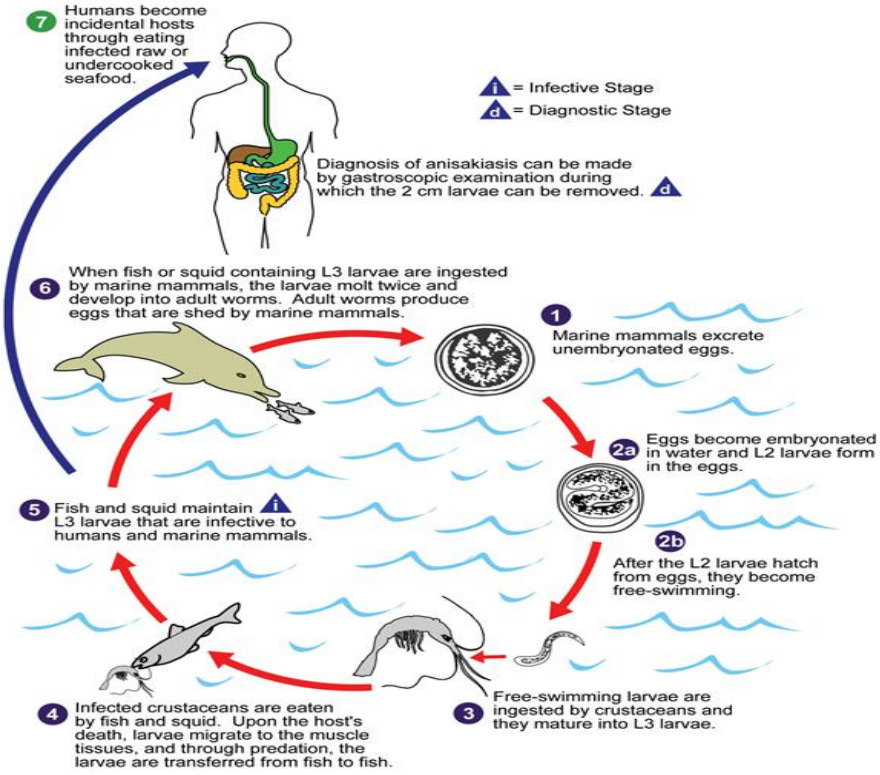


Figure 2.3 Life cycle of Anisakis simplex

Source: http://www.fepn.net/styled-11/styled-13/index.html

2.10 Genetics of parasite infections

2.10.1 Significance of parasites and studying genetic variation

Parasites represent a diverse range of invertebrate organisms, including species of protozoans, helminths or worms and arthropods (Taliaferro, 2009), and many cause significant diseases and major socio-economic losses globally (Roeber *et al.*, 2013). Parasitic infections influence human and animal health through clinical and subclinical disease (Randall and Gibbs, 1981). The control and prevention of parasitic disease depends on an adequate knowledge of interactions among factors such as genetic variation, life cycles of parasites, human behaviour and environment (Mata, 1982). Since then many studies have been done on complete sequencing of genetic variation in parasites and genetic analysis that provide a significant amount of valuable information in many areas such as parasite systematics (= taxonomy and phylogeny), diagnosis, gene expression and function, taxonomy, genetic structure of parasite population and epidemiology (Gasser and Newton,

2000, Gasser and NB, 2001, McManus and Bowles, 1996, Nadler, 1990, Nadler, 1995a, Nadler, 1995b). In order to identify the structure of the parasite genome, many new and various molecular approaches have been developed to understand how parasites are genetically related to each other. The variation within and between species, as well as changes in gene frequencies at the population or species level over time (evolution) is studied. Four major processes lead to microevolution: mutation, genetic drift, selection, and gene flow which cause changes in allele frequencies over time (genetic divergence), and generates adaptation of parasite to their environments and resistance genes to anti-parasitic drugs (Hufbauer and Roderick, 2005). Since the rate of sequence evolution varies extensively with gene or DNA segment, the evolutionary relationships of virtually all levels of classification of organisms (kingdoms, phyla, classes, families, genera, species, and intraspecific populations) could be studied. Therefore, phylogenetic analysis has become an important tool for providing information about the evolutionary process and evolution time history of parasite and depends on accurate analysis of genetic variation in specific DNA regions within and among taxa.

2.10.2 DNA regions used for studying genetic variation in parasites

Regions of genomes of organisms, over evolutionary time, accumulate mutations, but the rate of evolution may vary in sequence within and among species, or multicopy gene may vary within a single gene (McManus and Bowles, 1996). DNA sequence variation within and among species has been identified in many important parasite genes (McManus and Bowles, 1996, Grant, 1994). In addition, McManus *et al*. (1996) also stated that introns and non-coding regions would generally evolve more rapidly than coding regions as they are unlikely to be highly constrained by function. The lack of functionality in non-coding DNA allows significant variation between closely related species and even within species (Zeyl *et al*., 1996). Various DNA regions of parasites have been employed to analyse genetic variation within and among species, including those of the mitochondrial genome, and repetitive elements and ribosomal DNA (rDNA) of the nuclear genome.

DNA sequencing has been applied to amplify both nuclear (Roos and Grant, 1993 , Christensen *et al*., 1994a, Christensen *et al*., 1994b) and mitochondrial genomes (Blouin *et al*., 1992, Blouin *et al*., 1995, Blouin *et al*., 1998, Hu *et al*., 2003a, Hu *et al*., 2002, Hu *et al*., 2003b) for species identification and/or population genetic studies. Although the vast

majority of genetic information is contained in the nucleus, there are important functions encoded by DNA in mitochondria. Since the genomes of DNA in mitochondria are much less complex than nuclear genomes, restriction endonuclease digestion of the former generally yields fragments that are easily resolved and thus more amenable to the study of sequence variation (Barry *et al*., 1986).

2.10.2.1 Ribosomal DNA (rDNA)

In most eukaryotes, nuclear ribosomal RNA genes are known as ribosomal DNA (rDNA). Ribosomal DNA is found as a multigene family consisting of tandemly repeat units that are arranged in arrays of three of the four genes interspersed encoding nuclear ribosomal RNA (rRNA), located at the chromosomal sites known as nuclear organizing regions (NORs) (Long and Dawid, 1980, Prokopowich *et al*., 2003). Each repeat unit consists of a transcribed region (having genes for 18S, 5.8S and 28S rRNAs and the external transcribed spacers i.e. ETS1 and ETS2) and a non-transcribed spacer (NTS) region (Pereira *et al*., 2010). The 18S, 5.8S and 28S genes code for ribosomal RNA (rRNA) and are usually conserved within taxa. The external transcribed spacer (ETS) is located upstream of the 18S gene, while the first and second internal transcribed spacers (ITS-1 and ITS-2) are located among the 18S and 5.8S and 28S genes, respectively (Hillis and Dixon, 1991). The transcribed spacers are excised from rRNA during formation of the mature ribosome and typically exhibit higher DNA sequence variability than the coding regions (Hillis and Dixon, 1991). An advantage of studying repetitive genes, such as rDNA, is that they are abundant in the organism and therefore it permits specific detection and amplification by PCR from minute nanogram to pictogram quantities of parasite material (Gasser *et al*., 1993). Also, PCR amplification is easy with universal primers which can be designed for conserved regions flanking more variable regions, such as the internal transcribed spacer (ITS), and used for various kind of organisms without prior knowledge of their sequences (Poczai and Hyvönen, 2010). Therefore, Ribosomal DNA has been widely studied in different parasite species and used to investigate a variety of purposes, including evolutionary analysis, biogeographic investigations, diagnosis of parasitic disease and systematics (taxonomy and molecular phylogeny) (McManus and Bowles, 1996, Chilton *et al*., 1997, Gasser and Newton, 2000).

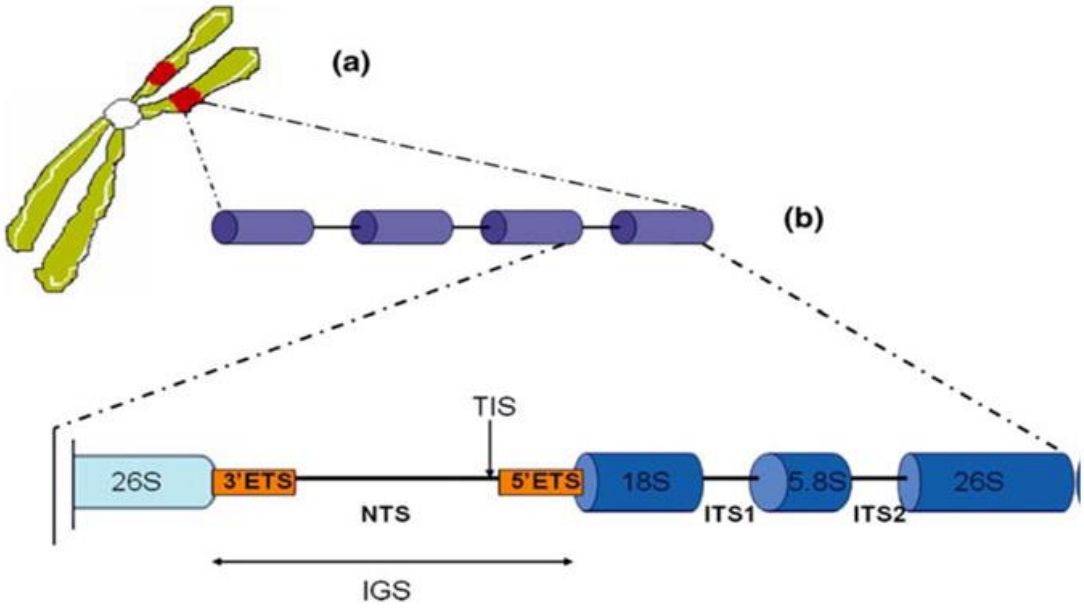


Figure 2.4 Structure of Ribosomal DNA in parasites

Source: Poczai and Hyvonen, 2010

2.10.2.2 Mitochondrial DNA

The characteristics of mitochondrial genomes vary extensively in size and gene content (from minute genomes to 20 times as many genes) across diverse parasites groups (Feagin, 2000). A typical nematode mtDNA genome is usually small (13–26 kb), circular, compact, maternally inherited, and evolves independently of the nuclear genome (Brown, 1985). They contain 12–13 protein genes (cox1–cox3, nad1–nad6, nad4L, cob, atp6 and/or atp8) that encode enzymes required for oxidative phosphorylation, two ribosomal (r) RNA genes (rrnS and rrnL) encoding the RNA components of the mt ribosome, and 22 transfer (t) RNA genes required for translation of the different mt proteins (Hu and Gasser, 2006). Recently mitochondrial genes have been extensively used to investigate the molecular evolution, phylogeny, biogeography, systematics and population genetics of parasite species and showed that mtDNA cox1 sequences are useful genetic markers to identify variation within parasite species (Bowles *et al*., 1992, Zhang *et al*., 1998, Bøgh *et al*., 1999) and between species (Black and Piesman, 1994, Bowles *et al*., 1995a , Bowles *et al*., 1995b , Blouin *et al*., 1998, Morgan and Blair, 1998). The presence of intraspecific variation in mitochondrial DNA sequences has meant that they are particularly amenable to investigation to detect

population variation using molecular approaches, including DNA sequencing (Gasser *et al*., 1998c, Bøgh *et al*., 1999).

2.10.3 Genetic variation of rDNA on the internal transcribed spacers (ITS) of nematodes

Due to the limited morphological features suitable for taxonomic identification and the frequent difficulty in assessing complete life cycles or knowledge of alternative hosts, genetic analysis have been applied as powerful tools to address some of these questions (Dorris *et al*., 1999, Gasser, 2001, Gasser, 2006a, Jones *et al*., 2012). Many parasite groups have been genetically identified using the first or second internal transcribed spacers (ITS) of nuclear ribosomal DNA (rDNA) (Gasser et al., 1996a, *Bott et al.*, 2009). Nematodes are among the most abundant group of parasites, with over 20,000 species currently described, of which at least one-third are found in vertebrates (Anderson, 1984, Anderson, 2000).

The first and/or second internal transcribed spacers (ITS-1 and/or ITS-2) of rDNA of ascaridoid nematodes have been amplified to provide useful genetic markers for genotypic identification, because they have been shown to be considerably more variable in sequence within a species.

2.10.4 Morphological and molecular approaches

2.10.4.1 Morphology-based approaches

Marine nematodes infesting fish have been identified only as L3 larvae of the genus Anisakis by a light microscope (Oshima, 1972) and scanning electron microscopic (Roongruangchai *et al*., 2012). The foremost problem of these methods is be able to describe distinct structural traits and morphological characterizations such as excretory system, number and distribution of caudal papillae, are often applicable only to adults (Fagerholm, 1991). Therefore, it's difficult to identifyAnisakis L3 e in fishes, especially between *A. simplex* (*sensu stricto* (*s.s*)) (Rudolphi, 1809) and *A. pegreffii* Campana Rouget and Biocca, 1955 (Quiazon *et al*., 2008). Instead, molecular genetic techniques have been successfully used to identify each larva species.

2.10.4.2 Molecular methods for studying genetic variation in parasites

Recently, several methods have been used for identification of different species of nematode such as Multilocus enzyme electrophoresis (MEE) or allozyme electrophoresis (Nadler, 1990, Andrews and Chilton, 1999) and DNA-based techniques (McManus and Bowles, 1996, Gasser, 1999 , Gasser and Chilton, 2001, Gasser and Newton, 2000). MEE technique can only be applied to live or frozen specimens, but not those fixed in alcohol, while genomic DNA methods can be used for both frozen or alcohol-fixed nematodes (Gasser *et al*., 1993). In addition, DNA-based approaches are better as DNA are more easily isolated, extracted, detected and sensitively amplified by the polymerase chain reaction (PCR) (Gasser *et al*., 1993). DNA is also more stable than protein (McManus and Bowles, 1996).

2.10.4.2.1 Protein-based approaches

Protein electrophoresis (e.g. multilocus enzyme electrophoresis, MEE) is a common molecular approach to examine proteins translated from coding genes. It detects variation among alleles by examining the movement of proteins through an electrical field and has been widely applied to the study of parasites over the last thirty years (Andrews and Chilton, 1999). However, this technique requires that specimens be compared on the gels at the same time (or using appropriate mobility controls) and requires live material that has been frozen on collection (Andrews and Chilton, 1999).

2.10.4.2.2 DNA-based approaches

DNA has become an increasingly popular target for studies of genetic variability, particularly because DNA is less susceptible to degradation during collection and storage of parasites than protein. There is a wide range of techniques that are applicable to DNA, and these offer greater sensitivity in the detection of genetic variation.

2.10.4.2.3 PCR-based approaches (Genetic analysis of PCR products)

Once amplification of target DNA product is generated by PCR, genetic variability of inter- or intra-population of organisms can be identified and characterized from DNA sequence analysis using different methods such as; enzyme electrophoresis (Lewontin and Hubby, 1966), restriction fragment length polymorphism (RFLP) analysis (Sambrook *et al*., 1989), random amplified polymorphic DNA (RAPD) analysis (Williams *et al*., 1990), denaturing gradient gel electrophoresis (DGGE) analysis (Fischer and Lerman, 1983), single-strand conformation polymorphism (SSCP) analysis (Orita et al., 1989, Fujita and Silver, 1994),

amplified fragment length polymorphism (AFLP) analysis or direct sequencing and random PCR approaches, but since then a number of new protocols have been widely applied in population genetic studies, such as minisatellite (Jeffreys *et al*., 1985) and microsatellite (Litt and Luty, 1989, Dietrich *et al*., 1996).

The method of choice depends very much on whether the focus of the study is nuclear or mitochondrial and inter- or intra-population variability.

2.10.4.2.3.1 PCR-SSCP analysis

The polymerase chain reaction-linked single-strand conformation polymorphism technique (PCR-SSCP) has been applied to overcome limitations of the morphological identification of parasites at different developmental stages to species (Gasser and Monti, 1997). SSCP has significant advantages than other nucleic acid techniques for the accurate analysis of allelic and mutational sequence variation. In addition, it is simple, low-cost, time efficient, has the potential to detect point mutations for DNA fragments sizes of up to 450–500 bp and also able to discriminate difference in sequence from large numbers of samples by a single base during 1–2 days (Sunnucks, 2000, Gasser, 2006b). Briefly, the SSCP analysis method includes serial steps starting from isolation of genomic DNA and PCR amplification of target sequences, through to the gel-based separation of amplicons and scanning for mutations by SSCP (Gasser, 2006b). SSCP has demonstrated utility to study a wide range of pathogens and diseases, and has been extensively applied in biomedical research to investigate different genes of many organisms (Gasser, 2006b).

2.10.5 Genetic variation in Anisakis spp. (Anisakidae family)

Anisakidae (Skrjabin and Karokhin, 1945) is a major family of parasitic roundworms in the superfamily Ascaridoidea (Railliet & Henry, 1915). This family contains a wide range of species including; *Anisakis* (Dujardin, 1845), *Contracaecum* (Railliet and Henry, 1912) and *Pseudoterranova* (Mozgovoi, 1950), among the most reported as larvae in fishes (Klimpel and Palm, 2001, Anderson, 2000). Adult Anisakidae parasitize aquatic mammals (dolphins, sea lions, and whales), piscivorous birds and aquatic reptiles as the definitive host, while the third larval stages (L3) of *Anisakis*, *Pseudoterranova*, and *Contracaectim* parasitize marine fishes and squid as intermediate host and they have been implicated in severe clinical disease in humans, when they consume raw or undercooked contaminated fish (Sakanari and McKerrow, 1989, Smith, 1999).

Traditionally, individual Anisakid adults and larvae (third-stage) are identified and distinguished on the basis of morphological features, the host they infect, their pathological effect(s) on the host or/and their geographical origin (Gasser, 2006b). However, these criteria are sometimes insufficient for an accurate identification, due to the lack of different morphological characters; therefore the taxonomy, epidemiology and ecology of anisakid nematodes has been redefined using phylogenetic methods to improve their population genetics (Shamsi *et al*., 2008, Shamsi *et al*., 2009a, Shamsi *et al*., 2009b). The basis for investigating population structures is the accurate analysis of genetic variation, which is known to be widespread in many parasitic nematodes, utilizing molecular markers with sufficient levels of intraspecific sequence variability. Accurate identification of genetic variation between interspecies and intraspecies are potential molecular control targets for the development of improved diagnostic keys and phylogenetic trees (Bindroo and Moorthy, 2014).

During the last three decades, population genetics and phylogenetic studies have been extensively investigated in Anisakid nematodes based on nucleotide polymorphism of the nuclear or mitochondrial genome.

2.11 Anisakid nematodes in Australia

In Australia, various species of anisakid nematodes have been reported and described and infest a broad variety of animals such as birds (Mawson *et al*., 1986), reptiles (Glazebrook and Campbell, 1990), and marine fish (Shamsi and Butcher, 2011). As shown in figure 15, Australian anisakids are known principally from early works by Johnston and Mawson (Johnston, 1910, Johnston, 1913, Johnston, 1937) and (Johnston and Mawson, 1939, Johnston and Mawson, 1940a, Johnston and Mawson, 1940b, Johnston and Mawson, 1941a, Johnston and Mawson, 1942b, Johnston and Mawson, 1942c, Johnston and Mawson, 1942d, Johnston and Mawson, 1943a, Johnston and Mawson, 1943b, Johnston and Mawson, 1944, Johnston and Mawson, 1945a, Johnston and Mawson, 1945b, Johnston and Mawson, 1947a, Johnston and Mawson, 1949, Johnston and Mawson, 1951a, Johnston and Mawson, 1952, Johnston and Mawson, 1953) and (Mawson, 1953, Mawson, 1957, Mawson, 1969), but there is still a scarcity of knowledge and findings about their life cycle and health impacts of their larval and adult stages on human and animals.

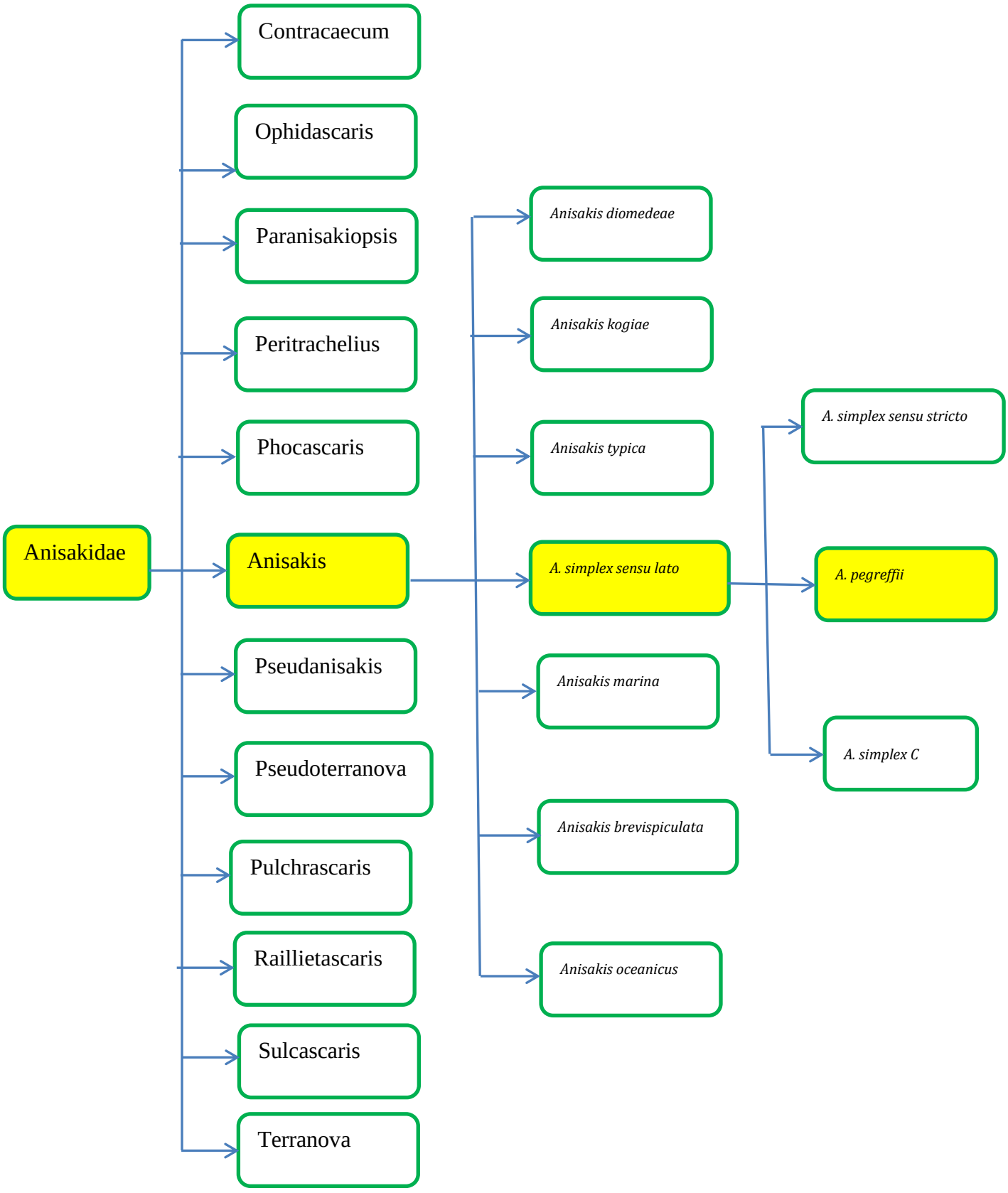


Figure 2.5 Summary of the current taxonomic status of anisakid nematodes reported in Australia.

Source: (This study)

2.12 *Anisakis pegreffii*

Anisakis larvae present major problems for commercial fishing industries (Mattiucci *et al*., 1997, Mattiucci and Nascetti, 2008) and are also identified as potential human health threats, both as causative agents of anisakiasis and as potential food-borne pathogenic allergens (D'Amelio *et al*., 1999, Umehara *et al*., 2007b, Mattiucci and Nascetti, 2007, Mattiucci *et al*., 2011, Fumarola *et al*., 2009, Kirstein *et al*., 2010). The third stage (L3) larva of *Anisakis simplex* belongs to *Anisakis* Type I. Previously, *Anisakis simplex* was considered to be the prime reason for Anisakiasis. However, recent studies on molecular genetics revealed that *Anisakis simplex* is composed of three siblings *A. simplex s.s* (Rudolphi, 1809) (Nascetti *et al*., 1986), *A. pegreffii* (Compana Rouget and Bioca, 1954) and *A. simplex C* (Mattiucci and Nascetti, 2007, Mattiucci *et al*., 2002, Nascetti *et al*., 1986). *A. pegreffii* and *A. simplex s.s* are widely distributed around the globe and can be characterised on the basis of their differences in terms of morphological and genetic structure as well as ecological traits, such as geographic distributions and definitive host preference. Both *A. pegreffii* and *A. simplex s.s* are common parasites of marine mammals worldwide and have been identified as the principal aetiologic agent of Anisakiasis (Abollo et al., 2003, Mattiucci et al., 1997, Mattiucci and Nascetti, 2008, Mattiucci and Nascetti, 2006b, Mattiucci *et al*., 2009).

2.12.1 Morphological characterization

Generally, L3, L4 and adult stages of *Anisakis* species are morphologically distinguishable based on morphological criteria (total body length, maximum body width, distance of nerve ring to anterior end, oesophagus length, ventriculus length, ventriculus width, ratio between oesophagus and ventriculus length, tail length and mucro length) as shown in Table 1 (Quiazon *et al*., 2008, Shamsi *et al*., 2011b, Shamsi *et al*., 2012). The *Anisakis* spp were divided into 4 types: Type I, Type II, and 2 other types that were similar to *Anisakis* Type III and Type IV described by (Shiraki, 1974). *Anisakis* Type II, Type III, and Type IV larvae all had a short ventriculus, but their tails are morphologically different (Murata *et al*., 2011). L3 larva of *Anisakis* Type I can be distinguished from Type II in the following manner: *Anisakis* Type I has the boring tooth at the anterior end, longer ventriculus joining obliquely with the intestine, oesophagous, three anal glands encircle the rectum tail which is short and rounded, ending with a distinct mucron and the blunt posterior at the end while

Anisakis Type II has also oesophagous, intestine, short ventriculus but has no mucron (Koyama, 1974, Shamsi *et al*., 2011b).

L3 of A. *pegreffii* Type I are usually found encysted in a coiled, spring-like state on the intestines, stomach, gonads and rarely in the liver of intermediate hosts, which are a variety of fish species and large crustaceans such as squid or cephalopods (Shamsi *et al*., 2011b, Quiazon *et al*., 2008). Moreover, the development of the L3 larvae of *A. pegreffii* to the adult stage is highly dependent on the distribution pattern of primary/definitive host (marine mammals) (Shamsi *et al*., 2012). However, it is difficult to classify *Anisakis* Type I based on only morphological characterization. Therefore, the usage of molecular genetic methods become indispensable in order to identity them (Abe *et al*., 2005, Valentini *et al*., 2006b, Umehara *et al*., 2006, Quiazon *et al*., 2008).

Table 2.7 Measurements of some morphological characters in A. pegreffii at different life-history stages

Morphological characterization	Fish species Tiger flathead (Shamsi *et al*., 2011b)	Fish species Alaska Pollock, Greater amberjack, Alaska Pollock, Japanese Spanish mackerel and Pacific cod (Quiazon *et al*. 2008)			
	L3 larvae (n= 53)	L3 larvae (n= 71)	L4 larvae (n= 4)	Adult Male (n= 11)	Adult Female (n= 10)
Body length	21.74 mm (14.99–27.12)	11.10–26.7	11.20–17.76	14.30–20.55	15.90–29.01
Body width	0.48 mm (0.39–0.59)	0.38–0.60	0.38–0.79	0.68–1.03	0.72–1.15
Muscular oesophagus	2.16 mm (1.67–2.66)	1.04–2.11	1.55–1.85	2.00–2.95	2.27–3.07
Ventriculus length	0.84 mm (0.36–1.30)	0.50–0.78	0.50–0.60	0.50–0.70	0.60–0.70
Ventriculus width	-	0.12–0.27	0.18–0.22	0.21–0.24	0.25–0.27
Distance of nerve ring to the anterior end	0.31 mm (0.08–0.37)	0.20–0.31	0.27–0.29	0.27–0.34	0.34–0.36
Distance between anus and posterior end	0.12 mm (0.08–0.15)	-	-	-	-
Ratio between oesophagus and ventriculus length	-	1:1.5–1:3.1	1:3.08–1:3.1	1:3.1–1:4.2	1:3.5–1:4.7
Tail length	-	0.05–0.1	0.12–0.14	0.14	0.17–0.21
Mucro length	-	0.02–0.03	Absent	Absent	Absent

2.12.2 Molecular characterization

Many techniques have been successfully applied for the identification and differentiation of ascaridoid nematodes, including random amplified polymorphic DNA (RAPD) (Martin-Sanchez *et al*., 2005), PCR single stranded conformational polymorphism (PCR-SSCP) (Zhang *et al*., 2007, Zhu *et al*., 2007, Gasser, 1999 , Hoste *et al*., 1998, Hung *et al*., 1996, Hung *et al*., 1997), PCR restriction fragment length polymorphism (PCR-RFLP) (Abe *et al*., 2006, Kijewska *et al*., 2002, Szostakowska *et al*., 2002, D'Amelio *et al*., 2000), and multiplex or specific PCR assay (Chen *et al*., 2008, Umehara *et al*., 2007a). Using those techniques derived from PCR to analyze DNA sequences, different suitable genes identified

were mitochondrial gene (cytochrome c oxidase subunit I [cox1], II [cox2]) and nuclear genes (internal transcribed spacer [ITS] region, including ITS1, 5.8S, and ITS2 of rDNA). These genes were further analyzed to identify the species of Anisakid nematodes (D'Amelio *et al*., 2000, Martin-Sanchez *et al*., 2005, Pontes *et al*., 2005).

Based on those tools and methods, the *Anisakis* genus has been identified as two clades. Clade I includes larvae of *Anisakis* type I (sensu Berland, 1961) with six species, three of which constitute the *A. simplex sensu lato* (*s.l*). complex (*A. simplex s.s.*, *A. pegreffii* and *A. simplex C*), *A. typica* and *A. ziphidarum* (Mattiucci and Nascetti, 2008), and *A. nascettii* (Mattiucci *et al*., 2009). Clade 2 includes larvae of *Anisakis* type II (sensu Berland, 1961) with three species; *Physeteris, A. brevispiculata* (Mattiucci *et al*., 2001), and *A. paggiae* (Mattiucci *et al*., 2005). *A. pegreffii* has been accurately identified using morphological and molecular analyses which included several genetic techniques and sequencing specific regions of informative genes as mentioned in Table 2.10.

Table 2.8 Distribution of *A. pegreffii* in different marine and fish species and identified by using genetic markers

Species/genotype (Taxa)	Larval stage	Host species	Location	Amplified region	Accession number	Reference
A. pegreffii	Adult	Delphinus delphis and Tursiops truncates	Australia	ITS-1 and ITS-2	FN391850–2, FN556997 and FR849715–7	Shamsi *et al*., 2011
	L4	Delphinus delphis	Australia	ITS-1 and ITS-2	FN391853–61 and	Shamsi *et al*., 2012
	L3		Poland	ITS-1,5.8S, ITS-2	AY603531	Kijewska *et al*.,2004 (unpublished)
	L3	*Trachurus trachurus*, Lepidopus caudatus and Engraulis encrasicolus	Italy	ITS-1,5.8S, ITS-2		Cavallero *et al*., 2012
	L3	Micromesistius poutassou	Italy	ITS-1,5.8S, ITS-2	AY826720	Nadler *et al*., 2005
	L3	Scomber japonicas	Japan	ITS-1,5.8S, ITS-2	AB277823	Umehara *et al*., 2006
	L3	Seriola dumerili	Japan	ITS-1,5.8S, ITS-2	AB246367	Yoshinaga *et al*., 2006
	L3	Astroconger myriaster,	China	ITS-1,5.8S, ITS-2	AB246367	Zhang *et al*., 2007
	L3	Clupea pallasi, Coryphaena		ITS-1,5.8S, ITS-2	AB246367	Zhang *et al*., 2007

	hippurus,				
L3	Lophius titulon, Mugil cephalus,		ITS-1,5.8S, ITS-2	AB246367	Zhang *et al*., 2007
L3	Pneumatophorus japonicus,		ITS-1,5.8S, ITS-2	AB246367	Zhang *et al*., 2007
L3	Scomberomorus niphonius		ITS-1,5.8S, ITS-2	AB246367	Zhang *et al*., 2007
L3	and Sebastiscus marmoratus		ITS-1,5.8S, ITS-2	AB246367	Zhang *et al*., 2007
L3	Neoplatycephalus richardsoni	Australia	ITS-2	FN556176	Shamsi *et al*., 2011
L3	Neoplatycephalus richardsoni	Australia	ITS-2	FN556177	Shamsi *et al*., 2011
L3	Neoplatycephalus richardsoni	Australia	ITS-2	FN556178	Shamsi *et al*., 2011
L3	*Theragra chalcogramma*	Japan	ITS-1,5.8S, ITS-2	EU624343 and EU624343	Quiazon *et al.*, 2009
L3	*Trachyrincus scabrous*	Morocco and Mauretania	ITS-1,5.8S,ITS-2	EU718479 and EU718479	Kijewska *et al.*, 2009
L3	Scomber japonicas	Japan	18S–28S	AB277823 and AB277823	Umehara *et al*., 2008
L3	Astroconger myriaster	China	ITS-2	AM706346 and AM706346	Zhu *et al*., 2007
L3	Gadus macrocephalus	Japan	ITS-1,5.8S, ITS-2	AB196670 and AB196670	Abe *et al*., 2005
L3	G. macrocephalus	Japan	ITS-1,5.8S,ITS-2	AB196671 and AB196671	Abe *et al*., 2005
L3	S. japonicus, S. australasicus, Arctoscopus japonicus, Takifugu poecilonotus,	Japan		HQ717730 and HQ717730	Quiazon *et al*., 2011
L3		Japan	mtDNA *cox*-2	DQ116428	Valentini *et al*., 2006
L3	*S. japonicus*	Japan	mtDNA *cox*-2	EU413958	Quiazon *et al*., 2008
L3	Scomber japonicas	Japan	mtDNA cox1		Umehara *et al*., 2006
L3	Theragra chalcogramma	Japan	mtDNA *cox*-2	EU624343 and EU624343	Quiazon *et al*., 2009

2.12.3 Prevalence of *A. pegreffii*

2.12.3.1 Fish and marine mammals

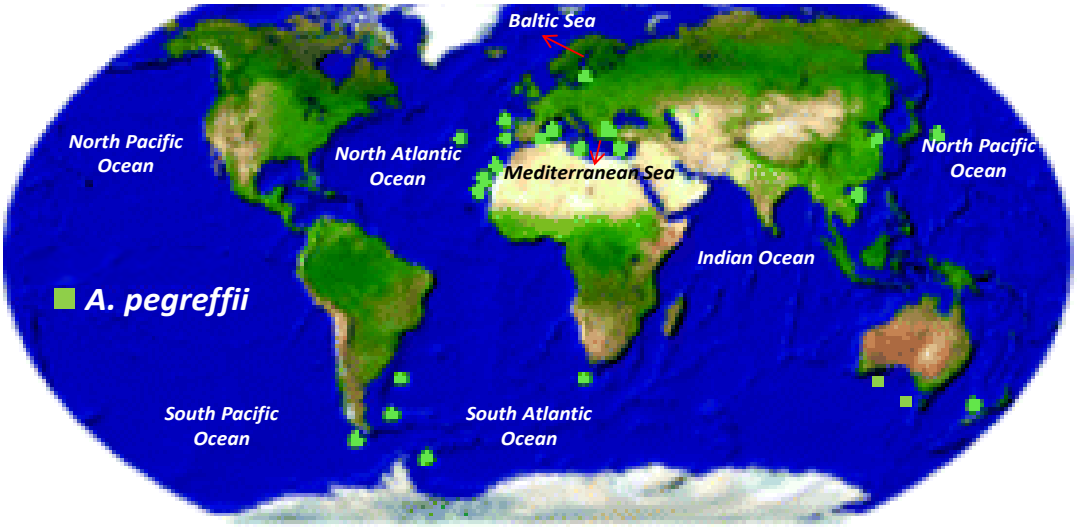


Figure 2.6 Distribution of *A. pegreffii* worldwide

Source: Klimpel and Palm, 2011

The presence of *Anisakis* sp. larvae infection varies with host species and their geographical location (Figure 4), and information presented in Table (2.8) provides information on the host species, and therefore the potential risk of human anisakiasis.

Table 2.9 Collecting data and frequency (%) of *A. pegreffii* (L3) identified by rDNA genetic markers in different hosts and localities.

Geographical location	Host species	*Np*	*Na*	*A. pegreffii* (%)	References
Korean sea	*Scomber japonicas*	34	32	94.1	Lee *et al*., 2009
Korean sea	*Trachurus pacificus*	5	2	40	Lee *et al*.,2009
Korean sea	*Trachurus lepturus*	21	13	61.9	Lee *et al*., 2009
Portugal	*Scomber japonicas*	34	7	20.6	Pontes *et al*., 2005
Portugal	*Trachurus picturatus*	31	8	25.9	Pontes *et al*., 2005
Portugal	*Aphanopus carbo*	48	6	12.5	Pontes *et al*., 2005
Kyushu	*Scomber japonicas*	11	10	91	Umehara *et al*., 2008
Fukuoka prefecture	*Scomber japonicas*	38	37	97	Umehara *et al*., 2006
Kagoshima Prefecture	*Seriola dumerilie*	10	9	90	Quiazon *et al*., 2008
Kyoto Prefecture	*Scomberomorus niphonius*	6	6	100	Quiazon *et al*., 2008
Kumamoto Prefecture	*Scomber japonicas*	67	66	98	Quiazon *et al*., 2008
Sea of Japan	*Scomber japonicas*	4073	3380.6	83	Suzuki *et al*., 2010
Greece	*Illex coindetii*	13	13	100	Abollo *et al*., 2003
Argentina and Falkland islands	*Illex argentines*	19	19	100	Abollo *et al*., 2003
Melbourne and Lakes Entrance, and South Australia	*Neoplatycephalus richardsoni*	53	4	28	Shamsi *et al*., 2011
South-eastern Australian waters	*Delphinus delphis*	2	2	100	Shamsi *et al*., 2012
South-eastern Australian waters	*Tursiops truncates*	1	1	100	Shamsi *et al*., 2012
Australia (Victoria)	*Sillago flindersi*	194	48	24	Abdul Jabbar *et al*., 2012
southern Western Australia	*Pseudocaranx dentex*	17	3	17.6	Abdul Jabbar *et al*., 2013
New Zealand	*Parapercis colias*	50	50	100	Mattiucci and Nascetti, 2007
New Zealand	*Pseudophycis bachus*	50	50	100	Mattiucci and Nascetti, 2007
Mediterranean Sea					
Tunisia	*Merluccius merluccius*	53	42	79.2	Farjallah *et al*., 2008
Tunisia	*Scomber scombrus*	87	68	78.1	Farjallah *et al*., 2008
Tunisia	*Trichiurus lepturus*	9	9	100	Farjallah *et al*., 2008
Tunisia	*Scomber japonicas*	5	5	100	Farjallah *et al*., 2008
Tunisia	*Lophius piscatorius*	9	9	100	Farjallah *et al*., 2008
Tunisia	*Micromesistius poutassou*	11	11	100	Farjallah *et al*., 2008
Tunisia	*Todarodes sagittatus*	13	13	100	Farjallah *et al*., 2008
Tunisia	*Muraena Helena*	14	14	100	Farjallah *et al*., 2008
Tunisia	*Phycis phycis*	9	3	33.3	Farjallah *et al*., 2008
Tunisia	*Phycis blennoides*	8	4	50	Farjallah *et al*., 2008
Tunisia	*Thunnus thynnus*	2	2	100	Farjallah *et al*., 2008
Tunisia	*Echiichthys vipera*	3	3	100	Farjallah *et al*., 2008
Tunisia	*Trachinus draco*	2	2	100	Farjallah *et al*., 2008
Tunisia	*Xiphias gladius*	2	2	100	Farjallah *et al*., 2008
Algeria	*Merluccius merluccius*	14	12	85.7	Farjallah *et al*., 2008
Libya	*Merluccius merluccius*	41	24	51.2	Farjallah *et al*., 2008
China (Yellow Sea)	*Astroconger myriaster*	18	4	22.2	Zhang *et al*., 2007
China (Yellow Sea)	*Clupea pallasi*	11	2	18	Zhang *et al*., 2007
China (Yellow Sea)	*Coryphaena hippurus*	9	3	33.3	Zhang *et al*., 2007
China (Yellow Sea)	*Lophius titulon*	41	3	7.3	Zhang *et al*., 2007
China (Yellow Sea)	*Mugil cephalus*	7	3	42	Zhang *et al*., 2007
China (Yellow Sea)	*Pneumatophorus japonicas*	30	2	6	Zhang *et al*., 2007
China (Yellow Sea)	*Scomberomorus niphonius*	52	3	5	Zhang *et al*., 2007
China (Yellow Sea)	*Sebastiscus marmoratus*	29	4	13	Zhang *et al*., 2007
Taiwan (coastal waters)	Taiwan (coastal waters)	5630	5630	93.6	Chou *et al*., 2011

Spanish Mediterranean Sea	*Micromesistius australis*	27	16	59	Martın-Sanchez *et al*., 2005
Atlantic coasts	*Micromesistius australis*	15	3	20	Martın-Sanchez *et al*., 2005
Eastern Mediterranean Sea					
Off Cyprus	*Merluccius merluccius*	22	0	0	Mattucci *et al*, 2004
Crete Sea	*Merluccius merluccius*	300	251	83.5	Mattucci *et al*, 2004
Aegean Sea	*Merluccius merluccius*	60	60	100	Mattucci *et al*, 2004
Ionian Sea	*Merluccius merluccius*	206	197	95.9	Mattucci *et al*, 2004
Adriatic Sea	*Merluccius merluccius*	150	150	100	Mattucci *et al*, 2004
Western Mediterranean Sea					
Tyrrhenian Sea	*Merluccius merluccius*	150	139	92.7	Mattucci *et al*, 2004
Ligurian Sea	*Merluccius merluccius*	150	140	93.3	Mattucci *et al*, 2004
Balearic Islands	*Merluccius merluccius*	40	6	14.3	Mattucci *et al*, 2004
Off Malaga	*Merluccius merluccius*	20	14	70	Mattucci *et al*, 2004
Off Alicante	*Merluccius merluccius*	20	4	23.1	Mattucci *et al*, 2004
North-east Atlantic Ocean					Mattucci *et al*, 2004
Atlantic coast of Morocco	*Merluccius merluccius*	202	124	61.3	Mattucci *et al*, 2004
Galician coast	*Merluccius merluccius*	200	11	10.5	Mattucci *et al*, 2004
Bay of Biscay	*Merluccius merluccius*	200	7	3.5	Mattucci *et al*, 2004
Great Sole Bank	*Merluccius merluccius*	232	49	21.4	Mattucci *et al*, 2004
Italian Tyrrhenian Sea and	*Trachurus trachurus*	76	73	96	Cavallero *et al*., 2012
Northwestern Mediterranean	*Lepidopus caudatus*	12	12	100	Cavallero *et al*., 2012
Northwestern Mediterranean	*Lampris guttatus*	28	0	0	Cavallero *et al*., 2012
Northwestern Mediterranean	*Engraulis encrasicolus*	61	23	37.7	Cavallero *et al*., 2012
Kyushu Japanese coast	*Scomber japonicas*	11	10	90	Umehara *et al*., 2008
Mainland Portugal	*Helicolenus dactylopterus*	363	339	93.5	Sequeira *et al*., 2010
Madeira Portugal	*Helicolenus dactylopterus*	60	6	10	Sequeira *et al*., 2010
Azores Portugal	*Helicolenus dactylopterus*	242	32	13.3	Sequeira *et al*., 2010
Northern Japan	*Gadus macrocephalus*	26	24	92	Abe *et al*., 2005
Central Mediterranean of southern Italy	*Caretta caretta*	38	10	26	Santoro *et al*., 2010

*N*p: number of parasites collected, *N*a: number of *A. pegreffii* examined

A. pegreffii has been characterized by different geographical distribution and definitive host preference. The relationship between the parasite and the host is generally adaptive (Audícana-Berasategui *et al*., 2007). Mattucci *et al*. (1997) discovered *A. pegreffii* for the first time in the digestive tract of a Mediterranean monk seal (*Monachus monachus*) from eastern Sardinia (Italy). Umehara *et al*. (2006) investigated the geographical distribution of *A. pegreffii* and *A. simplex sensu stricto* isolated from several hosts captured in Japanese waters. *A. pegreffii* was detected in 37 out of 38 (97%) *S. japonicas* caught in waters off Fukuoka prefecture. In contrast, *A. pegreffii* was not found in *S. japonicus* caught in the waters around Hokkaido (Japan). Pontes *et al*. (2005) detected *A. pegreffii* in seven out of

34 cases (20.6%) from *S. japonicus*, eight out of 31 (25.9%) from *Trachurus picturatus* and six out of 48 (12.5%) from *Aphanopus carbo* in Portugal.

Suzuki *et al*. (2010) identified *Anisakis* type I larvae in 74.3% (162/218) of the mackerels samples (fish), and 99.8% of the *Anisakis* type I larvae comprised of *A. pegreffii* and *A. simplex s.s*. The average number of *A. pegreffii* and *A. simplex sensu stricto* larvae per fish was 47 and 6, respectively. Zhang *et al*. (2007) collected 200 third-stage larvae (L3s) from eight different fish species at the Yellow Sea in China and then subjected them to morphological and molecular study. Larvae identified as *A. pegreffii* were (n /197) and *Hysterothylacium* sp. were (n/ 3). Choua *et al*. (2011) identified *A. pegreffii* and the recombinant genotype of *A. pegreffii* and *A. simplex s.s*., in the north eastern spotted mackerels fish with extremely different frequencies of 97% and 3%, respectively. While in the southwestern spotted mackerels samples, *Anisakis pegreffii* and the recombinant were also identified with extremely different frequencies of 63% and 9%, respectively in the Taiwanese coast. Farjallah *et al*. (2008) collected 282 *Anisakis* larvae (L3s) from 13 teleost fish species and one cephalopod species captured at different sites off the Algerian, Tunisian and Libyan coasts (North African coasts of the Mediterranean Sea) and were then subjected to molecular study. Seventy-nine percent of the larval forms examined were identified as *A. pegreffii* and 0.7% as a hybrid genotype between *A. pegreffii* and *A. simplex s.s*. species.

In Australia, tiger flathead have been described to be parasitized by *A. pegreffii* which is endemic and distributed from northern New South Wales to western Victoria, including Tasmanian waters as shown in Figure 2.4 (Kuiter, 2000)**.** Jabbar *et al*. (2012) examined 50 specimens of the commercially important fish, *Sillago flindersi*, from Bass Strait, Australia. *A. pegreffii* represented 24% of total collected larval Anisakid (194). Previous studies (Cannon 1977*b*; Lymbery *et al*. 2002) have reported infections of various species of fish in Australia with *Anisakis* Type I and II larvae. Jabbar *et al*. (2013) examined 108 commercially important fish, caught in the Indian Ocean off the coast of southern Western Australia. *A. pegreffii* represented 50% of total collected larval Anisakid from *Pseudocaranx dentex*. The presence of *A. pegreffii* in some of the commercial and recreational fish may raise human health concerns, because these anisakid nematodes have been found previously to be associated with human anisakidosis and allergic sensitization. Shamsi *et al*. (2011b) collected larval anisakid nematodes to estimate prevalence and intensity of infection. 53 Anisakis larval type I were collected from four *Neoplatycephalus*

richardsoni (tiger flathead). The alignment of the ITS-2 sequences revealed that they were identical to the sequence available for *A. pegreffii.* Martın-Sanchez *et al*. (2005) discovered that the total of 59.26% (16 out of 27) of the larvae collected from blue whiting specimens captured on Spanish Mediterranean coasts were identified by PCR-RFLP as *A. pegreffii*. In Atlantic waters, this percentage dropped to 20.00% while *A. simplex s.s.* represented 66.67%. Also, the percentage of hybrid genotypes between both members of the *A. simplex* complex (*A. simplex s.s.* and *A. pegreffii*) was 16.67%. Abattouy *et al*. (2011) found that the prevalence was generally higher in fish from the Atlantic (67.9%) than from the Mediterranean (57.0%), but they did not differ in the mean abundance, intensity, or prevalence of muscle parasitization. *A. pegreffii* was the predominant species (82.6% of larvae) followed by the hybrid *A. simplex s.s./A. pegreffii* (16.3%) in mackerel (*Scomber japonicus*) fish in Moroccan coast. Mattiucci *et al*. (2008) examined Atlantic horse mackerel and found *A. pegreffii* was the most prevalent species in the Mediterranean Sea.

A. simplex s.s. was the most common species found in all samples from NE Atlantic waters. Mixed infections of *A. pegreffii* and *A. simplex s.s* were found in all samples from the Portuguese and Spanish Atlantic coasts and in samples from south of Ireland. Cavallero *et al*. (2012) collected four fish species from the Tyrrhenian Sea, northwestern Mediterranean and 73 larvae from *T. trachurus*; 12 from *L. caudatus* and 23 from *E. encrasicolus* and showed the restriction profiles corresponding to *A. pegreffii*. Also, three individuals collected from *T. trachurus* and five from *E. encrasicolus* showed the heterozygote pattern between *A. pegreffii/A. simplex s.s*. Mattiucci *et al*. (2004) examined hake (*Merluccius merluccius*) from the north-east Atlantic Ocean and Mediterranean Sea. The larvae of *Anisakis* type I were collected and subjected to morphological and molecular examination. *A. pegreffii* exhibited the highest prevalence values in all hakes sampled from the Mediterranean Sea compared with other species. Santoro *et al*. (2010) examined thirteen loggerhead sea turtles (11 males and 2 females) from the Central Mediterranean of southern Italy. The larval nematodes recovered from the examined loggerhead sea turtles, belonged to the species *A. pegreffii,* which were detected through gross necroscopy from 7 turtles, while in the other 6 positive loggerhead sea turtles *A. pegreffii* larvae were revealed by histopathology. Lee *et al*. (2009) collected sixty L3 larvae of *Anisakis* spp from *Scomber japonicus*, *Trichiurus lepturus*, and *Todarodes pacificus* from a Korean sea, where forty-seven anisakid larvae (78.3%) were matched with a typical PCR-RFLP pattern for *A. pegreffii* and thirty-two of 47 *A. pegreffii* isolates were from *S. japonicus*, thirteen were

from *T. lepturus*, and two were from *T. pacificus*. Therefore, *A. pegreffii* was identified as the most prevalent species of Anisakis isolated from S. *japonicus*, *T. lepturus*, and *T. pacificus* in Korea.

Abe *et al*. (2005) recorded the existence of *A. pegreffii* with *A. simplex s.s.* from the same teleost host Gadus macrocephalus (pacific cod) in Japan. Sequeira *et al*. (2010) reported *A. pegreffii, A. simplex s.s.* and their recombinant genotype were the dominant anisakids in four fish from mainland Portugal (*Hepatoxylon trichiuri, Lecithochiriinae A, Hypohepaticola* sp. and *Lecithocladium* sp.). Shih *et al*. (2010) reported *A. pegreffii, A. simplex s.s* and recombinant genotype for first time in cobia, *Rachycentron canadum L*., in Taiwan.

In relation to a previous study (Mattiucci *et al*., 1997 and Marques *et al*. 2006) *A. pegreffii* is widely distributed in the Australian region and Southern Hemisphere. The presence of the fourth larval and adult stages of *A. pegreffii* in the stomach of *Delphinus delphis, Tursiops truncates* has been reported for the first time in Australian waters (Shamsi *et al.* 2012). The existence of the fourth larval and adult stages of *A. pegreffii* in Australian marine mammals supports the fact that various species of fish in Australia are infected with larval stages of *A. pegreffii* (Shamsi *et al.* 2011b).

Abollo *et al.* (2001) reported that *A. pegreffii* and *A. simplex s.s*. have been found together parasitizing seven fish species in Galician coast. Also, Abollo *et al*. (2001) reported that 52.71% of the larval forms examined were identified as *A. simplex s.s.*, 34.11% as *A. pegreffii* and 13.18% as the hybrid genotype. The highest prevalence of *A. simplex s.s.* larvae occurred in the north of the Iberian Peninsula reducing southwards. Inversely, a similar tendency was found in *A. pegreffii* larvae with the highest prevalence in the Alboran Sea and lowest in the Cantabrian Sea, whereas the hybrid genotype was more prevalent in fish caught in the areas close to the Gibraltar strait (Cadiz and the Alboran Sea). In addition, 100% of the larval forms examined from the lesser flying squid *Illex coindetii* in Salonika Gulf (Greece) and the short finned squid *Illex argentinus* in Argentina and Falkland islands were identified as *A. pegreffii*. In contrast, 100% of the larval forms examined from the European flying squid *Todarodes sagittatus* in Reykjanes (Iceland) were identified as *A. simplex s.s.* Paggi *et al.* (1998) identified three species of larvae and adults of *Anisakis* from the beaked whales *Mesoplodon layardii* and *Ziphius cavirostris* as *A.*

pegreffii, *A. simplex* C and *A. physeteris* from the Mediterranean Sea and South African waters by using molecular diagnostic keys. Umehara *et al*. (2007) published the molecular identification of one hundred larvae recovered from eighty five patients with anisakiasis, of which 98.8% of patients were parasitized by *A. simplex s.s.* and only one was infected by *A. pegreffii*. In contrast, the most frequent species recovered from the patients with anisakiasis in Italy is *A. pegreffii* (D'Amelio *et al*., 1999; Fumarola *et al*., 2009; Mattiucci *et al*., 2007, 2011; Moschella *et al*., 2004a), indicating the wide range of these species in Italian seas and their common raw or marinated consumption. It would be of great significance to further correlate the geographical and etiological relationship for human anisakiasis cases, since fish species, especially the anchovy *Engraulis encrasicholus*, is of high commercial value and is widely used for raw marinade frequently infected with *A. pegreffii* larvae in Italian seas and responsible for human anisakiasis (Mattiucci *et al*., 1997).

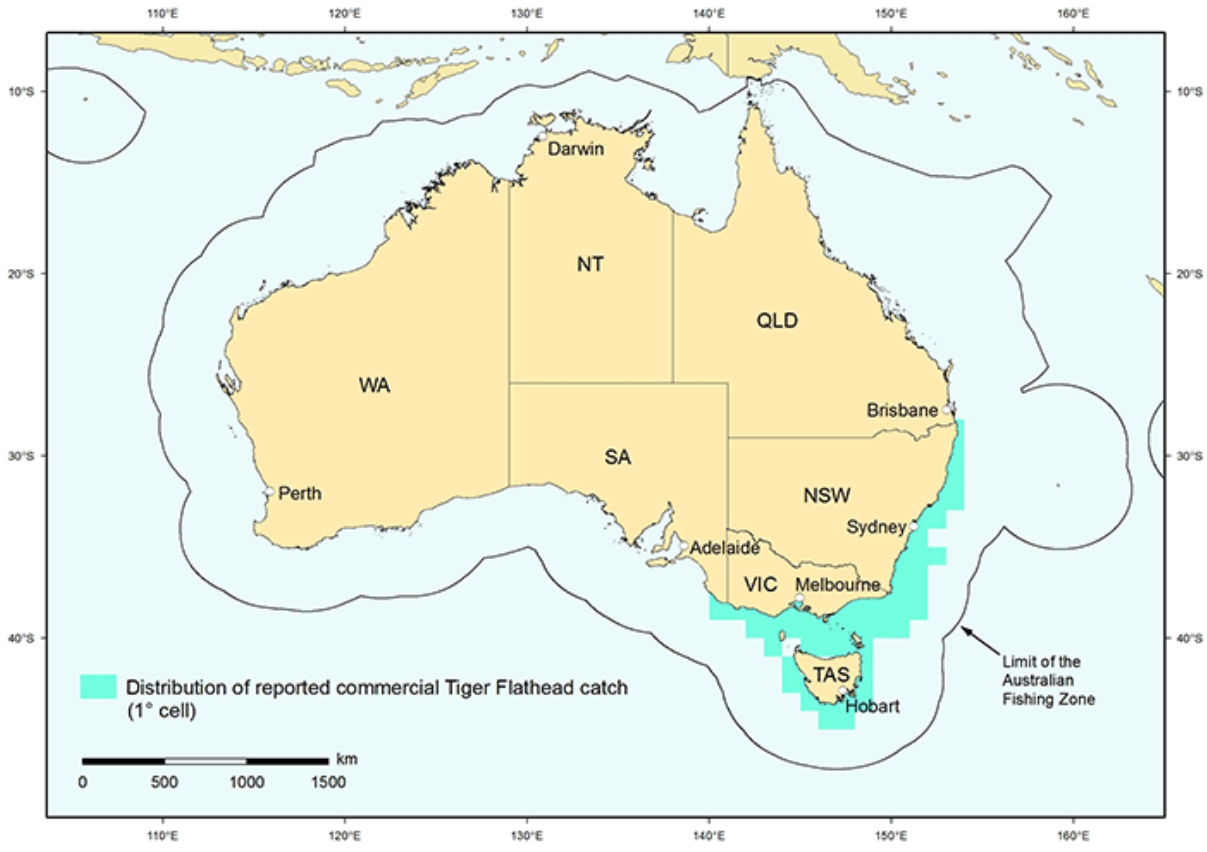


Figure 2.7 Distribution of reported commercial catch of Tiger Flathead in Australian waters.

Source: http://www.fish.gov.au/reports/finfish/flatheads/Pages/tiger_flathead.aspx

2.12.4 Epidemiology of human anisakiasis

Rapid changes in world demand and trade for seafood and fish along with improved endoscopic diagnostic techniques may be associated with increasing world reports of anisakiasis infections. The term anisakiasis has been used to designate not only the human disease caused by L3 of Anisakis spp. but also the human disease caused by L3 of other members of the Anisakidae family as shown in Figure 2.6. Furthermore, in 1988, a group of experts on the standardized nomenclature of animal parasite diseases suggested the use of three different terms for human disease caused by L3: (i) anisakidosis for human disease caused by any member of the family Anisakidae, (ii) anisakiasis for human disease caused by members of the genus Anisakis, and (iii) pseudoterranovosis for human disease caused by members of the genus Pseudoterranova (Kassai *et al*., 1988).

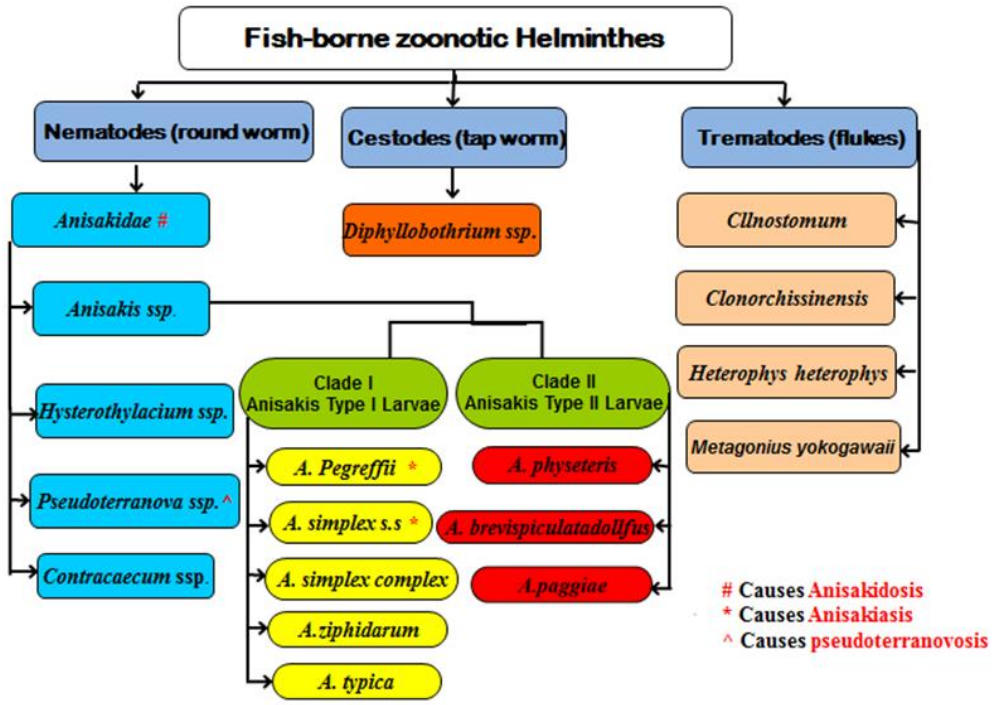


Figure 2.8 Different genera of anisakid nematodes causing human Anisakidosis identified.

Source: (This study)

Anisakiasis is related to traditions of ingestion of raw or undercooked fish infected with *Anisakis* sp. larvae (Audicana *et al*., 2002a). Table 2.12 shows some of the most popular seafood dishes that have a significant risk to harbor L3 of anisakids.

Table 2.10 Popular seafood dishes at risk of harboring infectious anisakid larvae (Eisenbarth, 2009).

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

Human anisakiasis caused by the third-stage larvae (L3) of *Anisakis* spp has been known since the early 60s. Since then, about 20,000 cases of anisakiasis have been reported globally, from which, 90% are from Japan (where approximately 2,000 cases are now diagnosed annually), and the rest mainly from Europe (Audicana *et al*., 2002a, Bouree *et al*., 1995, Chai *et al*., 2005). Recently, there has been a marked increase in the reported prevalence of anisakiasis from all five continents (Audicana and Kennedy, 2008). There are many factors that play important roles in acquiring human Anisakiasis. For instance; increase in the frequency of travelling and eating native food such as raw fish often represents a risk for humans to acquire food-borne parasitic infections. It has been reported that most cases of anisakiasis are caused by two species of the genus *Anisakis* in humans: *A. simplex s.s* and *A. pegreffii* (Mattiucci and Nascetti, 2008).

2.12.4.1 *A. pegreffii* and human anisakiasis

As mentioned above, Anisakiasis is a parasitosis disease caused by the ingestion of raw fish contaminated with *A. simplex or A. pegreffii* (Romero *et al.,* 2013). Human infections were first described in the Netherlands in 1960 and then in different countries. Larval anisakid nematodes in marine fish have been morphologically identified as L3 larvae of the genus Anisakis by a light microscope and at species level by molecular techniques based on genetic variation (Jabbar *et al.,* 2012). In the early study of Anisakis species in Japan, the most larval anisakis cause Anisakiasis were isolated from fish in Japanese waters and identified as *A. simplex s.s* using molecular approaches (Arai *et al*., 2014). However, Quiazon *et al* (2011) have found the distribution of *A. simplex* to be more predominant in Northern Japan to Pacific sides, while *A. pegreffii* has been predominantly isolated from fish in Northern Japan to Pacific sides. Although over 98.8% of patients were found

infected with *A. simplex s.s* (Umehara *et al.,* 2007), several cases of Anisakiasis have also been caused by *A. pegreffii* in Italy and Japan in recent years. However, in Italy *A. pegreffii* is widespread in commercial fish species such as marinated anchovies in coastal areas, or fashion foods (sushi, sashimi, etc.) in inland areas and responsible for many cases Anisakiasis in Italy, in contrast one previous case was reported in Japan. These findings could be associated with fish species and the predominant pathogenic species or genotype of Anisakidae and geographical area.

As it mentioned later, the detection, diagnosis and treatment of *A. pegreffii* infection have been demonstrated that rely on several laboratory methods in addition to clinical symptoms, clinical history, travel history, and geographic location of patient. Because larvae can become spoiled or fragmented, making it impossible to identify them at the genus level using morphological features, when they infest humans. Therefore, the molecular tools have to be used to identify them at the species level (Mattiucci *et al*., 2013). For instance, during diagnosis and treatment, DNA obtained from nematodes or nematode fragments removed from the stomach and embed in the tissue of human patients were identified using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and showed 99% identity to that of the *A. pegreffii* sequence obtained from the GenBank database (Mattiucci *et al*., 2011, Mattiucci *et al*., 2013). *A. pegreffii* nematodes can also induce gastroallergic anisakiasis associated with hypersensitivity reaction symptoms rang from complaining of acute abdominal pain in the lower right quadrant, nausea to emesis lasting several hours those patients. These symptoms are probably due to the penetration site of the submucosal layer of the gastric wall by larvae (Mattiucci *et al*., 2011). The above findings have allowed the identification of *A. pegreffii* as a zoonotic agent of human anisakiasis.

2.12.4.2 Seafood related to *A. pegreffii* and Anisakiasis

Anisakiasis is a human disease common to areas with accidental ingestion raw or undercooked of contaminated fish or squid with live or dead larval nematodes belonging to the family of Anisakidae (Daschner et al., 2002, Audicana et al., 2002c). Humans gain the infection by eating raw seafood dishes such as sushi, sashimi, ceviche, and lomi-lomi or undercooked fish and squid dishes. The prevalence and intensity of Anisakis infection ranges from 1% to 100% according to fish species, fishing area, season and species of the Anisakidae family (Mattiucci and Nascetti, 2006b). Therefore, the higher presence of the parasite in the body of fish increases the risk of infection with contracting anisakiasis with

frequent consumption of raw or undercooked fish. The source of Anisakiasis depends on the species of fish being consumed. For example, anchovies (*Engraulis encrasicolus*) are the main sources of Anisakiasis in Italy and Spain, chub mackerel (*Scomber japonicus*) and common squids are thought to be the main sources of Anisakiasis in Japan. Pacific salmon (*Oncorhynchus spp.*) and herring (*Clupea harengus*) are associated with human infections in the United States and other European countries, respectively (Audicana and Kennedy, 2008).

In Italy, the European anchovy (*Engraulis encrasicolus*) represents one of the most attractive Mediterranean fisheries products (Solas *et al*., 2009), but is a common paratenic host for A. *pegreffii* in the Adriatic anchovy (Mladineo *et al*., 2012). Generally, the zoogeographical occurrence of *A. pegreffii* in the Adriatic is not at all surprising, because of it being the most frequent anisakid species in the Mediterranean and its adjacent seas (Mladineo *et al*., 2012). As mentioned previously, *A. pegreffii* is the dominant species of Anisakis detected in anchovies (as intermediate hosts) in the Mediterranean Sea as well as the Atlantic Ocean, this might probably be due to the occurrence of various dolphin species, such as *Tursiops truncatus*, as the main definitive hosts (Mattiucci and Nascetti, 2006b). Therefore, anchovies currently represent a significant potential source of infection for humans in Italy.

Since Anisakis type I including (A. *pegreffii* and A. *simplex s.s*) have been successfully confirmed by molecular markers (D'Amelio *et al*., 1999) in anchovies, several cases of Anisakiasis have now been recognised in Italy and Spain due to the consumption of *Italian acciughe marinate* infected with *A. pegreffii* and *Spanish boquerones en vinagre* contaminated with *A. simplex s.s*., respectively. In Italy, further findings revealed that marinated anchovies contaminated with *A. pegreffii* represent the most common food that causes sensitization rather than fried anchovies. The connection between Anisakis hypersensitivity and marinated seafood are also confirmed by Spanish authors (Valinas *et al*., 2001, Garcia and Pozo, 1997). Maximum human anisakiasis was detected in people living along the Adriatic and Tyrrhenian coasts of Italy where marinated anchovies are frequently consumed as a traditional food and are either prepared at home or restaurants (AAITO-IFIACI Anisakis Consortium). Furthermore, it's also confirmed that people living in the large cities had higher hypersensitivity and reactions rather than in small towns for the following two reasons; 1) immigration from 'endemic areas' and 11) the increasing

presence of fish restaurants present or delivery of raw or uncooked fish dishes (AAITO-IFIACI Anisakis Consortium).

2.12.5 Diagnosis of Anisakiasis allergenic proteins

Diagnosis of allergy due to Anisakis currently relies on several pathological data, clinical history and clinical data. For example; pathological data is related to the type of parasites, the host, timing of exposure, infection intensity and the environment. Clinical history is related to travel history and geographic location of the patient (Ndao, 2009, Caraballo and Acevedo, 2011), while clinical data is related to the detection of serum IgE antibodies to allergenic proteins (Nieuwenhuizen and Lopata, 2013). In recent times two species of the genus Anisakis have been found to cause infections in humans (Anisakiasis): *A. simplex s.s and A. pegreffii* (Mattiucci *et al*., 2008b). Both human epidemiological studies and experimental animals (rat and mouse) models support the idea that infection by helminths is characterized within hours of ingesting contaminated fish with L3 by gastrointestinal symptoms, which may or may not be associated with acquired protective antibody immunity and allergy sensitization in the host (Finkelman *et al*., 1997, Finkelman *et al*., 2004, Allen and Maizels, 2011, Audicana *et al*., 1995, Daschner *et al*., 2000, Dominguez-Ortega *et al*., 2001).

Traditionally, diagnosis of anisakiasis has been deemed as either being invasive or non-invasive (or luminal). Invasive anisakiasis occurs when Anisakis larvae are attached to, embedded in, or penetrate through the host tissues. Therefore, they have been found in the mucosa or submucosa of the gastric stomach and intestine and have migrated to extraintestine tissues such as the omentum, pancreas, liver, and probably the lung (Kobayashi *et al*., 1985, Rushovich *et al*., 1983, Yokogawa and Yoshimura, 1967). In addition, cases of gastric anisakiasis (95%) are more common than those of enteric anisakiasis (Kang *et al*., 2010, Lee *et al*., 2009). Non-invasive anisakiasis is generally asymptomatic and involves no tissue penetration by the worm. This form gives rise to the "tingling throat syndrome," which occurs when worms migrate back up the esophagus into the oropharynx. These worms are often coughed up or felt wiggling around in the mouth between the gums and cheek 1 day to 2 weeks after ingestion of the infected fish or seafood (Deardorff *et al*., 1987, Kliks, 1983b). Generally, larvae belonging to the genus Pseudoterranova are non-invasive, but penetration into tissues has also been reported (Ishikura et al., 1988, Kwee and Sautter, 1987, Little and MacPhail, 1972).

Medically, anisakiasis has been divided into acute and chronic stages, based on the pathological features and the location of the larvae and the time of infection. The clinical symptoms of acute stage include sudden epigastric pain, nausea, vomiting, diarrhea, urticaria and, subsequently, an eosinophilic granuloma for a period ranging from 2–3 hours to 2 days after eating. These symptoms are likely facilitated by a hypersensitivity reaction in those patients. During this stage, anisakis larvae can be detected with gastroscopy, and using 0.1% indigo carmin solution, which shows a good contrast with the larvae, making it more visible. Therefore, gastro endoscopy represents an urgent examination to diagnose and remove larvae from the stomach, and observe oedema and hyperaemia of the mucosa during acute disease. When diagnosis and treatment are undertaken within a few hours of the onset of the infection, the epigastric pains disappear immediately (Fujino *et al*., 1984, Bouree *et al*., 1987, Ikeda *et al*., 1989). In contrast, in the chronic stage it is more difficult to collect the whole *Anisakis* body, because it is embedded into the tissue. Therefore, diagnosis is established through the histopathology of the lesion, showing eosinophilic granulomas and cross sections of Anisakis larvae, with the characteristic Y lateral cords (Dooley and Neafie, 1976). The serological tests are very important in chronic Anisakidosis.

Anisakiasis with digestive tract symptoms depends on the time of intermission between intake of an intermediate host (fish and squid) contaminated by its third-stage larvae (L3) and the onset of symptomatic Anisakiasis. For example; the time interval between intake of fish and onset of gastric Anisakiasis is generally 1–12 h after ingestion of raw fish (Kakizoe *et al*., 1995). The time period between intake of fish and onset of intestinal Anisakiasis is generally 5–7 days after ingestion of the anisakid larvae (Ishikura *et al*., 1990). The time difference between intake of fish and onset of developments of allergic symptoms is generally ≤ 5 h (range, 1 h to 24 h) after the ingestion of infected fish (Lopez-Serrano *et al*., 2000, Foti *et al*., 2002), and patients may have signs of allergic response before gastrointestinal symptoms, which leads to misdiagnosis of the reaction to Anisakis fish. Therefore, it is considered that anisakid larvae may be responsible for the four clinical forms of illness in humans: gastric, intestinal, ectopic Anisakidosis (or extra-gastrointestinal), and allergic forms (Audicana and Kennedy, 2008, Liang et al., 2013).

Victims of acute gastric anisakiasis usually complain of acute gastric pain, nausea, meteorism, vomiting or bloody vomiting within 2–3 hours to 2 days or several months

which may develop to gastro allergic disease after eating raw fresh marine fish (marinated anchovies) (Mattiucci *et al*., 2013, Fumarola *et al*., 2009). Untreated gastric disease may cause chronic ulcer-like symptoms possibly persisting for months and can pose a challenge for diagnostic purposes (Ito *et al*., 2007). Anisakis and anisakis fragments can be detected and removed from the stomach by means of biopsy forceps, during the esophagogastroduodenoscopy (EGDS) test (Mattiucci *et al*., 2013). Gastroallergic anisakiasis associated with *A. pegreffii* induces allergic reactions with different manifestations in affected patients; the hypersensitivity reaction mechanism is induced in the submucosal layer of the gastric wall around the penetration site of the *A. pegreffii*. Surgical or histological examination is performed to remove any eosinophilic granuloma surrounding the larva of the nematode (Mattiucci *et al*., 2011).

During the cases of chronic intestinal anisakiasis of *A. pegreffii* after ingestion of the anisakid larval stage, patients complained of acute abdominal pain, nausea and emesis lasting for several hours. This can potentially develop into abdominal peritonitis or intestinal occlusion and, hence, become a surgical problem.

Studies of anisakid parasitization in Italy have recorded several cases of gastric or intestinal anisakiasis or both at the same time in some patients (Table 2.13), which may be associated with mild to severe immunological responses, after the *A. pegreffii* larvae penetrate the digestive mucosal tissues as shown in table 2.14.

Table 2.11 Parasitic episodes without allergic symptoms

Country	Year	Author	Diagnosis and number of patients
Italy	2011-2012	(Mattiucci *et al*., 2013)	1 Submucosal layer of gastric wall 1 Gastric mucosa 6 Lumen of stomach
Italy	2009	(Fumarola *et al*., 2009)	2 Gastric mucosa
Italy	2011	(Mattiucci *et al*., 2011)	1 intestinal wall
Italy	2004	(Moschella *et al*., 2004)	1 intestinal wall

Table 2.12 *A. pegreffii* allergy reports worldwide with a special mention of anaphylaxis.

Country	Year	Author	Numbers of allergic patients	
			Total	With anaphylaxis
Italy	2011-2012	(Mattiucci *et al*., 2013)	8	0
Italy	2009	(Fumarola *et al*., 2009)	2	0
Italy	2011	(Mattiucci *et al*., 2011)	1	1
Italy	2004	(Moschella *et al*., 2004)	1	0

In Japan, gastric Anisakidosis is more common than intestinal anisakidosis (95% of cases), whereas in Europe intestinal anisakidosis is predominant. These differences might have resulted from a reporting bias, differences in diagnostic methods or epidemiological factors (Van Thiel, 1976). However, more than 10% of gastrointestinal anisakiasis cases may be accompanied by allergic symptoms (Repiso Ortega *et al*., 2003, Ohtaki and Ohtaki., 1989, Kakizoe et al., 1995). In addition, several studies have detected the presence of anti-Anisakis IgE antibodies in more than 10% of healthy subjects who frequently consume raw fish (Puente *et al*., 2008, Anadon *et al*., 2009), suggesting the presence of a large number of infected patients who do not develop clinical allergic symptoms.

A. pegreffii has less penetrative power towards the muscle of certain fish and solid agar. Also, it's chances of survival in artificial gastric juice is lower than *A. simplex s.s.* (Suzuki *et al*., 2010b, Quiazon *et al*., 2011, Arizono *et al*., 2012, Romero *et al*., 2013), However, *A. pegreffii* has been justified to be an aetiological agent of anisakiasis because It has been demonstrated to be responsible for 14.8% of the penetrations of the gastric mucosa in rats (Romero *et al*., 2013). It has been found to also cause human anisakiasis (Umehara *et al*., 2007b). Hence, the above mentioned results/findings have allowed the identification of *A. pegreffii* as a zoonotic agent of a human gastric or intestinal case of anisakiasis within the stomach or an eosinophilic granuloma formation in the mucosa with severe allergic symptoms.

2.12.6 Clinical and symptomatic manifestations of Anisakiasis caused by *A. pegreffii*

Detection and diagnosis of parasite infections has improved during the past 54 years since human anisakiasis was first described in 1961. However, as yet there is currently no available test to diagnose Anisakiasis disease in an appropriate fashion with a high performance of sensitivity and specificity. After human ingestion of contaminated fish, the

larva can either be expelled with vomit or stools or can penetrate the wall of the gastrointestinal tract and be embedded into the tissue where they eventually die. However, alive larvae have been extracted hours and days after the infection and there are indications that they may be able to persist for weeks or even months within the human host (Asami *et al*., 1965, Kliks, 1983b, Deardorff *et al*., 1986, Deardorff *et al*., 1987, Spehn *et al*., 1988, Alonso *et al*., 1997).

Infection with *Anisakis* sp. larvae causes serious illness with symptoms of gastric or allergic reaction, if not treated in time. Due to the vagueness and/or the diversity of symptoms present during the early stages of the anisakiasis disease it can often lead to misdiagnosis as appendicitis, peritonitis, gastric ulcer, cancer or tumor, ileitis, cholecystitis, diverticulitis, tuberculosis, peritonitis, cancer of the pancreas, or Crohn's disease (Oshima, 1972, Yokogawa and Yoshimura, 1967, Hayasaka et al., 1971). Various laboratory methods have been developed and evaluated for detection of *A. simplex* infectious disease (Anisakiasis) such as; Anisakis-specific IgE, positive Skin Prick tests (SPT), complement fixation (CF), hemagglutination (HA) test, immunoelectrophoresis, Latex agglutination (LA) test, indirect immunofluorescence assay (IFA or DFA), enzyme-linked immunosorbent assay (ELISA), radioallergosorbent (RAST) test, and rapid diagnostic tests (RDTs) (Tsuji, 1989, Akao and Yoshimura, 1989b, Ndao, 2009).

Worldwide increasing evidence from both human and animal data studies show that the frequent exposure to allergic proteins from Anisakis-infested fish by ingestion, handling and vapor inhalation routes produced during fish processing (cleaning, cooking, or fish meal production) is an important risk factor for allergic sensitization and skin reactions (urticaria and/or angioedema) to Asthma which may develop to anaphylactic reactions and immunomodulatory responses (Nieuwenhuizen *et al*., 2006, Mattiucci *et al*., 2011). Upon investigation more than 90% of infection cases described worldwide was caused by a single larva (Audicana and Kennedy, 2008, Smith, 1999).

In heavy infections, the clinical symptoms correlate with the biologic stages of L3 or L4 larva as it completes its life cycle. Because it is difficult to recover adult and larvae in a stool specimen, normally diagnostic infection and treatment of L3 or L4 larva is based on the combination of patient’s history, clinical symptoms, imaging, surgical removal procedures and molecular technique. The morphological and molecular identification are made after extracting them from intestinal tract (mucosa or submucosa of the gastric

stomach and intestine or extraintestine) during endoscopic examination or in tissue sections of eosinophilic granulomas (Siles *et al*., 1997).

Therefore, anisakis initially cause discomfort, acute pain (gastric pain, nausea, vomiting) and subsequently an eosinophilic granuloma for a period ranging from 2–3 hours to 2 days after eating. These symptoms elicit hypersensitivity reaction in those patients. Therefore, the high levels of IgE as observed in the serum specimens from patients with allergic reaction were due to cross-reactivity between antibodies and panallergens from dead or live larval *Anisakis*. Several cases of human anisakiasis have been reported in gastric (Stallone *et al*., 1996), intestinal (Moschella *et al*., 2004b) (Moschella *et al*., 2004a, Moschella *et a*l., 2005), extra-intestinal (Cancrini *et al*., 1997) and gastrointestinal mucosa and submucosa (Maggi *et al*., 2000, Pampiglione *et al*., 2002). Most of these cases were diagnosed based on histopathological findings of the presence of the Anisakis larva or location of the granulomatous lesions by surgery or endoscopy (Marquardt, 2000). There is no drug for the treatment of infected patients. Patients are treated with endoscopic removal of the worm using biopsy forceps; the pain disappears within a few hours of treatment (Bogitsh et al., 2005).

2.12.7 Characterization of allergenic proteins

Identified and biochemically characterised allergens are named using the systematic nomenclature of the Allergen Nomenclature Sub-Committee of the World Health Organizaion (WHO) and International Union of Immunological Societies (IUIS) (Chapman *et al*., 2007). The system uses abbreviated genus and species names and an Arabic number to indicate the chronology of allergen purification. For example; Ani s 3 (Ani) refers to the third-stage Anisakis larvae; (s) refers to the genus anisakis simplex and (3) refers to the number of allergen. Recently it has been shown that exposure to larval somatic proteins or heat-killed larvae (L3) of Anisakis (*A. pegreffii* and *A. simplex)* and excretory products can also cause allergic reactions (Kirstein *et al*., 2010, Caballero *et al*., 2008, Moneo *et al*., 2005). Therefore, the heat-stable components of fish contaminated with the third larvae (L3) of *A. pegreffii and A. simplex* may be sufficient to cause allergic reactions to somatic and excretory/secretory (ES) allergens despite cooking. Hence, purification, identification and characterisation of individual allergic proteins are required to improve allergy diagnosis and to investigate the impact of food processing or digestion on the allergenicity of Anisakis allergens and to analyse the relevant IgE binding.

Currently 12 allergens have been identified in *A. simplex* as shown in Table 2.4. Human patients are exposed primarily to excretory–secretory (ES) antigens that are secreted from live larvae or somatic antigens from dead larvae in food, or both, and in cases where the larva penetrates the tissue, it is killed by the host, and subsequently degenerates inside the host (Audicana and Kennedy, 2008). Many allergens of Anisakis are heat- and/or pepsin-resistant (Caballero *et al*., 2008, Caballero and Moneo, 2004, Moneo *et al*, 2005), and most of them are present in ES products. Major allergens of Anisakis are recognised in more than 50% of patients analysed and minor allergens are recognised by less than 50% of analysed patients.

According to various authors, viability of the larvae and anisakis antigenic proteins may be resistant to cooking and deep freezing. Therefore, ingestion of cooked fish and /or freezing does not prevent allergic manifestations. For example; the heat-stable allergen Ani s 4 was detected by immunohistochemistry in the fish muscle after heating at 70°C, this suggests that Ani s 4 allergens were released from the larvae into the surrounding tissue and that the tissues retained their allergenicity even after the larvae were killed by heat treatments. Thus, cooking at this specific temperature will not ensure the safety of fish consumption by individuals already sensitized to Anisakis heat-resistant allergens (Vidaček *et al*., 2011). Antigenic protein Ani s 4 and *A. simplex* crude antigens were detected in the larvae heated at 94± 1^0C for 3 min. Again, this indicates that allergic symptoms could be provoked in previously sensitized consumers, even if the larvae were killed by heat treatment (Vidacek, 2010, Vidaček *et al*., 2009).

The knowledge of the characteristics of sensitising AS antigens (structural antigens of the parasite or antigens secreted by it), their potency, lability or stability in contact with enzymes, heat, acid environments, and also those treated allergens on the function and efficiency of the immune system and allergenicity have not been investigated well. Studies aimed to investigate findings which can be deduced such as; many allergens of Anisakis are heat stable, exposure to Anisakis proteins in fish on an ongoing basis can also cause symptoms such as chronic urticaria, contact dermatitis, asthma and rhinoconjunctivitis (Kasuya et al., 1990, Carretero Anibarro et al., 1997, Montoro et al., 1997, Anibarro and Seoane, 1998, Armentia et al., 1998, Scala et al., 2001, Daschner et al., 2005). Allergen-specific IgE antibody can be detected in the blood even in the absence of a viable infection. The anisakis antigens involved in IgE production are not affected by freezing or cooking temperatures. Therefore, these antigens can induce the formation of IgE antibodies if

anisakis is consumed even after it has been killed by cooking or freezing (Akao et al., 1989). However, this fact has also been disputed in the past (Garcia *et al*., 2001).

2.12.8 Immune reactions and allergy to A. *pegreffii*

Anisakiasis is caused by two main mechanisms: allergic reactions and direct tissue damage (Nieuwenhuizen and Lopata, 2013). The former ranges from isolated urticaria and angioedema to life-threatening anaphylactic shock associated with gastrointestinal symptoms. Allergic reactions can occur after primary infection with Anisakis from ingestion of contaminated fish and exposure to allergens of the parasite. Direct tissue damage is due to parasite invasion of the gut wall, development of eosinophilic granuloma, or perforation (Choi *et al*., 2009). It has been demonstrated that an increased risk of an allergic response to A. *pegreffii* is associated with frequent fish consumption and occupational exposure (eg, fish processing) (Mattiucci *et al*., 2013). These symptoms are common in the regions where people consume anchovy and bony fish as their main dish (Mattiucci *et al*., 2013), and generally occur within 1 to 24 h after the ingestion of the infected fish (Mattiucci *et al*., 2013). Manifestations of allergic symptoms lead to a clear development to urticaria accompanied by gastrointestinal symptoms. The above findings suggest that eating and direct exposure to allergenic proteins in infested fish through skin contact, or oral and/or inhalational of aerosolised *A. pegreffii* proteins, enhances Th2 immune responses during anisakiasis (Nieuwenhuizen *et al*., 2006).

Nieuwenhuizen *et al*., 2006 demonstrated the prevalence of sensitization to bony fish (pilchard and anchovy) contaminated with A. *pegreffii* in fish processing worker factories was 8%, higher than the 6% prevalence of sensitization to fish. Sensitization by the inhalation route of allergen from A. *pegreffii* had a higher risk of allergic reactions, in particular skin symptoms and increased nonspecific bronchial hyperreactivity on methacholine challenge while epicutaneous exposure to A. *pegreffii* proteins can lead to dermatitis. Overall, *A. pegreffii* specific IgE reactivity was associated with bronchial hyperreactivity and dermatitis was significantly increased with contaminated fish consumption. In experimental animal samples, it has been found that *A. pegreffii* (L3) proteins induced oral hypersensitivity in sensitized mice by activation of T_H2/type 2 immune response such as IL-4/IL-13 cytokine production, which is responsible for IgE production by the host B cells. The earliest antibody responses were found after approximately 1 week, with production of the IgM isotype. For all infections,

immunoglobulin production consisted primarily of IgM and IgG1, and a Th2 response was indicated by the obtained cytokine pattern (Nieuwenhuizen *et al*., 2009).

The relationships between parasite infections and allergic diseases have many features, and one of them is allergens from L3 that enhance the immune hypo reactivity in both the innate and adaptive systems and allergic responses diseases in the host. Therefore, allergens produced by L3 can induce many parameters during the time of infection such as levels of IgE, Th2 cytokines, eosinophils, and cytotoxic mediators. These chemicals cause a range of allergic reactions and symptoms from intermediate urticaria to severe anaphylaxis (Audicana and Kennedy, 2008). Gastro-allergic anisakiasis has been known to be accompanied by Th2 memory response which provokes a strong specific immune response by antibody isotypes, the immunoglobulin (Ig) IgE, IgG, IgA and IgM (Daschner *et al*., 2002, Kennedy, 2000, Cho, 2006, Audicana and Kennedy, 2008). Daschner and colleagues (2002) have also reported that the allergic IgE mediated reaction in the course of gastro-allergic anisakiasis involves a parallel secondary Th2 type memory response and a primary immunologic stimulation of both Th2 and Th1 lymphocyte subsets against previously unrecognized antigens. It was observed that the high levels of IgE remained in the serum specimens, due to cross-reactivity between antibodies against panallergens from dead or live larval Anisakis.

Anisakiasis are commonly associated with high levels of IgE but usually during the first 7 days of anisakiasis there are no signs of disorders in blood tests (Petithory *et al*., 1993). Demonstrable antibody is seen from as early as 10 days to as late as 35 days during the infection pattern (Akao and Yoshimura, 1989a). There is a considerable cross-reactivity in polyclonal test systems (using extracted larval antigens or antigens defined by antisera produced in rabbits). Additionally, the number of blood eosinophil cells in peripheral blood increased while stool examination for parasitic infection remained negative (Petithory *et al*., 1993). Immunodiagnostic tests become positive after the tenth day and were negative between 6 and 12 months (Petithory *et al*., 1986). An increase in specific IgE, detected with the RAST test (Desowitz *et al*., 1985) has been noticed over a period of about 2 months (Deardorff *et al*., 1986).

An ELISA assay using a monoclonal antibody against defined antigen An2 is highly specific for A. *simplex* (Namiki, 1989, Iglesias *et al*., 1997). The An2-defined antigen is a heterodimer of 40-kDa and 42-kDa molecules and represents an excretory/secretory

product of the worm (Akao and Yoshimura, 1989b, Iglesias *et al*., 1997). IgE antibodies are detectable as early as a week, and the IgG, IgA and IgM antibodies are detectable at 4 to 5 weeks using ELISA assay. The level of IgE detection by antigens in serum specimens of patients is not affected by freezing or cooking temperatures and not all specimens had IgE specific for the allergen. Ani s1 at 24 kDa was a major secretory/excretory antigen detected using the western blotting (WB) method.

Chapter three: Identification of *A. pegreffii* in tigerflathead "Neoplatycephalus richardsoni" and presence of tropomyosin protein in raw extracts

Abstract

Background

A. pegreffii a nematode parasite in fish has been reported in association with human Anisakidosis. In recent years, it has also been recognized that allergenic responses to allergic proteins can occur in humans against both live anisakids and anisakids killed in contaminated food during cooking or freezing.

Aim

Tiger flathead is a particularly popular fish in the Australian cuisine, however, this fish demonstrates considerable infections with potentially zoonotic parasites. Few data exist on the possibility of hypersensitivity and allergic reaction to Anisakis parasite in Tiger flathead fish. Therefore, the aim of this current chapter is to establish *A. pegreffii* nematodes as a parasite in Tiger flathead from Victoria (Melbourne and Lakes Entrance) and to evaluate the antigenicity of tropomyosin in *A. pegreffii* extracts.

Methods

Larval anisakids collected from tiger flathead were characterised by employing a combined morphological examination and molecular approaches including; Genomic DNA, sequencing and phylogenetic analysis of the ITS-1 and ITS-2 regions. Morphological identification alone is known to not allow complete species-specific identification, particularly of larval stages, highlighting the need for molecular approaches. The molecular identification is frequently used to identify nematode groups. Furthermore, monoclonal anti-tropomyosin antibody, polyclonal anti-tropomyosin raised against crustaceans tropomyosin, in addition to anti-crustacean protein antibodies were used in this study to identify the presence of natural tropomyosin obtained from the L3 larvae of *A. pegreffii*.

Results

Both ITS-1 and ITS-2 regions were PCR-amplified from genomic DNAs from all *Anisakis* larval morph types and amplicons subjected to SSCP analysis. One amplicon from the same profiles (ITS-1 and ITS-2) were selected for sequencing. The alignments of the ITS-1 and ITS-2 sequences revealed that they were identical to the sequence available for *A. pegreffii* from the database.

Protein extract of *A. pegreffii* was prepared according to established protocols and proteins separated by SDS-PAGE. Tropomyosin protein was detected at 41 kDa MW using polyclonal anti-tropomyosin and anti-crustacean antibodies raised in rabbit by immunoblotting technique. However, tropomyosin from *A. pegreffii* did not respond to the monoclonal anti-tropomyosin antibody.

Conclusions

In conclusion, the results of this study have shown that morphological characterization does not allow for the reliable identification of Anisakis nematodes. However coupling morphology with genetic characterization using ITS-1 and ITS-2 of nuclear ribosomal DNA has provided suitable genetic markers for the accurate identification of the L3 larvae of *A. pegreffii* in this study. The polyclonal anti-tropomyosin and anti-crustaceans reacted with the tropomyosin of *A. pegreffii*. This indicates that there are similar epitopes on the tropomyosin protein of A. pegreffii and crustaceans, which allows for cross reactivity. However, there was no reaction with the monoclonal anti-tropomyosin of crustaceans by the tropomyosin of *A. pegreffii,* implying that the epitope against which this antibody was raised might not be present on *A. pegreffii* tropomyosin. This is an indication of variation in the antibody epitopes of the same protein in two different organisms.

3.1 Introduction

Ascaridoid nematodes of the genus *Anisakis* are zoonotic parasites infecting marine fishes worldwide. Human anisakiasis is caused by the consumption of raw, salted, pickled, smoked, or improperly cooked infected fish with third-stage *Anisakis* larvae (L3) that play the role of intermediate hosts (Couture *et al*., 2003). Recently, it has also been recognized that anisakiasis and/or allergic responses can occur in humans against live L3 or food in which worms have been killed by cooking or freezing (Moreno-Ancillo *et al*., 1997, Audicana *et al*., 2002c). Australia is a country of great diversity of fish and fishing seasons. The most popular fish targeted by fishermen due to its numbers and relative ease of capture is flatheads, which is distributed from Northern New South Wales to Western Victoria, including Tasmanian waters and a particularly popular fish in the Australian cuisine. Tiger flathead has considerable infections with high prevalence and intensity of anisakid nematodes including *Contracaecum type II, Hysterothylacium type IV, Hysterothylacium type VIII* and *A. pegreffii* (Shamsi *et al*., 2011b). Several studies have revealed that the first and second internal transcribed spacers (ITS-1 and ITS-2, respectively) of nuclear ribosomal DNA (rDNA) provide suitable genetic markers for the identification of anisakid species and PCR-SPSS of both ITS-1 and ITS-2 with phylogenetic sequence analysis (Jabbar *et al*., 2012) provides a powerful approach for exploring the genetic composition of anisakid populations and for investigating their biology.

Recently, in Italy and Japan it was shown that L3 of *A. pegreffii* is capable of infecting humans to cause Anisakidosis, which is in some cases accompanied by allergic reactions (Umehara *et al*., 2007; Fumarola *et al*., 2009). Symptoms of anisakiasis include nausea, intense abdominal pain and vomiting (Sakanari *et al*., 1989). This infection with L3 can also induce hypersensitivity IgE-mediated reactions with several clinical manifestations ranging from urticaria: angioedema to anaphylaxis (Audicana *et al*., 1995) These signs may be exposed primarily to somatic and excretory–secretory (ES) antigens from dead larvae in food or from the larva when it penetrates the tissue termed (gastroallergic anisakiasis), and produce excretory–secretory (ES) antigens against host or both. Currently 12 allergens have been identified in *A. simplex* (Table 2.4). Tropomyosin is a stable somatic muscle protein to resist heat treatment, which attributed to its exceptionally stable alpha helical coiled-coil secondary structure (Lopata *et al*., 2010). Tropomyosin has been identified from *A. simplex* and called (Ani s 3) (Asturias et al., 2000, Perez-Perez *et al*., 2000), and it has been

suggested that protein from *Anisakis* could be involved in human IgE response (Pascual *et a*l., 1997a, Martinez *et al*., 1997). Recently, patients with specific IgE antibodies to tropomyosin of shrimp, dust-mite, cockroach and snail also recognised *A. simplex* tropomyosin (Ani s 3) (Lopata, unpublished data). The aim of this chapter is to identify *A. pegreffii* in its third larval stage (L3), which parasitizes tiger flathead species, using combined morphological and molecular approaches and to confirm the presence of tropomyosin as a somatic protein, which could be associated with allergic reaction in human and IgE response.

3.2 Materials and methods

3.2.1 Collection of parasites

Tiger Flathead "*Neoplatycephalus richardsoni"* were purchased from the local seafood market in Melbourne and nematodes species were collected from the surface of internal cavity and organs (Liver, gut, stomach, gonads and pyloric caecum) as shown in Fig. 2.1.
The live nematodes were washed several times as follows: First with sterile water then with 0.9% saline solution, followed by 2% acetic then with sterile water and finally with 0.9% saline solution. They were then preserved in 0.9% saline solution and stored at 4°C for molecular sequencing and stored at -20°C for protein extraction.

3.2.2 Identification of parasite

Each worm was cut into three pieces namely, interior, posterior and middle parts. The interior and posterior parts from each nematode were then cleared in lactophenol for morphological examination and some for crude protein extraction, while the middle part was stored at -70°C for extraction genomic DNA and identification of species.

3.2.2.1 Morphological identification

Initial identification was made using light microscope to differentiate third stage larvae (L3) of *Anisakis larval type I* from other related nematodes based on several characters such as; size, the excretory and digestive systems (Cannon 1977, Deardorff, Overstreet 1981, Shamsi *et al*, 2009).

3.2.2.2 Extraction of total genomic DNA

Each Mid-body sections from individual nematodes were transferred to individual 1.5ml Eppendorf tubes each containing 500µl of extraction solution (containing 350µg/ml Proteinase K in extraction buffer (20mM Tris-HCL, pH 8.0, 100mM EDTA and 1%SDS)). The tubes were incubated for 18 hours. Genomic DNA was isolated using a standard sodium dodecyl sulphate (SDS)- Proteinase K method (Gasser et al., 1993) and purified directly using Wizard TM DNA Clean-Up columns (Promega) according to the manufacturer's recommendations. Control DNA samples were prepared from host tissue using the same method as for parasites.

3.2.2.3 Preparing λ DNA (PstI) marker

It was a mixture of 900 µL sterile dH_2O, 107.53 µLλDNA (50 µg), 90 µL buffer H (10X) and 18 µL *Pst*I (100U/ µL). The mixture was then incubated overnight at 37 °C. The

mixture was added with 90 µL of loading dye prior to use. The whole mixture was aliquoted into 100 ul using 1.5 ml tubes and stored in -20°C.

3.2.2.4 Amplification of nuclear ribosomal DNA regions (ITS-1 and ITS-2) by polymerase chain reaction (PCR)

The first and second internal transcribed spacers of nuclear ribosomal DNA (ITS-1 and ITS-2, respectively) were chosen for use in this study as they provide species-specific markers for anisakid nematodes (e.g., D´Amelio et al., 1994; Zhu et al., 1998a). Based on the rDNA sequences of anisakid nematodes in the GeneBank™ database, two primers were designed to amplified ITS-1 and ITS-2 genes. The ITS-1 was amplified by PCR using the primer set SS1: 5´-GTTTCCGTAGGTGAACCTGCG-3´ (forward) and NC13R: 5´-GCTGCGTTCTTCATCGAT-3´ (reverse), while ITS-2 was amplified using primer set SS2: 5´-TTGCAGACACATTGAGCACT-3´ (forward) and NC2: 5´-TTAGTTTCTTTCCTCCGCT-3´ (reverse). 2µl of genomic DNA (20ng) were added directly to 48µl PCR mix (overlaid with paraffin oil) containing 250µM of each dNTP, 3.5Mm MgC12, 25pmo1 of each primer and 0.5U of Taq polymerase (promega), placed immediately on a freeze block and then subjected to PCR in a 480 thermal cycler (Perkin Elmer). The cycling conditions were: initial denaturation in 94°C for five minutes, then 94°C for 30 seconds (denaturation), 55°C (ITS-1) or 53°C (ITS-2) for 30 seconds (annealing) and 72°C for 30 second (extension) for 35 cycles, followed by 72°C for 5 minutes. The same conditions were used to amplify ITS-2, expect that the annealing temperature was 53°C. PCR products were separated in 1.5% agarose gel (Sambrook et al., 1989) at 100V in Tris/Boric Acid/EDTA Buffer (TBE: 65mM Tris-HCL, 27mM boric acid, 1mM EDTA, pH 9; Bio-Rad) for 1 hour and stained with ethidium bromide. 174-Hae (Promega) was used as the molecular weight marker on orange gel. A control negative sample without DNA was included in each PCR, and host DNA was subjected to the same amplification procedure as for the parasite DNA.

3.2.2.5 Single-Strand Conformation Polymorphism (SSCP) analysis

Given the large number of anisakid nematodes, larvae collected and examined in this study, sequencing of all specimens would have been time consuming and expensive. Besides, it could have resulted in numerous identical sequences. Hence, single strand conformation polymorphism (SSCP) analysis (Orita et al., 1989) was used for screening ITS-1 and ITS-2 amplicons for sequence variation among all individuals of anisakid nematodes collected. In this technique, single-strand PCR products run on a non-denaturing gel can be

differentiated not only based on their size but also their structure (Gasser and Chilton, 2001). The SSCP method used followed that described previously by Gasser et al., (2004) with some modification: 5µl of distilled water was mixed with 10µl of DNA sequencing stop solution; after denaturation at 94°C for 15 minutes and immediate snap freezing on a freeze block (-20°C), 12µl of the products were loaded on a SSCP gel (Elchrom Scientific) and run for 18 hours for ITS-1 and ITS-2, the gel was stained with super gold for 30 minutes and destained in distilled water for 5 minutes. Samples were selected for sequencing based on the variation shown in their profiles, host species and their geographical location.

3.2.2.6 Purification of amplicons

PCR product of 50 µL reaction was loaded to agarose gel and allowed to run by electroporation as mentioned in 3.2.2.1. Purification of DNA from agarose gel and PCR reaction was performed using Isolate II PCR and Gel kit (Bioline, Australia) following the manufacturer's instructions. Briefly in gel purification, the band was detected by visualising the gel under UV. The desired band was excised using a clean scalpel and loaded into the 1.5 Eppendorf tube. The excised band mass was then determined by weighing the tube before and after the gel slice. Each 100 mg of the slice was dissolved in 200 binding buffer by incubating the tube at 50 ^{0}C for 5-10 minutes with vortexing every 2-3 min until the gel completely melted. For PCR reaction purification, all 50 µL of PCR was added to 100 µL of binding buffer. After these steps, both the dissolved gel and PCR were then preformed with same procedure. The mixture was loaded into gel columns set on collection tubes and centrifuged at 11000xg for 30 sec. The filtrated solution was discarded and the column was washed twice with 700 µL of washing buffer, the filtrate was also discarded after centrifuging the column at 11000xg for 30 sec each time. The column's silica membrane was dried by centrifugation at 11000 for a minute. The purified DNA was eluted with 25 µl of elution buffer or H_2O, centrifuged at 11000 xg for a minute and then stored at - 20°C.

3.2.2.6.1 Preparation of purified amplicons for sequencing

The sequencing was performed by the Australian Genome Research Facility (AGRF). Purified DNA concentration was measured by Nanodrop 2000 as mentioned above. The DNA amount was adjusted according to the instructions of AGRF based on the DNA length. The amount of the DNA was made in a volume of 11 µL Nuclease-Free Water (Qiagen) and added to 1 µL of either forward or reverse primer. The total mixture of the

DNA and primer (12 µL) was sent to AGRF by Australian express post. It can be received by AGRF within 16 – 22 hours.

3.2.3 Preparation of raw extracts (protein)

3.2.3.1 0.1M Tris 0.5M Glycine buffer (pH=8.7)

About 30 posterior and interior of third stage larvae (25mg in total) were placed in liquid nitrogen and grinded thoroughly with motor and pestle. After that, the "worm" powder was homogenized in 2.5ml of 0.1M Tris (hydroxymethy1) Aminomethane Tris 0.5M Glycine buffer (pH=8.7). This extract was transferred to 15ml falcon tubes.

Disruption and agitation of this extract were achieved by sonication, followed by centrifugation for 5 minute at full speed. Subsequently, a pure supernatant of *Anisakis* extract was obtained and aliquoted into four 1.5ml microcentrifuge tubes.

3.2.3.2 Protein estimation by Bradford method

Bradford assay was used to determine the concentration of protein contents in the extracts. In this assay, the standard protein is usually Bovine serum Albumin (BSA) of known concentration that binds to Coomassie brilliant blue G-250 dye, which converted the red form to a stable blue form upon binding to the protein. After that the colorimetric reaction was measured in terms of absorbance at 595nm using spectrophotometer and based on these absorbance values, a standard curve is generated, which was subsequently used to calculate the concentration of the sample proteins (Bradford, 1976).

Quick start Bradford protein assay kit (Bio-Rad, USA) was used to quanttify the proteins in the raw extracts of *Anisakis* spp, this kit contains a set of seven concentrations of BSA (2, 1.5, 1, 0.75, 0.5, 0.25, 0.125mg/ml) as standard proteins. Serial dilutions of the samples (1:2, 1:4, 1:8, 1:16) were prepared using PBS buffer, which was also used as a blank. 5µl of the seven concentrations of BSA, diluted samples and PBS were pipetted into 96 wells 300 µl microplate in duplicates, then 200µl of Bradford regent was added to these wells using a multichannel pipette. Then Thermo Scientific Multiskan Ascent Microplate Photometer (Bio-Rad, USA) was used to read the absorbance values at 595nm.

Also, the same methods have been applied to quantify the proteins in the Black tiger prawns (*Peraeus Monodon*) heat extracted and codfish (*Gadus morhua*) heat extracted, which served as positive and negative controls, respectively in the immunoblotting assay.

3.2.4 Simples's preparation for SDS-PAGE.

To avoid denaturation of the proteins, the samples were prepared just 10 minutes before applying them onto the gel. Depending on the result of the Bradford assay, different protein amounts of each extract were prepared by diluting them in PBS to obtain a final volume of 20µl of a 2 mg/ml of denatured protein per sample well, then 5µl of 5x sample buffer were added and heated in a water bath or heater at 100°C for 5 minutes. After that, a volume of 25µl of the sample was loaded into wells using special loading tips. 5µl of protein molecular marker, Precision Plus Protein Dual Color Standards (Bio-Rad, USA) was loaded in the first well of the gel.

3.2.4.1 SDS-PAGE: Running conditions and Staining

Electrophoresis apparatus was connected to the power supply and the voltage set in two stages, first stage at 80 volts for 10 minutes until the protein reached the top of the resolving gel and the second stage at 160 volts for 60 minutes until the protein reached the bottom of the gel glasses.

Following the electrophoresis, the gel stained with fresh Coomassie blue stain solution for 30 minutes at room temperature under gentle shaking and destaining with shaking in destaining solution for 90 minutes with changing this solution every 20 minutes. To visualize the proteins bands, the stained gel is scanned in Gel Doc EQ system (Bio-Rad, USA).

3.2.4.2 Coomassie blue staining

When the electrophoresis was completed, the stacking gel region was cut out using a scalpel blade and the gel removed from the glass plates. The gel was stained with about 20ml Coomassie blue stain for 1 hour on a rotating shaker at room temperature. Afterwards, the stain was removed and the gel was washed with distilled water, the gel was then placed on transparency paper and scanned.

3.2.4.3 Immunblotting Analysis

The SDS-PAGE electrophoresis was performed again for the protein samples using the Kaleidoscope pre-stained marker. After that, the proteins were transferred from SDS-PAGE gel to a nitrocellulose membrane using the iBlot TM Dry Blotting System (Invitrogen). First, the anode stack was placed on the tray, and the SDS-PAGE gel was overlaid on the top. Second, a pre-soaked iBlot TM filter paper in deionized water was placed on the top, and air bubbles were removed using the blotting roller. Third, the cathode was placed with

the electrode side facing up, and the disposable sponge was positioned on the top with the metal contact on the upper side. Finally, the lid was closed on the assembled stacks and the blotting process started.

3.2.4.4 Immunological Analysis of tropomyosin of *Anisakis Pegreffii*

3.2.4.4.1 Immunoblotting with anti-tropomyosin monoclonal antibody

After removing the nitrocellulose membrane from the device, it was placed in a plastic container bag and blocked with 50ml in 5% (w/v) skim milk in TBS for 1 hour on the shaker at room temperature, followed by washing three times with TBS for 5 minutes each time. Then, the membrane was placed in a plastic bag and incubated overnight at 4°C or 2 hours at room temperature with 10ml of the rat monoclonal anti-tropomyosin antibody diluted 1:6000 with 1% (w/v) skim milk in TBS 0.5 Tween20 (primary antibody). Subsequently, the membrane was placed in a plastic container bag and washed three times with 20ml TBS-T for 5 minutes each time, then incubated for 2 hours at room temperature with 10ml of the rabbit anti mouse antibody conjugated to biotin (Abcam, USA) and diluted 1:8000 with 1% (w/v) skim milk in TBS 0.5 Tween (secondary antibody). The membrane was washed 3 times with TBS containing 0.05% Tween20, and then the immunoblot was developed using enhanced chemiluminescent (ECL) method.

The membrane blot was incubated with chemiluminescent Peroxidise Substrate-3 (sigma-Aldrich, (USA) in a dark room for 3-5 minutes. Than it was placed between transparent sheets inside a photographic cassette. An X-ray film, Amersham Hyperfilm ECL (GE healthcare, UK) was then placed over the membrane. During this step, several exposure times (1-20 min) were tested to obtain optimal results. The exposed film was then placed in a developer solution, Kodak GBX developer replenisher (sigma-Aldrich, USA) for 1 min and then rinsed with water. Then it was placed in the fixer solution, Kodak GBX fixer replenisher (sigma-Aldrich, USA) for 3-5 min, then rinsed with water and allowed to dry and scanned.

To ensure the successful transferring of the protein, the immunblot membrane was stained in Coomassie Blue for 20 seconds and destained for 12 minutes then scanned.

3.2.4.4.2 Immunoblotting with anti-tropomyosin polyclonal antibody

After removing the nitrocellulose membrane from the device, it was placed in a plastic container bag and blocked with 50ml in 5% (w/v) skim milk in TBS for 1 hour on the shaker at room temperature, followed by washing three times with TBS for 5 minutes each time. Then, the membrane was placed in a plastic bag and incubated overnight at 4°C or 2 hours at room temperature with 10ml of the rabbit polyclonal anti-tropomyosin antibody produced in mouse diluted 1:40,000 with 1% (w/v) skim milk in TBS 0.5 Tween20 (primary antibody). Subsequently, the membrane was placed in a plastic container bag and washed three times with 20ml TBS-T for 5 minutes each time, then incubated for 2 hours at room temperature with 10ml of the goat anti rabbit IgG, HRP conjugate polyclonal antibody, (DAKO, USA) and diluted 1:40,000 with 1% (w/v) skim milk in TBS 0.5 Tween (secondary antibody). The membrane was washed 3 times with TBS containing 0.05% Tween20, and then the immunoblot was developed using enhanced chemiluminescent (ECL) method.

The membrane blot was incubated with chemiluminescent Peroxidise Substrate-3 (sigma-Aldrich, (USA) in the dark room for 3-5 minutes. Than it was placed between transparent sheets inside a photographic cassette. An X-ray film, Amersham Hyperfilm ECL (GE healthcare, UK) was then placed over the membrane. During this step, several exposure times (1-20 min) were tested to obtain optimal results. The exposed film was then placed in a developer solution, Kodak GBX developer replenisher (sigma-Aldrich, USA) for 1 min and then rinsed with water. Then it was placed in the fixer solution, Kodak GBX fixer replenisher (sigma-Aldrich, USA) for 3-5 min, then rinsed with water and allowed to dry and scanned.

To ensure the successful transfer of the proteins, the immunoblot membrane was stained in Coomassie Blue for 20 seconds and distained for 12 minutes then scanned.

3.2.4.4.3 Immunoblotting with anti-crustacean polyclonal antibody

After removing the nitrocellulose membrane from the device, it was placed in a plastic container bag and blocked with 50ml in 5% (w/v) skim milk in TBS for 1 hour on the shaker at room temperature, followed by washing three times with TBS for 5 minutes. Then, the membrane was placed in a plastic bag and incubation at 4°C overnight or 2 hours at room temperature with 10ml of the rabbit polyclonal anti-crustacean antibody produced in mouse diluted 1:40,000 with 1% (w/v) skim milk in TBS 0.5 Tween20 (primary

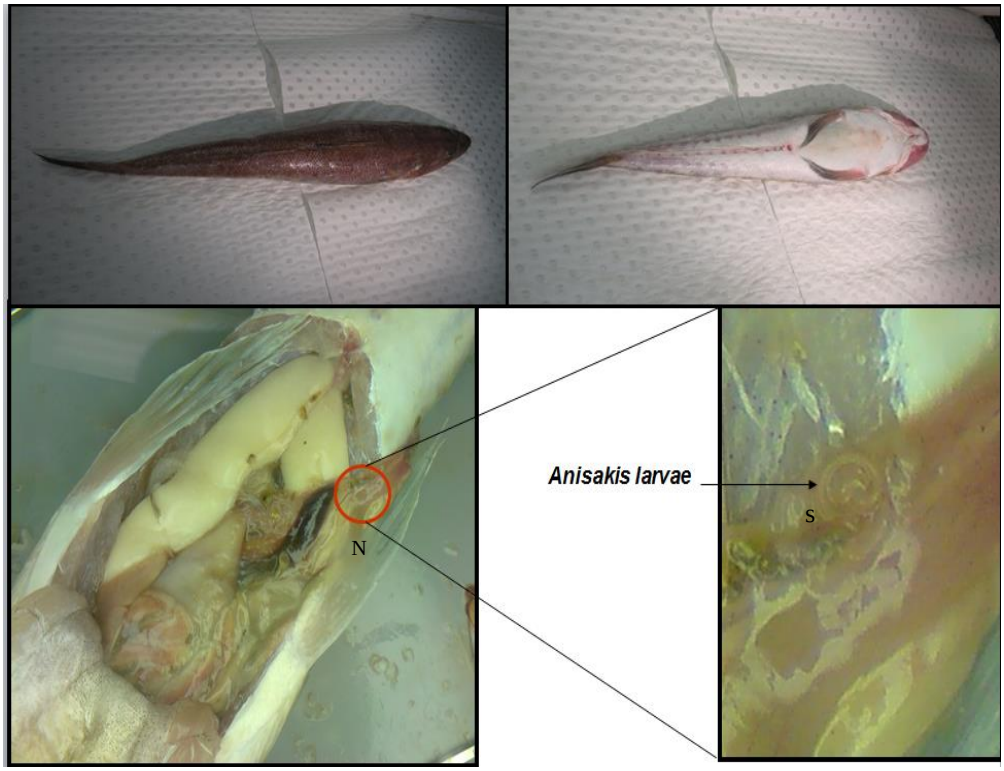
antibody). Subsequently, the membrane was placed in a plastic container bag and washed three times with 20ml TBS-T for 5 minutes each time, then incubated for 2 hours at room temperature with 10ml of the Goat anti rabbit IgG, HRP conjugate polyclonal antibody, (DAKO, USA) and diluted 1:40,000 with 1% (w/v) skim milk in TBS 0.5 Tween (secondary antibody). The membrane was washed 3 times with TBS containing 0.05% Tween20, and the immunoblot developed using enhanced chemiluminescent (ECL) method.

The membrane blot was incubated with chemiluminescent Peroxidise Substrate-3 (sigma-Aldrich, (USA) in the dark room for 3-5 minutes. Than it was placed between the transparent sheets inside a photographic cassette. An X-ray film, Amersham Hyperfilm ECL (GE healthcare, UK) was then placed over the membrane. During this step, several exposure times (1-20 min) were tested to obtain optimal results. The exposed film was then placed in developer solution, Kodak GBX developer replenisher (sigma-Aldrich, USA) for 1 min and rinsed with water. Then it was placed in the fixer solution, Kodak GBX fixer replenisher (sigma-Aldrich, USA) for 3-5 min, then rinsed with water and allowed to dry and scanned.

To ensure the successful transfer of the proteins, the immunoblot membrane was stained in Coomassie Blue for 20 seconds and destained for 12 minutes and then scanned.

3.3 Results

3.3.1 *Anisakis* third stage larvae (L3) in Tiger flathead fish "*Platycephalus richardsoni*".



Keys:
N= Anisakis nematodes on the surface of internal organs
S= Anisakis nematodes inside the mesenteric membrane

Figure 3.1 Anisakid nematodes on the surface of internal organs and inside the mesenteric membrane of Tiger flathead fish.

3.3.2 Identification of *Anisakis* species using different methods

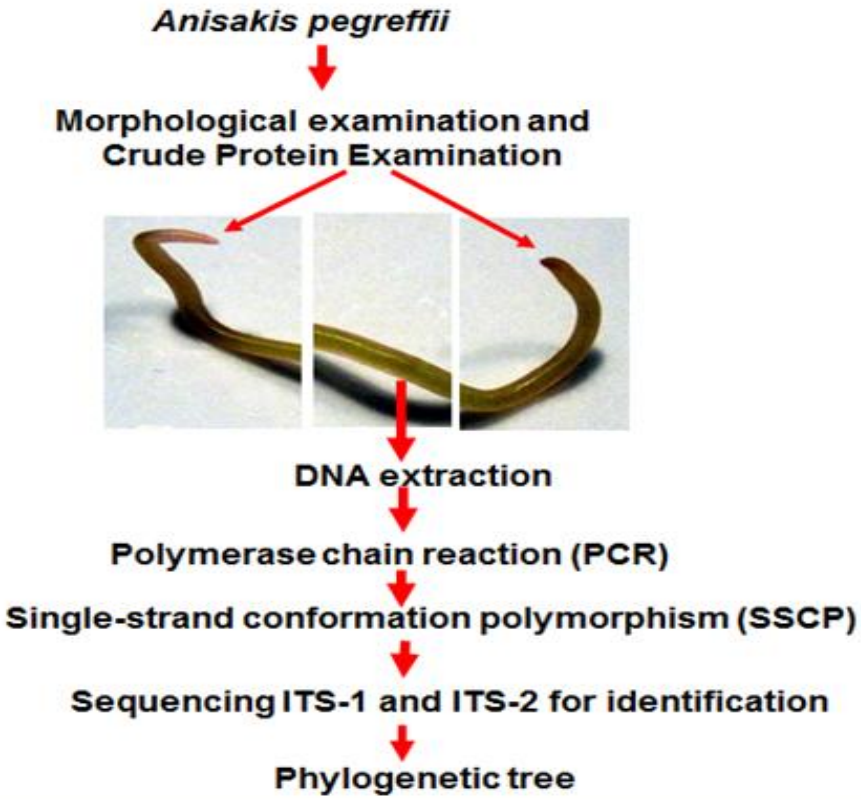


Figure 3.2: Methods for identifying Anisakis third stage larvae (L3)

3.3.3 The sequential steps of the molecular method of identifying *Anisakis* third stage larvae (L3)

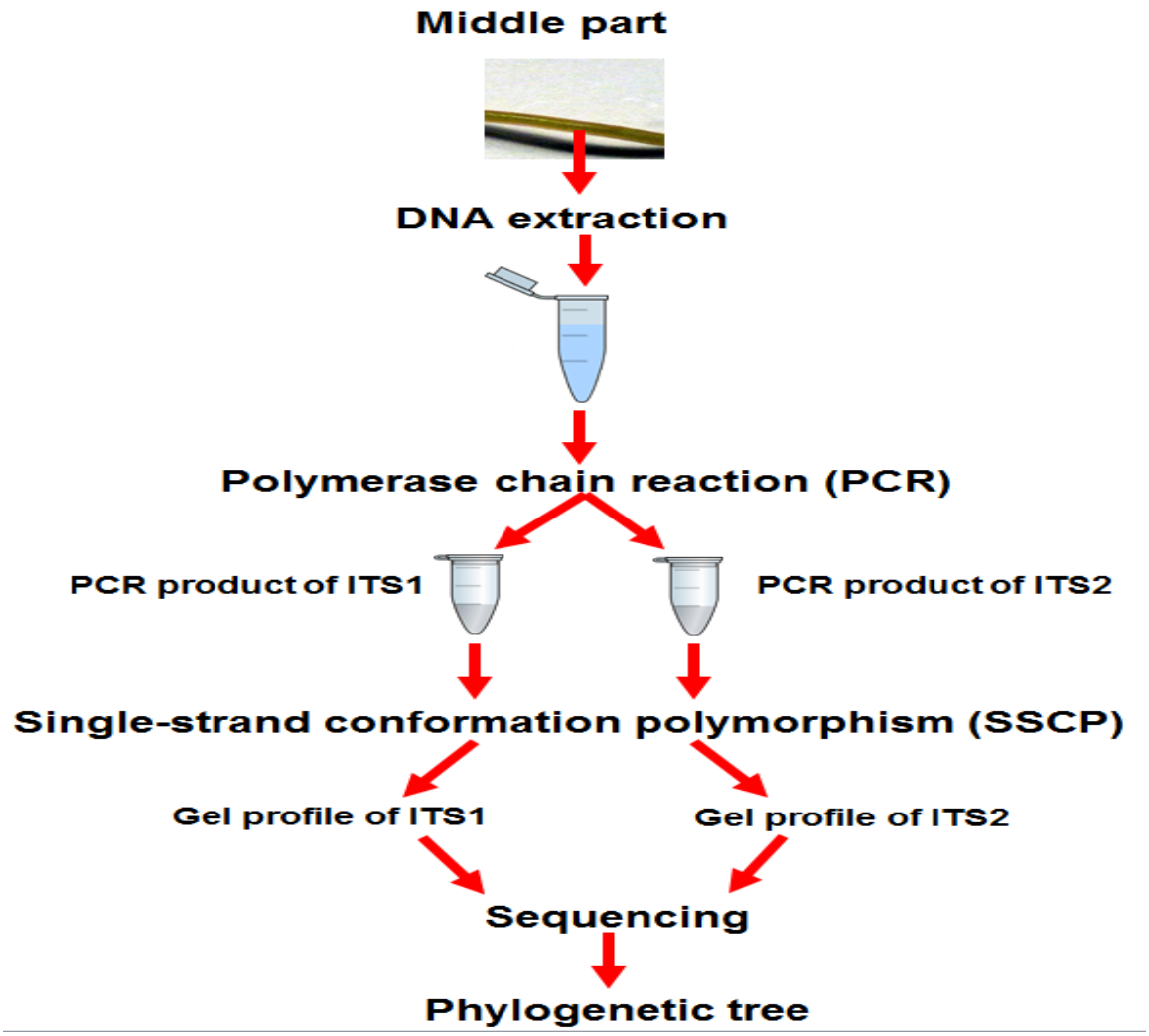


Figure 3.3 Steps in molecular identification of Anisakis third stage larvae (L3) using the mid-section of the larvae.

3.3.4 Morphological identification of *Anisakis* species

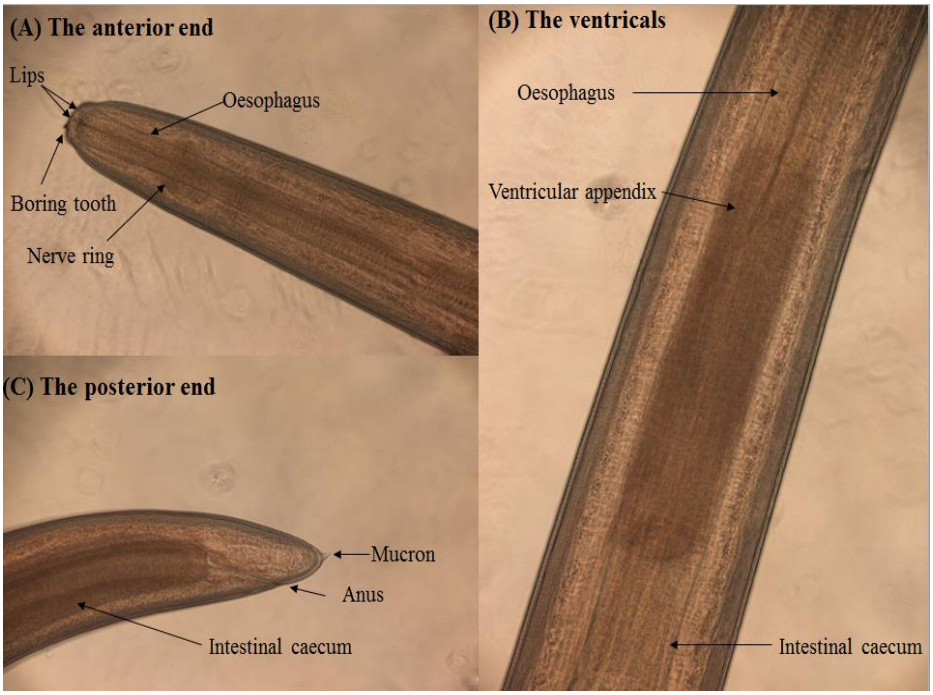


Figure 3.4 The morphological characterization of Anisakis spp larva using Fluorescent Microscope (10x times magnification).

3.3.5 Molecular identification

3.3.5.1 Lambda (λ) DNA (PstI) marker

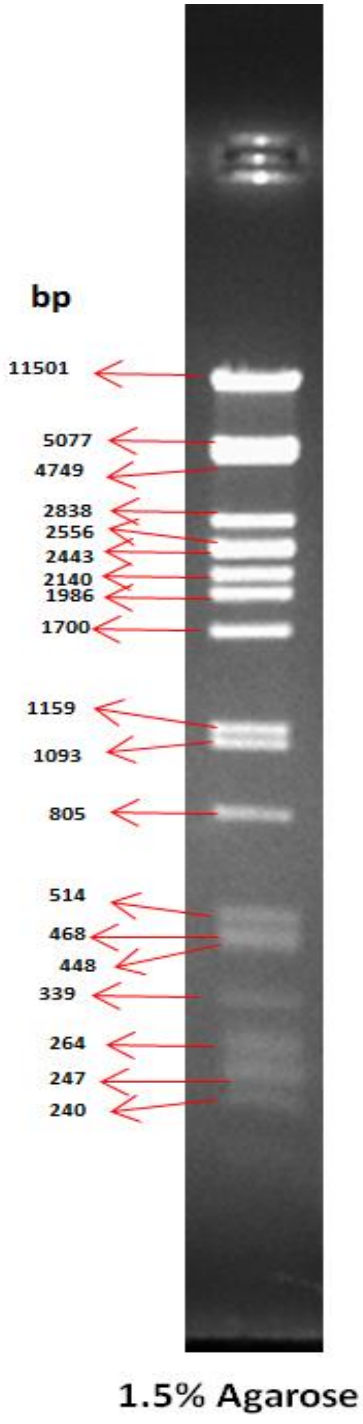


Figure 3.5 Lambda(λ) DNA (PstI) marker

3.3.5.2 Amplification of nuclear ribosomal DNA regions (ITS-1 and ITS-2) by polymerase chain reaction (PCR)

The first and second internal transcribed spacers of nuclear ribosomal DNA (ITS-1 and ITS-2) from *A. pegreffii* were amplified using PCR as shown in Figure 3.6.

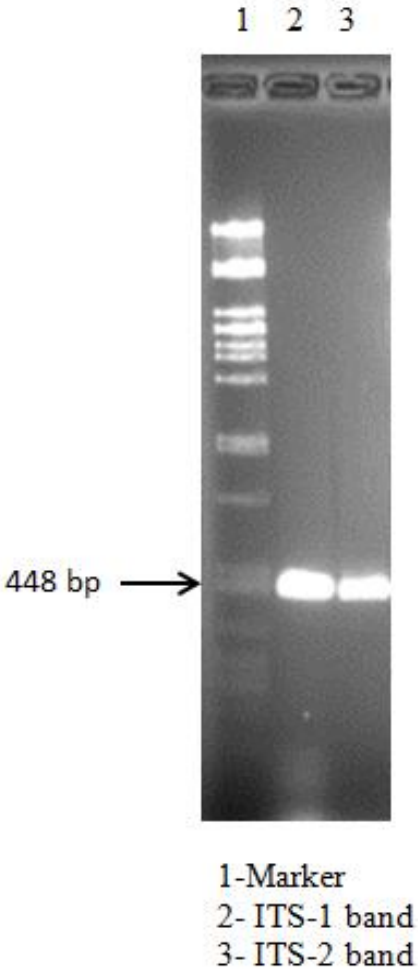


Figure 3.6: Full ITS-1 and ITS-2 PCR products amplified from *A. pegreffii* with ITS-1 and ITS-2 primers.

3.3.5.3 Single-Strand Conformation Polymorphism (SSCP) analysis

Representatives of ITS-1 and ITS-2 amplicons from each sample were screened for sequence variation using SSCP analysis for the *Anisakis* nematodes collected. (Figures 3.7). Also, one representative sample from each similar group band of ITS-1 and ITS-2 were selected for sequencing.

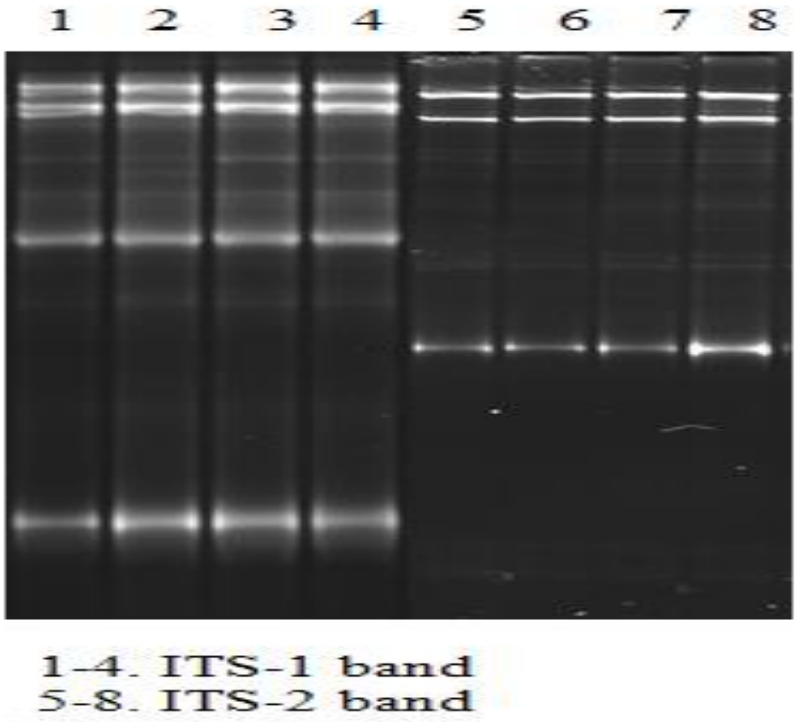


Figure 3.7 Full ITS-1 and ITS-2 PCR products amplified from *A. pegreffii* with SSCP.

3.3.5.4 Comparison of the ITS-1 and ITS-2 sequences of *A. pegreffii* from tiger flathead fish with the ITS-1 and ITS-2 sequences of allergenic *A. pegreffii* from two other fish species

It was known that marinated anchovies and Chub mackerel infected with *A. pegreffii* cause Anisakisis disease. Therefore, alignment of *A. pegreffii* ITS1 and ITS-2 sequences from tiger flathead was compared with alignment of *A. pegreffii* ITS1 and ITS-2 sequences from marinated anchovies and Chub mackerel as shown in Figure 3.8. Result shows the similarity between three sequencings were 99% percent.

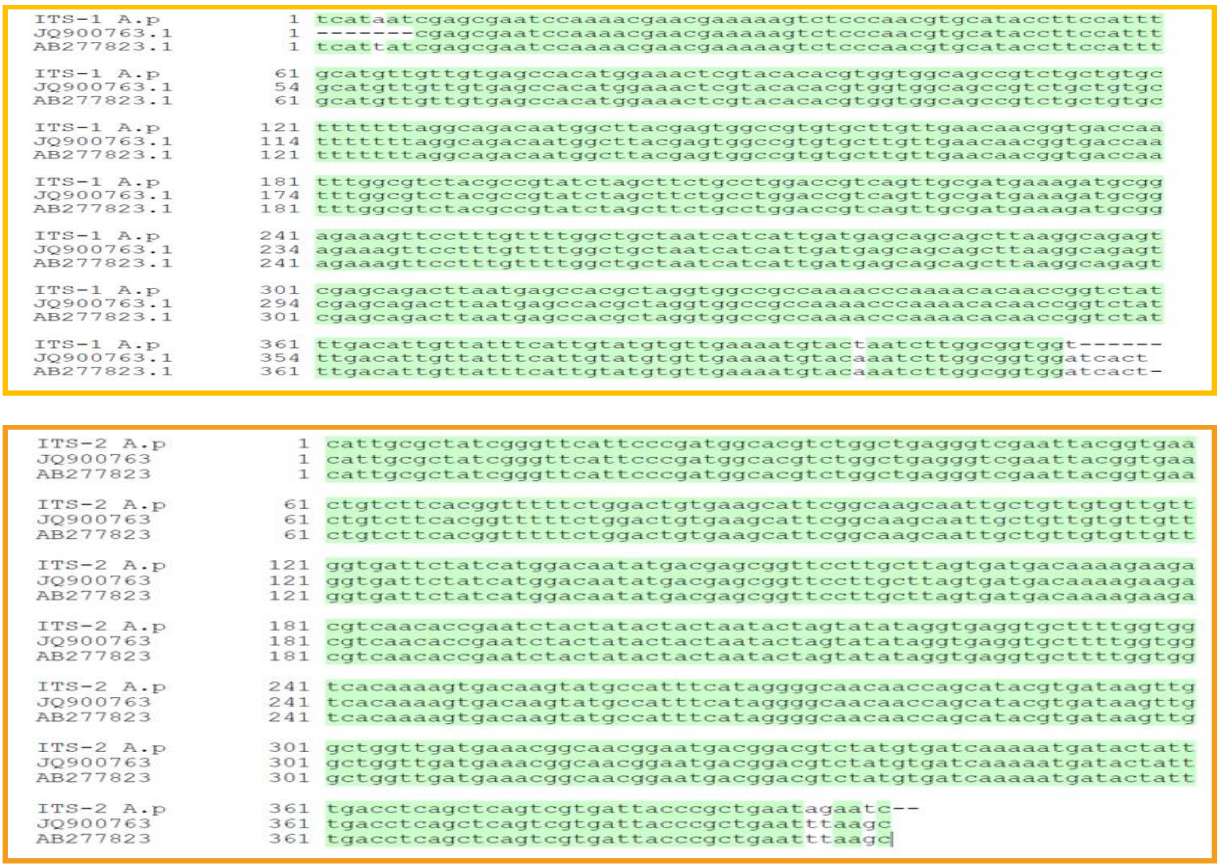


Figure 3.8 Alignment of ITS1 and ITS-2 sequences of *A. pegreffii* from tiger flathead with ITS1 and ITS-2 sequences of *A. pegreffii* from marinated anchovies and Chub mackerel.

3.3.5.5 Sequencing of Anisakis pegreffii ITS-1 and ITS-2 amplicons and phylogenetic tree

Sequences of ITS-1 and ITS-2 of *Anisakis pegreffii* from tiger flathead were compared and aligned to closely matching sequences in Genbank using BioEdit (version 7.0) and CLUSTER W (version 1.7, Thompson et al. 1994). The results show that ITS-1 and ITS-2 of *Anisakis pegreffii* were close to *Anisakis simplex* C and *A. simplex s.s*, as indicated in the phylogenetic tree in figure 3.9a,b.

A- *Anisakis pegreffii* ITS-1 sequencing and relationships between ITS-1 from *Anisakis pegreffii* with ITS-1 from other *Anisakis* parasitic nematodes.

TCATAATCGAGCGAATCCAAAACGAACGAAAAAGTCTCCCAACGTGCATACCT
TCCATTTGCATGTTGTTGTGAGCCACATGGAAACTCGTACACACGTGGTGGCAG
CCGTCTGCTGTGCTTTTTTTAGGCAGACAATGGCTTACGAGTGGCCGTGTGCTT
GTTGAACAACGGTGACCAATTTGGCGTCTACGCCGTATCTAGCTTCTGCCTGGA
CCGTCAGTTGCGATGAAAGATGCGGAGAAAGTTCCTTTGTTTTGGCTGCTAATC
ATCATTGATGAGCAGCAGCTTAAGGCAGAGTCGAGCAGACTTAATGAGCCACG
CTAGGTGGCCGCCAAAACCCAAAACACAACCGGTCTATTTGACATTGTTATTTC
ATTGTATGTGTTGAAAATGTACTAATCTTGGCGGTGGTTCAAC

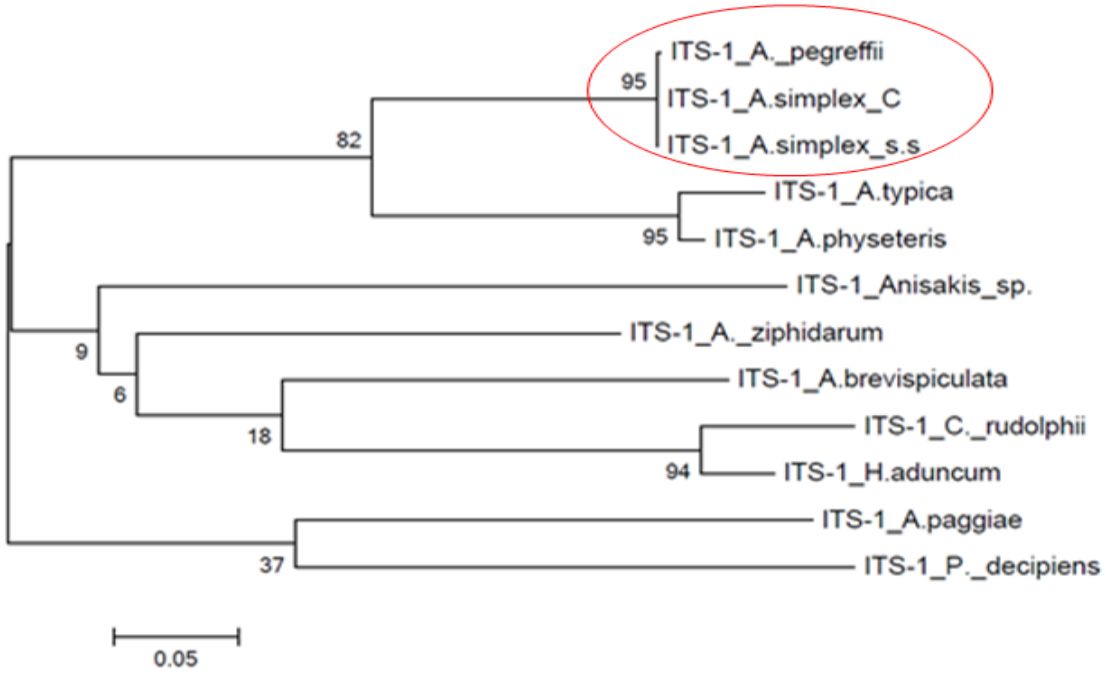


Figure 3.9a ITS-1 sequence of *A. pegreffii* from tiger flathead fish and the phylogenetic tree derived from alignment of sequences of ITS-1 of Anisakids using MEGA6 and BioEdi software.

B- *Anisakis pegreffii* ITS-2 sequencing and relationship between ITS-2 from *Anisakis pegreffii* with ITS-2 from other *Anisakis* parasitic nematodes.

ACGACATTGCGCTATCGGGTTCATTCCCGATGGCACGTCTGGCTGAGGGTCGAA
TTACGGTGAACTGTCTTCACGGTTTTTCTGGACTGTGAAGCATTCGGCAAGCAA
TTGCTGTTGTGTTGTTGGTGATTCTATCATGGACAATATGACGAGCGGTTCCTTG
CTTAGTGATGACAAAAGAAGACGTCAACACCGAATCTACTATACTACTAATAC
TAGTATATAGGTGAGGTGCTTTTGGTGGTCACAAAAGTGACAAGTATGCCATTT
CATAGGGGCAACAACCAGCATACGTGATAAGTTGGCTGGTTGATGAAACGGCA
ACGGAATGACGGACGTCTATGTGATCAAAAATGATACTATTTGACCTCAGCTCA
GTCGTGATTACCCGCTGAATAGAATC

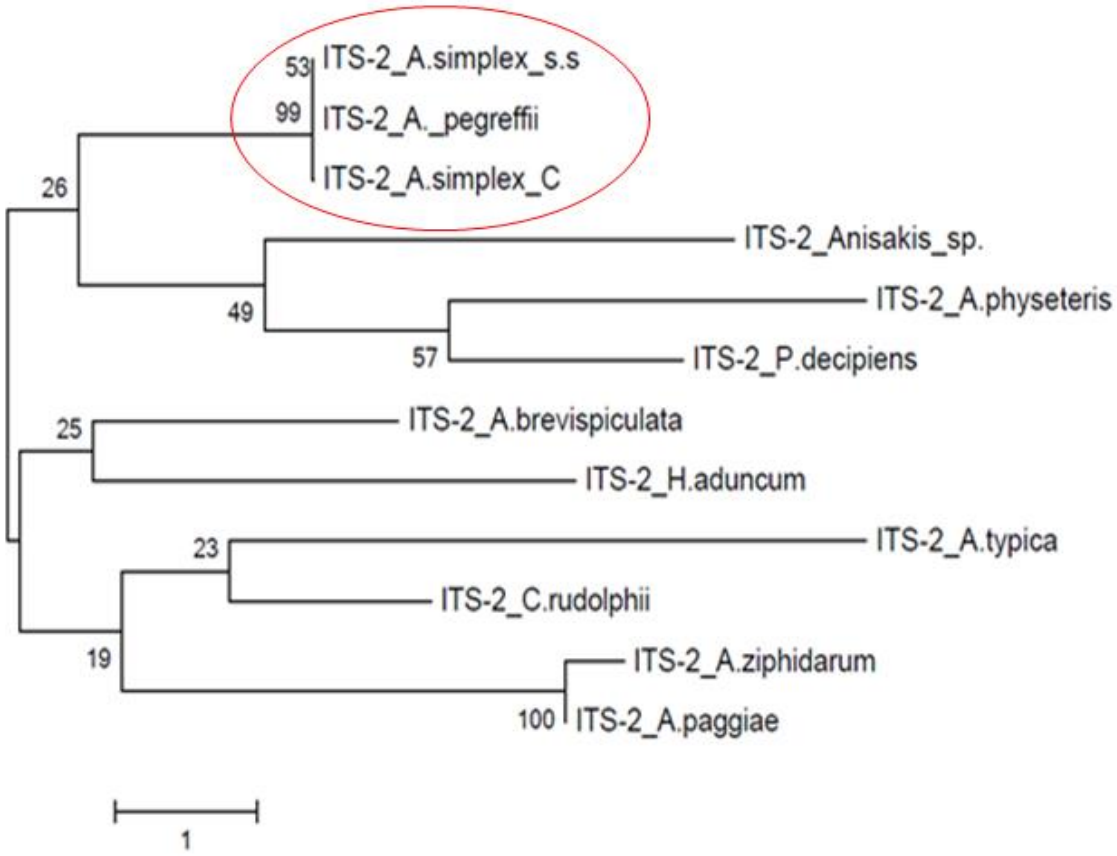
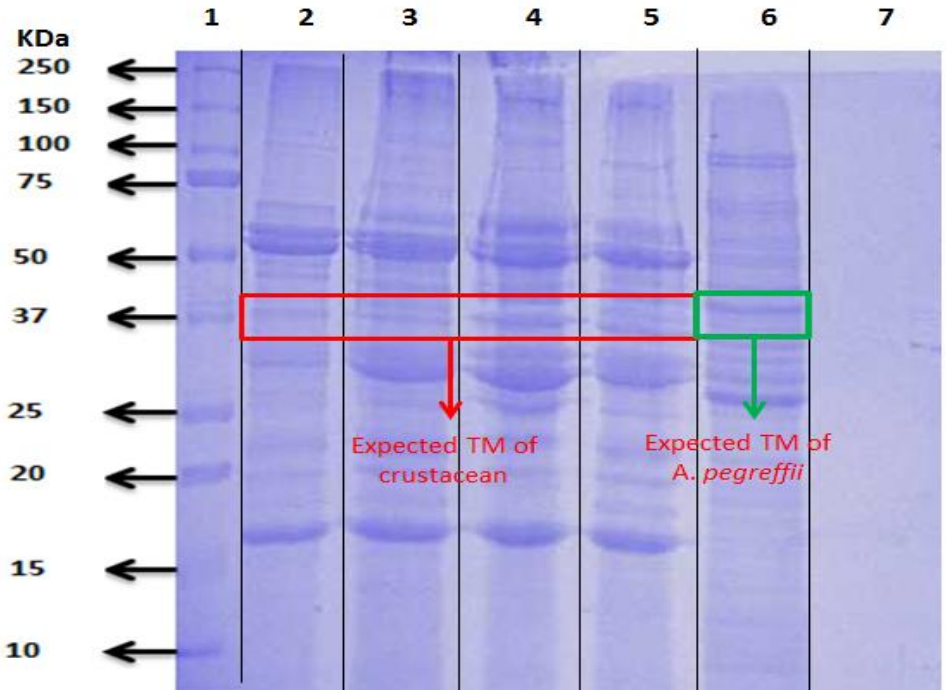


Figure 3.10b ITS-2 sequence of *A. pegreffii* from tiger flathead fish and the phylogenetic tree derived from alignment of sequences of ITS-2 of Anisakids using MEGA6 and BioEdi software.

3.3.5.6 Shellfish and *A. pegreffii* protein analysis

The extract of whole proteins from shellfish and *A. pegreffii* in 0.1M Tris 0.5M Glycine buffer showed many darker and wider bands particularly in the size between 30-50 kDa on the typical SDS-PAGE mini gel as shown in figure 3.10.

Also, it is clear that native tropomyosin of this nematode have higher molecular weight (~41kDa- green box) than the crustacean native tropomyosin (~ 37kDa- red box).



Keys:

L= Lane

L1=Marker

L2 =Black Tiger (positive control)

L3=King Prawn (positive control)

L4=Green Prawn (positive control)

L5=Banana Prawn (positive control)

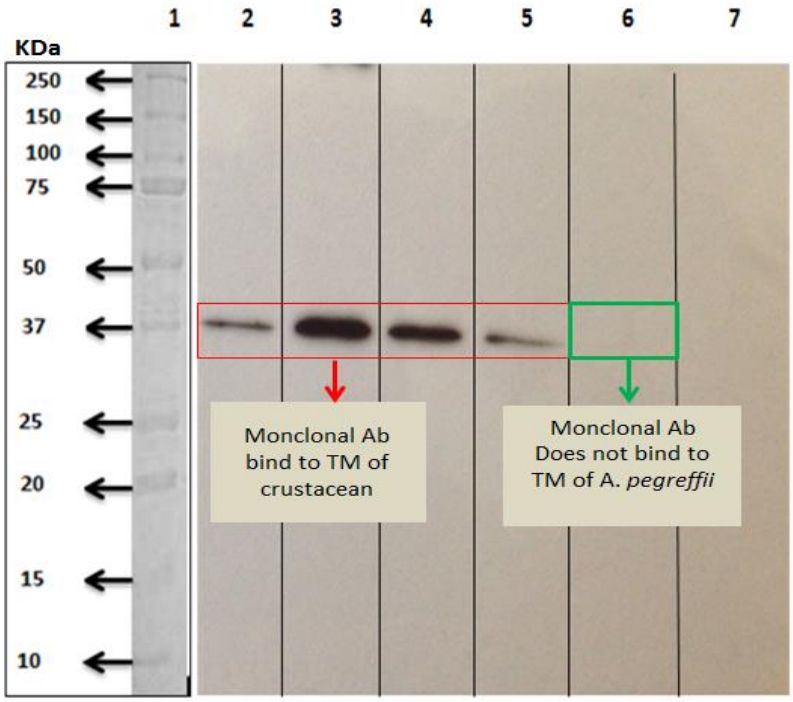
L6=*Anisakis pegreffii*

L7=PBS (negative control).

Figure 3.11 Comparison of crustacean native tropomyosin (37kDa) inside the red box with possible native tropomyosin band of *A. pegreffii* (41kDa) inside the green box on SDS-PAGE.

3.3.5.7 Immunoblotting with anti-tropomyosin monoclonal antibody

10 µg of extracted proteins from shellfish and *Anisakis pegreffii* were loaded onto the gel and then electro-transferred to the blotting membrane. Using the monoclonal tropomyosin antibody, a distinct dark band at 37kDa was observed as a positive control in the lanes 2, 3, 4 and 5 (inside green box). However, none of the tested *Anisakis pegreffii* extracted protein has revealed reactivity with this antibody (lane 6) as shown inside the red box (Figure 3.11), even with prolonged exposure period (2 days).



Key:

L= Lane

L1= Marker

L2= Black Tiger (positive control)

L3= King Prawn (positive control)

L4= Green Prawn (positive control)

L5= Banana Prawn (positive control)

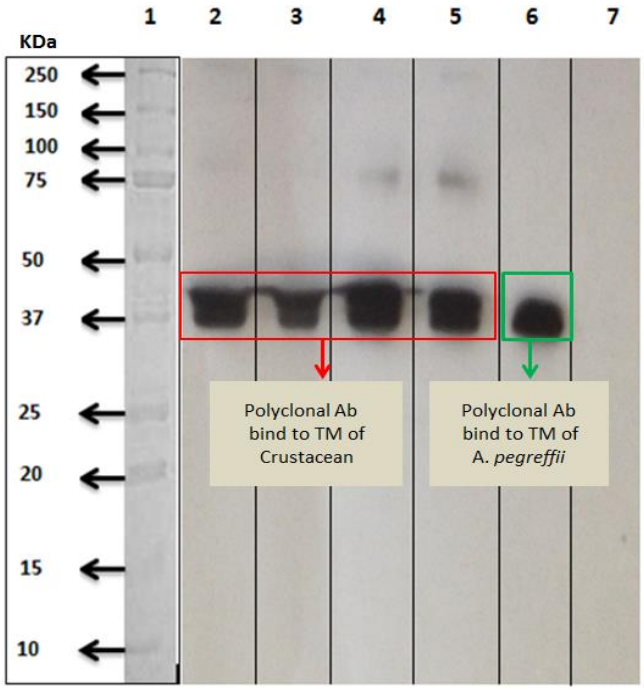
L6= *Anisakis pegreffii*

L7= PBS (negative control)

Figure 3.12 Immunoblotting profiles using mouse anti tropomyosin monoclonal antibody against native crustacean tropomyosin and *A. pegreffii* tropomyosin.

3.3.5.8 Immunoblotting with anti-tropomyosin polyclonal antibody

Extracted proteins (10 µg), from shellfish and *A. pegreffii*, were loaded onto the gel and then electro-transferred to the immunoblot membrane. Using anti- tropomyosin polyclonal antibody, a distinct dark band at 37kDa was observed as a positive control in the lanes 2, 3, 4 and 5 (inside red box). Also, the tested *A. pegreffii* extracted protein has revealed reactivity with this antibody and showed a single band of approximately 41kDa (lane 6) as shown inside green box (Figure 3.12).



Key:

L= Lane

L1= Marker

L2= Black Tiger (positive control)

L3= King Prawn (positive control)

L4= Green Prawn (positive control)

L5= Banana Prawn (positive control)

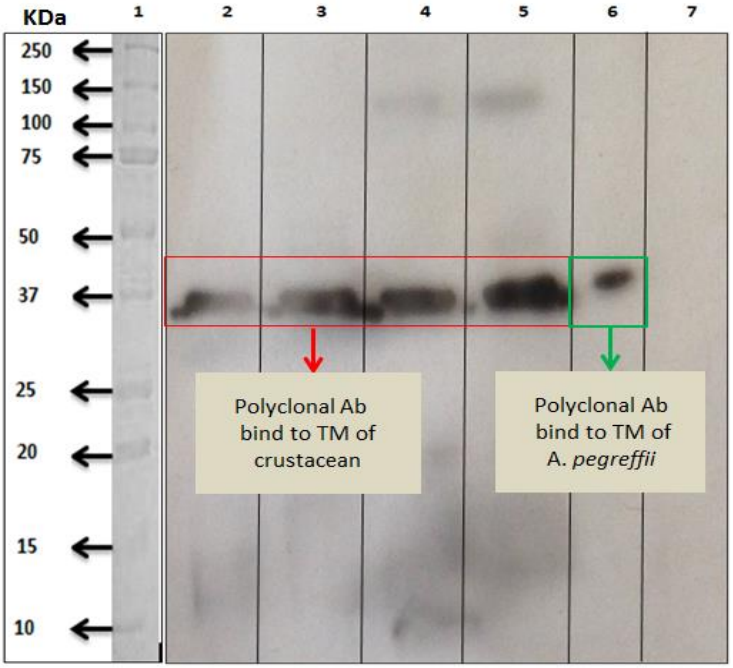
L6= *Anisakis pegreffii*

L7= PBS (negative control)

Figure 3.13 Immunoblotting profiles using mouse anti tropomyosin polyclonal antibody against native crustacean tropomyosin and *A. pegreffii* tropomyosin.

3.3.5.9 Immunoblotting with anti-polycrustacean antibody against native tropomyosin protein

Extracted proteins (10 µg), from shellfish and *A. pegreffii*, were loaded onto the gel and then electro-transferred to the immunoblot membrane. Using anti-crustacean polyclonal antibody, a distinct dark band at 37kDa was observed as a positive control in the lanes 2, 3, 4 and 5 inside green box. Also, the tested *A. pegreffii* extract protein has revealed reactivity with this antibody and showed a single band of approximately 41kDa (lane 6) as shown inside red box (Figure 3.13).



Key:

Lane =L

L1= Marker

L2= Black Tiger (positive control)

L3:=King Prawn (positive control)

L4= Green Prawn (positive control)

L5= Banana Prawn (positive control)

L6:=*Anisakis pegreffii*

L7= PBS (negative control)

Figure 3.14 Immunoblot profiles using mouse anti crustacean polyclonal antibody against native crustacean tropomyosin and *A. pegreffii* tropomyosin.

3.4 Discussion

Australian marine including pinnipeds and cetaceans have been reported relative frequently with various adult species of anisakids nematodes, while larvae of *Anisakis* spp. have been reported in marine fish such as *Sillago flindersi* (Eastern school whiting), mackerels and tiger flathead during their life cycle (Anderson, 2000; Shamsi *et al*., 2011b; Jabbar *et al*., 2012).

Although, only one report of human anisakidosis acquired in Australia was due to *Contracaecum* larval type (Shamsi and Butcher, 2011a) and two cases were diagnosed in Australia but acquired abroad (unpublished data), the risks of acquiring human anisakidosis in Australia still exists for many reasons:

- ✓ The prevalence of *A. pegreffii* in *S. flindersi* and mackerels has been reported in association with human Anisakidosis (Umehara *et al*., 2007; Mattiucci *et al*., 2011). However, tiger flathead has been demonstrated to be contaminated with *A. pegreffii.* Therefore, the potential risk to human's anisakidosis relating to the consumption of tiger flathead still exists.

- ✓ Tiger flathead (*Neoplatycephalus richardsoni*) dominates the retained catch in South East zones because it is the most popular fish species consumed in Australia.

- ✓ Many fish are exported from southern Australian waters to Japan; there is a possibility of Japanese consumers acquiring anisakidosis or allergies related to anisakids by eating *S. flindersi* infected with parasite.

- ✓ Australia is a multicultural country with different cuisines. Therefore, consumers eating raw fish, including sushi and sashimi, and undercooked finfish and shellfish are common.

Therefore, these findings make this study particularly important to investigate the molecular and immunological characterization of *A. pegreffii* parasite tiger flathead (*Neoplatycephalus richardsoni*).

Several species of *Anisakis* nematodes are very common fish parasites with a world-wide distribution (particularly *Anisakis* and *Pseudoterranova decipiens*). It has been reported that cases of anisakiasis are caused by *A. simplex s.s* and *A. pegreffii*.

A number of fish species such as mackerel, European hake, blue whiting, halibut and turbot, salmon and tiger flathead have been distributed worldwide to be parasitized by *Anisakis* nematodes to complete part of their life cycle such as the Anisakidae family. Tiger flathead (*Neoplatycephalus richardsoni*) is endemic to Australia and distributed from northern New South Wales to western Victoria, including the Tasmanian waters. At the same time it is also widely consumed in Australian cuisines and it has been reported and described previously to by highly parasitized with some genera of anisakid nematodes (Shamsi *et al* 2011, unpublished data). Conversely, the knowledge of the diversity, prevalence and genetic composition of anisakid nematodes parasitized tiger Australian flathead fish is limited. Therefore, it has been chosen as the main source of sample collection in this investigation.

During this study, all fish samples (tiger flathead) were highly infected with *Anisakis type I larva* as the infection intensity per individual fish ranged from 5 to 44 nematodes. The effect may be attributable to two factors; one immune system of tiger flathead is weak to resistance through parasite infection, second the L3 became so adapted to life in the host and continues their parasitic lifestyle. They are usually found encysted in a coiled, spring-like state on the walls of intestines, stomach, gonads and rarely in the liver. Therefore, this fact agrees with Shamsi *et al*. (2011 & unpublished data). All this indicates that tiger flathead is the perfect choice for the parasite larvae of *Anisakis* to survive.

In the past, *Anisakis type I larva* was considered indistinguishable based solely on morphological characteristics. Currently, based on genetic analysis, it is believed that *Anisakis* type I larva of cannon 1977, belongs to the clade I which include five species: *A, typica*, *A. ziphidarum* and *A. simplex* comprises of three genetically distinct species, including *A. pegreffii, A. simplex s.s* and *A. simplex C* (Nascetti *et al*., 1983; Mattiucci *et al*., 1997, Mattiucci *et al*., 2008; Shamsi *et al*., unpublished data).

Based on the genetic methods, the collected *Anisakis* larvae from tiger flatheads from the Melbourne areas in this study were identified using PCR amplification of the ITS-1 and ITS-2 fragments in ribosomal DNA, SSCP analysis of ITS-1 and ITS-2 amplicons and subsequent phylogenetic analyses. The analysis clearly revealed one unique species and it is

more likely to be *A. pegreffii*. Therefore, it is the main distributed species in tiger flathead, in agreement with Shamsi *et al*. (2011b), who collected Anisakis larvae from southern Australian waters. Moreover, *A. pegreffii*. has been distributed in the Mediterranean sea and in the Southern hemisphere (Abollo *et al*., 2003) with different species of fish such as *Sillago flindersi* (Australia), *Gadus macrocephalus, G. macrocephalus, S. japonicus, S. australasicus, Hippoglossoides dubius, Arctoscopus japonicus, Takifugu poecilonotus, Theragra chalcogramma, Scomber japonicas* (Japan), *Trachyrincus scabrous* (Morocco and Mauretania), *Astroconger myriaster, A. myriaster, Clupea pallasi, Coryphaena hippurus, Lophius titul, Mugil cephalus, Pneumatophorus japonicas, Scomberomorus niphonius* and *Sebastiscus marmoratus* (China) (Jabbar *et al*., 2012). Furthermore, it has been reported that 97% of *Anisakis* larvae collected from the East China Sea and the sea of Japan were identified as *A. pegreffii* (Umehara *et al*., 2006; Suzuki *et al*., 2010). Also, it has been confirmed that 100% of anisakis spp larvae collected form tiger flathead were identified as *A. pegreffii* (Shamsi *et al*., unpublished data).

One aim of this study was to compare the ITS-1 and ITS-2 sequences of *A. pegreffii* from tiger flathead with ITS-1 and ITS-2 sequences of *A. pegreffii* from marinated anchovies and Chub mackerel. It is known that marinated anchovies and Chub mackerel infected with *A. pegreffii* cause Anisakisis disease for consumers. The similarity of alignment sequencing was 99% between them. Therefore, anisakisis and allergic reaction could also be caused by tiger flathead contaminated with *A. pegreffii*. However, the phylogenetic trees of ITS-1 sequences among *A. pegreffii, A. simplex* C and *A. simplex s.s* showed 95% similarity. Furthermore, ITS-2 sequences showed the same percentage of similarity between the three species. In agreement with another study (Mattiucci *et al*., 2008a) the identity of sequences among the three species is that they belong to *Anisakis* type I.

Based on the previous data of the molecular weight of tropomyosin in parasite, the strong protein band in whole *A. pegreffii* on SDS-PAGE at 41kDa is suspected to be tropomyosin. Therefore, this size is in agreement with previous *A. simplex* and *A. lumbricoides* tropomyosin, which are known to be 41kDa and 40kDa, respectively (Asturias *et al*., 2000; Acevedo *et al*., 2009), which is higher than tropomyosin from crustacean, cockroach (*Periplaneta americana*) and fruitfly (*Drosophila melanogaster*), which are known to be 38kDa (Leung *et al*., 1996)

The tropomyosin from *A. pegreffii* was confirmed by immunoblotting using the anti-tropomyosin and anti-crustacean polyclonal antibodies (PAbs), which both reacted equally with A. *pegreffii* tropomyosin present in the extracted proteins. Also, for *A. pegreffii* extract, high molecular weight protein bands were detected by anti-crustacean (PAbs) with Tris-glycine extract. However, nematode tropomyosin from *A. pegreffii* was not identified when using anti-tropomyosin monoclonal antibodies (MAbs), while it was detected in crustacean extract protein at 37kDa.

The results of immunoblotting suggested that both tested nematodes tropomyosin share one or more epitope with crustacean tropomyosin, as the polyclonal antibodies recognise multiple epitope on the antigen. In contrast the anti-tropomyosin monoclonal seem to recognise one specific epitope in crustacean tropomyosin but not in Anisakis.

Chapter four: Larval anisakid nematodes in teleost fishes from Lizard Island, northern Great Barrier Reef, Australia (published 2012)

Abstract

Background

The larvae third stage (L3) of the Anisakid nematode parasitizes marine organisms, such as fish and squids. Anisakidosis is an initial human zoonotic disease associated with nematodes of the family Anisakidae. The majority of Human anisakiasis cases have been caused by frequent consumption of raw or undercooked fish and squids contaminated with L3 of the *Anisakis* species. Furthermore, *Anisakis* protein extract from L3 frequently causes allergic reactions in sensitized individuals. Despite a wide range of anisakid nematodes having infected a broad variety of invertebrate and vertebrate hosts in Australia, there is still little information about many aspects of the biology and ecology of these important parasites. In the past, 32 species of anisakid nematodes have been reported and described from a broad range of Australian animals (Johnston and Mawson, 1910 to 1969). Since then, many of the anisakid nematodes species have been detected in various fish species at different parts of Australian waters employing morphological characters and molecular techniques. In this study, ITS-1 and ITS-2 sequences of rDNA larval stages of anisakid nematodes were identified by sequences of Gene bank database and compared with sequences of anisakid nematodes.

Aim

The aim of the study is to identify larval anisakids nematodes present in teleosts at Lizard Island, on the northern Great Barrier Reef, Australia.

Methods

Using a combined morphological and molecular approach in a large range of teleosts. It was hoped that such data would indicate the pattern of infection in coral reef fishes and by attempting to relate the data to the ecology of the reef fish community would provide basic information on possible pathways in the food-webs that might facilitate the completion of parasitic life cycles.

Results

More than 107 species of teleost fishes, 450 fishes in total, were sampled during this study. Larvae of anisakid nematodes were identified using molecular-phylogenetic and morphological approaches in fishes collected from a single location, Lizard Island. Three

species of larvae of anisakid nematodes have been identified during this study; *Anisakis*, *Terranova* and *Hysterothylacium*. The epidemiological survey revealed eight morphotypes representing; *A. typica, Terranova* larval Types I and II, *Hysterothylacium* larval Types IV, Vb, Vc, VI and X. Mutation scanning analysis, following sequencing, revealed 20 profiles representing 20 genotypes.

Conclusions

The data presented during this study suggest that larval anisakid infections only occurred in very large numbers in members of Scombridae (mackerels) and Sphyraenidae (barracudas), although the numbers of fish sampled in the latter families were small. In the case of the scombrids and sphyraenids, the infestations were too heavy to determine the precise numbers of nematodes present.

4.1 Introduction

Various groups of marine internal parasites (nematodes, cestodes, trematodes) utilise trophic transmission to complete their complex life cycles, with definitive hosts being vertebrates near the apex of the food chains and feeding on smaller vertebrates or invertebrates at lower trophic levels (Lafferty *et al*., 2008, Marcogliese, 2002). In the case of anisakid nematodes, larval stages may be present in small crustaceans, which are ingested by small fishes, then by larger fishes, finally reaching their definitive hosts, which include sharks, birds and marine mammals. For example, for species of *Anisakis* the definitive hosts are dolphins and whales, for species of *Contracaecum*, fish-eating birds and pinnipeds, for species of *Terranova*, sharks and for species of *Hysterothylacium*, large predatory pelagic teleosts (Anderson, 2000).

The life cycles of parasites are potentially informative in establishing trophic pathways within marine systems, as well as discovering how parasites themselves may influence host abundance in invertebrate and fish communities in marine systems (Marcogliese, 2002, Lafferty *et al*., 2008). Parasites constitute a significant but frequently unrecognised component of marine biodiversity (Marcogliese, 2004). However, the life cycles of anisakid nematodes have been difficult to elucidate using traditional techniques of experimentally infecting hosts raised worm-free, although some anisakids cause severe gastroenteritis in humans if ingested in raw seafood (Van Thiel, 1962, Kasuya *et al*., 1990, Yagi *et al*., 1996, Audicana *et al*., 2002a, Shamsi and Butcher, 2011) In addition these parasites contribute to allergic reactions to seafood (Audicana et al., 2002a, Nieuwenhuizen *et al*., 2006, Audicana and Kennedy, 2008, Lopata and Lehrer, 2009).

In Australia, information on the prevalence and abundance of different species of anisakid nematodes is extremely limited. Although larval stages are found commonly in species of teleosts, frequently in species used for human consumption, identification beyond the level of genus is virtually impossible using morphological methods, resulting in the larvae being assigned to various morphotypes designated by Roman numerals (Cannon, 1977a, Cannon, 1977b, Bruce and Cannon, 1989, Bruce and Cannon, 1990, Bruce, 1990a, Bruce, 1990b, Nash, 1998, Lymbery *et al*., 2002, Doupé *et al*., 2003, Munõz *et al*., 2007, Shamsi, 2007, Shamsi *et al*., 2008, Shamsi and Butcher, 2011, Shamsi *et al*., 2011b, Shamsi *et al*., 2012, Jabbar *et al*., 2012). The more recent introduction of molecular techniques has allowed the identification of some larval stages in teleosts and, as a consequence, the identification of

life cycle patterns (Mattiucci and Nascetti, 2006b, Mattiucci and Nascetti, 2008). In addition to being able to elucidate the life cycles of parasites, the ability to specifically identify the larval stages of anisakid nematodes also provides an opportunity to investigate how these nematodes utilise intermediate hosts at various trophic levels to complete their life cycles. Cannon (1977a, b) provided initial data on the biology of larval anisakid nematodes in teleost fishes in south-eastern Queensland, but was hampered by the limitations of morphological methods, describing his morphological forms as different numerical types but being unable to confidently establish their relationships with adult forms. Nonetheless, this pioneering work suggested substantial ecological differences among the various genera of anisakid nematodes described (*Anisakis*, *Contracaecum*, *Terranova* and *Hysterothylacium* (as *Thynnascaris*)). Subsequently, it has been shown that some of the morphotypes identified by Cannon (1977b) in fact constitute more than one species (Shamsi *et al.* 2011b). This information indicates that molecular techniques of larval identification can provide considerable insights into nematode life cycles and in addition into the complex food webs that exist in marine systems, by elucidating the various intermediate host species in a food chain.

In this study, we surveyed larval anisakid nematodes present in teleosts at Lizard Island, on the northern Great Barrier Reef, Australia. The aim of the study was to identify larval anisakids using a combined morphological and molecular approach and in a large a range of teleosts. It was hoped that such data would indicate the pattern of infection in coral reef fishes and by attempting to relate the data to the ecology of the reef fish community would provide basic information on possible pathways in the food-webs that might facilitate the completion of parasite life cycles.

4.2 Materials and methods

4.2.1 Study area and collection of fishes

Fish were collected during two trips to Lizard Island (14° 40'E 145° 28'S) in the northern Great Barrier Reef, Queensland, Australia, in April 2008 and August-September 2010. Teleosts were collected by line fishing, seine netting, spear fishing and by anaesthetising small fish with clove oil. An attempt was made to examine as many fish species as possible as part of a preliminary study of anisakids infecting reef fishes.

Fish were photographed to confirm identifications and, in most instances, tissue samples were collected for genetic identification. Representatives of any fish species, which proved difficult to identify in the field, were preserved and returned to the Queensland Museum, Brisbane for definitive identification.

4.2.2 Isolation of anisakid larvae and their morphological identification

Nematodes were removed from the body cavity and were fixed in ethanol for subsequent examination. In cases where relatively small numbers of nematodes were present, all nematodes were collected. In fish infected with hundreds of nematodes (*Scomberomorus commerson* and *Grammatocyrnus bicarinatus*), a subsample of 100-200 nematodes was collected. Prevalence (the percentage of fish infected with parasites) and intensity of infection (the numbers of parasites in infected hosts) are reported following the definitions of Bush *et al.* (1997).

In the laboratory, all nematodes were prepared in a similar fashion. The anterior and posterior ends of each nematode were excised using a scalpel and preserved in lactophenol for morphological identification, while the mid-section of the body was utilised for molecular studies.

Each nematode was examined morphologically and assigned to a larval "Type" following Cannon (1977b) and Shamsi (2007). Representatives of each morphological nematode type, from each host species were retained and have been deposited in the Queensland Museum, Brisbane (G233918-79).

Drawings of each morphological type of nematode, and brief descriptions are provided. More detailed descriptions of each of these nematode types can be found in Cannon (1977b) and Shamsi (2007). Drawings were made with a drawing tube attached to an

Olympus BH microscope and measurements were made with an ocular micrometer; the latter are presented in mm, as the mean followed by the range; the number of measurements made is represented by 'n'. As the mid-region of the nematodes had been removed for molecular studies, no total lengths were available.

4.2.3 Extraction of total genomic DNA

Total genomic DNA from each Mid-body section of individual nematodes was extracted by the following steps; each section was transferred to individual 1.5ml Eppendorf tubes containing 500µl of extraction solution (containing 350µg/ml Proteinase K in extraction buffer (20mM Tris-HCL, pH 8.0, 100mM EDTA and 1%SDS)). The tubes were incubated for 18 hours at 37°C. Genomic DNA was isolated using a standard sodium dodecyl sulphate (SDS)-Proteinase K method (Gasser *et al*., 1993) and purified directly using Wizard TM DNA Clean-Up columns (Promega) according to the manufacturer's recommendations.

4.2.4 Amplification of nuclear ribosomal DNA regions (ITS-1 and ITS-2) by polymerase chain reaction (PCR)

The first and second internal transcribed spacers of nuclear ribosomal DNA (ITS-1 and ITS-2, respectively) were chosen for use in this study as they provide species-specific markers for anisakid nematodes (e.g., D´Amelio et al., 1999; Zhu et al., 1998a). Based on the rDNA sequences of anisakid nematodes in the GeneBank^{TM} database, nuclear ribosomal DNA (rDNA) were amplified from 10-50 ng of genomic DNA by PCR using two designed primer sets for ITS-1 and ITS-2 (Zhang *et al*. 2007; Shamsi *et al*. 2008), respectively. The ITS-1 was amplified by PCR using the primer set SS1: 5´-GTTTCCGTAGGTGAACCTGCG-3´ (forward) and NC13R: 5´-GCTGCGTTCTTCATCGAT-3´ (reverse), while ITS-2 was amplified using primer set SS2: 5´-TTGCAGACACATTGAGCACT-3´ (forward) and NC2: 5´-TTAGTTTCTTTCCTCCGCT-3´ (reverse). 2µl of genomic DNA (20ng) were added directly to 48µl PCR mix (overlaid with paraffin oil) containing 250µM of each dNTP, 3.5Mm MgC12, 25pmo1 of each primer and 0.5U of Taq polymerase (promega), placed immediately on a freeze block and then subjected to PCR in a 480 thermal cycler (Perkin Elmer). The cycling conditions were: initial denaturation in 94°C for five minutes, then 94°C for 30 seconds (denaturation), 55°C (ITS-1) or 53°C (ITS-2) for 30 seconds (annealing) and 72°C for 30 second (extension) for 35 cycles, followed by 72°C for 5 minutes. The same conditions were used to amplify ITS-2, expect that the annealing

temperature was 53°C. A control negative sample without DNA was included in each PCR run and known positive controls were included. Following PCR, 5 µl of each amplicon was separated in 1.5% agarose gel (Sambrook *et al*., 1989) at 100V in Tris/Boric Acid/EDTA Buffer (TBE: 65mM Tris-HCL, 27mM boric acid, 1mM EDTA, pH 9; Bio-Rad) for 1 hour and stained with ethidium bromide, then photographed. 174-Hae (Promega) was used as the molecular weight marker on orange gel.

4.2.5 Single-Strand Conformation Polymorphism (SSCP) analysis

Given the large number of anisakid nematodes, larvae collected and examined in this study, sequencing of all specimens would have been time consuming and expensive. Besides, it could have resulted in numerous identical sequences. Hence, single strand conformation polymorphism (SSCP) analysis (Orita *et al*., 1989) was used for screening ITS-1 and ITS-2 amplicons for sequence variation among all individuals of anisakid nematodes collected. In this technique, single-strand PCR products run on a non-denaturing gel can be differentiated not only based on their size but also their structure (Gasser and Chilton, 2001). The SSCP method used followed that described previously by Gasser et al., (2006; protocol B) to display sequence variation within and among amplicons, as described previously (Jabbar *et al.* 2012). Briefly: 5µl of distilled water was mixed with 10µl of DNA sequencing stop solution; after denaturation at 94°C for 15 minutes and immediate snap freezing on a freeze block (-20°C), 12µl of the products were loaded on an SSCP gel (Elchrom Scientific) and run for 18 hours for ITS-1 and ITS-2, the gel was stained with super gold for 30 minutes and destained in distilled water for 5 minutes. Samples were selected for sequencing based on the variation shown in their profiles, host species and their geographical location.

4.2.6 Purification of amplicons and sequencing

For each locus, amplicons representing each unique SSCP profile were selected, treated with shrimp alkaline phosphatase and exonuclease I (Fermentas Inc., USA), and subjected to bi-directional, automated sequencing (BigDye® Terminator v.3.1, Applied Biosystems, Foster City, California, USA) using (separately) the same primers employed in PCR. The quality of each sequence was assessed by appraising its electropherogram using the program BioEdit (Hall, 1999). Polymorphic sites were designated using International Union of Pure and Applied Chemistry (IUPAC) codes.

Table 4.1 Fish collected and examined for larval anisakid nematodes on Lizard Island, Queensland, including prevalence (no. of individuals infected) of infection.

Order	Family	Genus and species	Common name	No. examined	Prevalence
Perciformes	Acanthuridae	*Ctenochaetus binotatus*	Twospot surgeon fish	1	0
		Zebrasoma veliferum	Sailfin tang	6	0
		Acanthurus sp. 14366		1	0
				(8)*	
	Apogonidae	*Apogon compressus*	Ochre-striped cardinal fish	5	0
		Apogon exostigma	Narrowstripe cardinalfish	2	0
		Apogon angustatus	Broadstriped cardinalfish	2	0
		Zoramia leptacantha	Threadfin cardinalfish	117	0
		Apogon properuptus	Southern orange-lined cardinal fish	2	0
		Apogon rubrimacula	Redspot cardinalfish	5	0
		Archamia fucata	Orangelined cardinalfish	8	0
		Archamia zosterophora	Blackbelted cardinalfish	1	0
		Cheilodipterus artus	Wolf cardinalfish	10	0
		Cheilodipterus intermedius	Intermediate cardinalfish	8	1
		Cheilodipterus quinquelineatus	Five-lined cardinalfish	17	0
		Nectamia fusca	Samoan cardinalfish	19	0
		Rhabdamia gracilis	Luminous cardinalfish	10	0
				(206)	
	Atherinidae	*Atherinomorus endrachtensis*	Eendracht Land silverside	11	5
				(11)	
	Blenniidae	*Ecsenius stictus*	Great Barrier Reef blenny	1	0

	Plagiotremus tapeinosoma	Piano fangblenny	1	0
	Salarias alboguttatus	White-spotted blenny	1	0
	Salarias fasciatus	Jewelled blenny	2	0
			(5)	
Bothidae	*Bothus pantherinus*	Leopard flounder	1	0
			(1)	
Carangidae	*Caranx papuensis*	Brassy trevally	2	2
			(2)	
Chaetodontidae	*Chaetodon plebeius*	Blueblotch butterflyfish	2	0
	Chaetodon citrinellus	Speckled butterflyfish	10	1
	Chaetodon baronessa	Eastern triangular butterflyfish	1	0
	Ctenochaetus binotatus	Twospot surgeonfish	1	0
	Chaetodon auriga	Threadfin butterflyfish	6	0
	Chaetodon lineolatus	Lined butterflyfish	3	0
	Chaetodon ephippium	Saddle butterflyfish	3	0
	Chaetodon rafflesii	Latticed butterflyfish	3	0
	Chaetodon lunulatus	Oval butterflyfish	2	0
	Chaetodon pelewensis	Sunset butterflyfish	2	0
	Chaetodon ulietensis	Pacific double-saddle butterflyfish	8	1
	Chaetodon melannotus	Blackback butterflyfish	8	0
	Chaetodon unimaculatus	Teardrop butterflyfish	2	0
	Chaetodon kleinii	Sunburst butterflyfish	2	0
	Chaetodon vagabundus	Vagabond butterflyfish	9	0
	Chelmon rostratus	Copperband butterflyfish	4	0

	Forcipiger flavissimus	Longnose butterfly fish	3	0
	Heniochus chrysostomus	Threeband pennantfish	4	0
	Heniochus varius	Horned bannerfish	2	0
			(75)	
Cirrhitidae	*Paracirrhites forsteri*	Blackside hawkfish	1	1
Lethrinidae	*Lethrinus atkinsoni*	Pacific yellowtail emperor	4	0
	Lethrinus harak	Thumbprint emperor	6	0
	Lethrinus nebulosus	Spangled emperor	4	3
			(14)	
Lutjanidae	*Caesio cuning*	Redbelly yellowtail fusilier	3	3
	Lutjanus carponotatus	Spanish flag snapper	2	2
	Lutjanus ehrenbergii	Blackspot snapper	4	0
	Lutjanus fulviflamma	Dory snapper	1	1
	Lutjanus fulvus	Blacktail snapper	1	0
	Lutjanus monostigma	One-spot snapper	1	0
	Lutjanus russellii	Russell's snapper	2	0
			(14)	
Microdesmidae	*Gunnellichthys monostigma*	Onespot wormfish	1	0
			(1)	
Mullidae	*Parupeneus trifasciatus*	Doublebar goatfish	4	0
	Parupeneus ciliatus	Whitesaddle goatfish	1	0
	Parupeneus indicus	Indian goatfish	1	0
	Mulloidichthys vanicolensis	Yellowfin goatfish	2	0
			(8)	
Muraenidae	*Gymnothorax pseudothyrsoideus*	Highfin moray	1	0

			(1)	
Nemipteridae	*Scolopsis monogramma*	Monogrammed monocle bream	4	1
	Scolopsis margaritifera	Pearly monocle bream	1	0
	Scolopsis affinis	Peters' monocle bream	2	0
			(7)	
Pinguipedidae	*Parapercis hexophthalma*	Speckled sandperch	1	0
			(1)	
Pomacanthidae	*Centropyge bicolor*	Bicolor angelfish	1	0
			(1)	
Pomacentridae	*Abudefduf septemfasciatus*	Banded sergeant	1	0
	Abudefduf sordidus	Blackspot sergeant	1	0
	Acanthochromis polyacanthus	Spiny chromis	1	1
	Dascyllus reticulatus	Reticulate dascyllus	1	0
			(4)	
Pseudochromidae	*Pseudochromis fuscus*	Brown dottyback	1	0
	Stegastes apicalis	Australian gregory	1	1
			(2)	
Scaridae	*Scarus oviceps*	Dark capped parrotfish	1	0
			(1)	
Scombridae	*Grammatorcynus bicarinatus*	Shark mackerel	6	6
	Scomberomorus commerson	Narrow-barred Spanish mackerel	2	2
			(8)	
Serranidae	*Cephalopholis boenak*	Chocolate hind	3	1
	Cephalopholis cyanostigma	Bluespotted hind	5	2
	Epinephelus ongus	White-streaked grouper	1	1

		Plectropomus areolatus	Squaretail coral grouper	1	0
		Plectropomus leopardus	Leopard coral grouper	8	6
				(18)	
	Siganidae	*Siganus corallinus*	Blue-spotted spinefoot	1	0
		Siganus doliatus	Barred spinefoot	1	0
		Choerodon schoenleinii	Blackspot tuskfish	1	0
		Coris batuensis	Batu coris	1	0
		Halichoeres scapularis	Zigzag wrasse	1	0
		Oxycheilinus diagrammus	Cheeklined wrasse	1	0
		Thalassoma lunare	Moon wrasse	2	0
				(8)	
	Sillaginidae	*Sillago sp. 14308*		1	0
				(1)	
	Sphyraenidae	*Sphyraena jello*	Pickhandle barracuda	1	0
		Sphyraena forsteri	Bigeye barracuda	2	2
				(3)	
Beloniformes	Belonidae	*Tylosurus crocodilus*	Hound needlefish	4	1
				(4)	
	Hyporhamphidae	*Hyporhamphus affinis*	Tropical halfbeak	7	1
				(7)	
Mugiliformes	Mugilidae	*Valamugil buchanani*	Bluetail mullet	1	0
		Liza vaigiensis	Squaretail mullet	8	1
				(9)	
Gonorynchiformes	Chanidae	*Chanos chanos*	Milkfish	3	0
				(3)	
Tetraodontiformes	Balistidae	*Balistapus undulatus*	Orange-lined triggerfish	1	0
		Rhinecanthus aculeatus	White-banded triggerfish	16	0
		Sufflamen chrysopterus	Halfmoon triggerfish	4	0
				(21)	

	Monacanthidae	*Oxymonacanthus longirostris*	Harlequin filefish	1	0
		Paraluteres prionurus	False puffer	1	0
				(2)	
	Ostraciidae	*Ostracion cubicus*	Yellow boxfish	3	0
				(3)	
	Tetraodontidae	*Arothron hispidus*	White-spotted puffer	3	0
		Arothron manilensis	Narrow-lined puffer	2	0
		Arothron mappa	Map puffer	1	0
		Arothron nigropunctatus	Blackspotted puffer	1	0
		Canthigaster bennetti	Bennett's sharpnose puffer	2	0
		Canthigaster solandri	Spotted sharpnose	1	0
		Canthigaster valentini	Valentin's sharpnose puffer	2	0
				(12)	
Beryciformes	Holocentridae	*Neoniphon sammara*	Sammara squirrelfish	2	0
				(2)	
Total:				464	46 (9.9%)

4.2.7 Phylogenetic analyses

Prior to phylogenetic analyses, sequence types defined herein for each locus (ITS-1 and ITS-2) were subjected (separately) to BLASTn analysis (http://blast.ncbi.nlm.nih.gov) to establish the 'top hits' to all nucleotide sequences available in the current databases and identities (in %) calculated by pairwise comparisons. Subsequently, the consensus sequence was aligned with a selected subset of closely related reference sequences (*Anisakis typica*, *Hysterothylacium* spp., *Pseudoterranova* spp., *Terranova* spp. and *Raphidascaris trichiuri*) using the program Clustal X (Thompson *et al.* 1997) and alignments were adjusted manually. Phylogenetic analyses were performed on individual or concatenated sequence datasets. Each concatenated (ITS-1+ITS-2) sequence was derived from the same individual nematode. Phylogenetic analysis of nucleotide sequence data was conducted by Bayesian inference (BI), employing the Markov chain Monte Carlo (MCMC) method in MrBayes

3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). The likelihood parameters for BI were based on the Akaike Information Criteria (AIC) test in Modeltest v3.7 (Posada and Crandall 1998). The "best" model for the ITS-1 dataset using AIC was the general time-reversible model of evolution, with gamma-distribution and a proportion of invariable sites (GTR+Γ+I) while that for ITS-2 as well as for concatenated (ITS-1+ITS-2) sequence datasets was the transversion model of evolution, with gamma-distribution and a proportion of invariable sites (TVM+Γ+I). Estimates of the base frequencies, the substitution rate model matrix and the proportion of invariable sites were fixed. Posterior probabilities (pp) were calculated using 2,000,000 generations, employing four simultaneous tree-building chains, with every 100^{th} tree being saved. At this point, the potential scale reduction factor approached one, and the standard deviation of split frequencies was <0.01. A consensus tree (50% majority rule) was constructed based on the final 75% of trees generated by BI. Phylogenetic trees constructed using different datasets were examined for concordance in topology

4.3 Results

4.3.1 Initial identification of parasite species.

Initial identification was made using the light microscope to differentiate third stage larvae (L3) of *Anisakis* spp from other related nematodes based on several characters.

4.3.2 Sample preparation for identification using different methods

All the samples were cut to three pieces, interior, posterior and middle parts. The interior and posterior parts were used for morphological identification and the middle part for molecular identification as shown in figure 4.1.

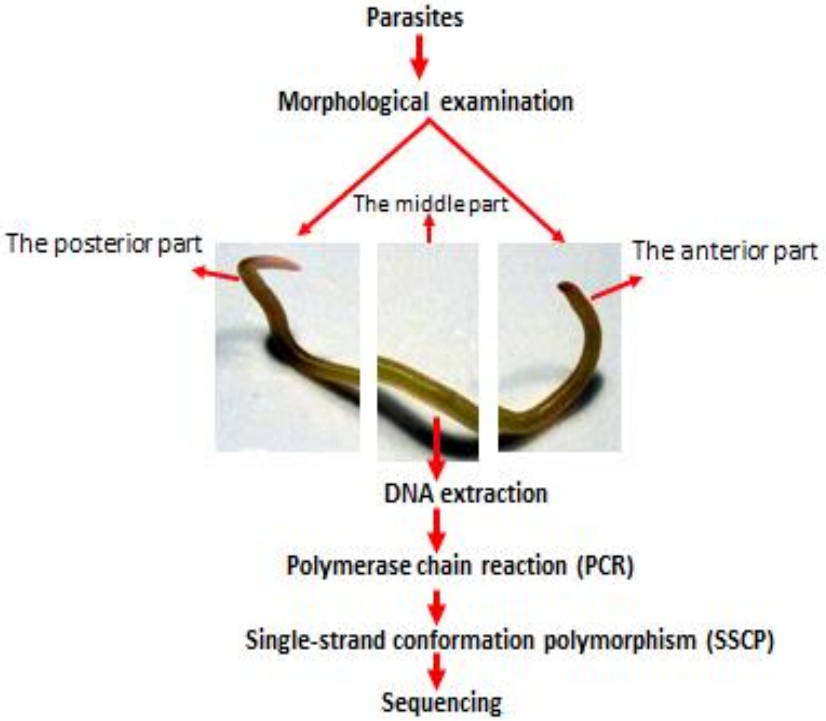


Figure 4.1 Steps in morphological and molecular Identifcation of *A. pegreffii*

4.3.3 Amplification of nuclear ribosomal DNA regions (ITS-1 and ITS-2) by polymerase chain reaction (PCR)

The first and second internal transcribed spacers of nuclear ribosomal DNA (ITS-1 and ITS-2) from each sample were amplified using PCR as shown in figures 4.2 and 4.3. Figures 4.2 and 4.3 are represntatives of the ITS-1 and ITS-2 amplicons from over 300 samples processed/amplified.

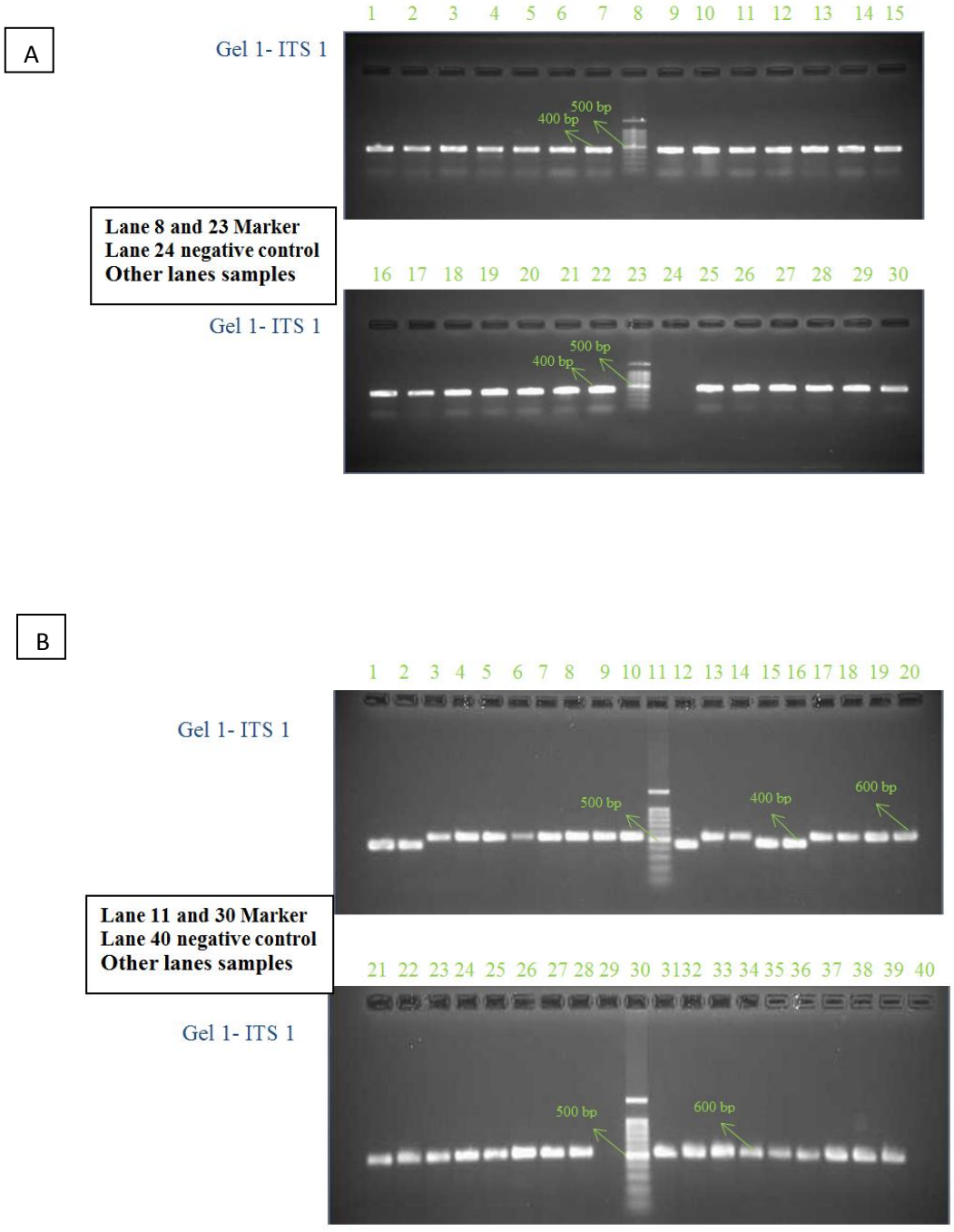


Figure 4.2 A, B Gels A and B showing full ITS-1 PCR products amplified from different Anisakidae species with ITS-1 primers.

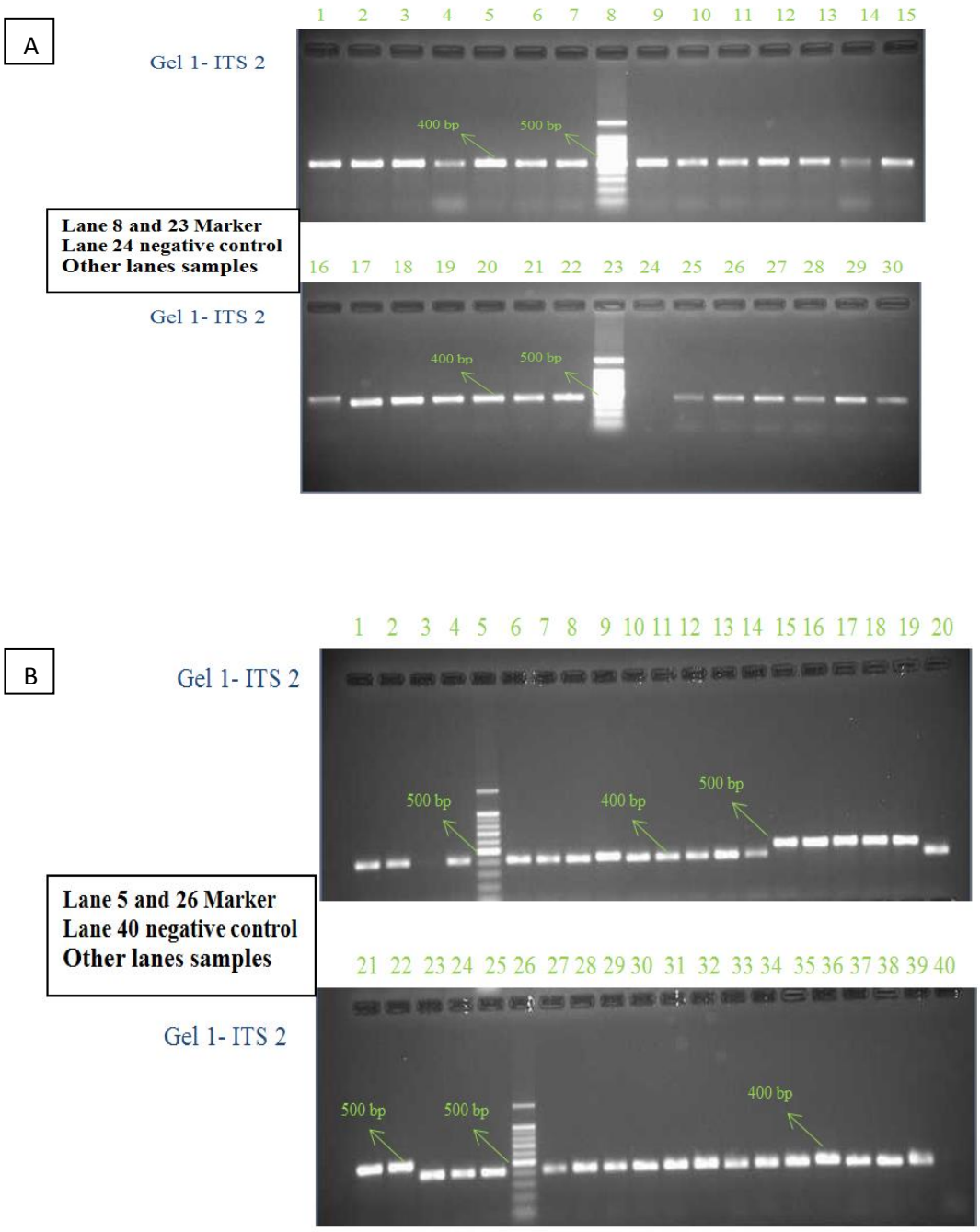


Figure 4.3: A, B Gels A and B showing full ITS-2 PCR products amplified from different Anisakidae species with ITS-2 primers.

4.3.4 Single-Strand Conformation Polymorphism (SSCP) analysis

ITS-1 and ITS-2 amplicons from each sample were screened on SSCP gels to analysis sequence variation among all individuals of anisakid nematodes collected. Since more than 300 amplicons for ITS-1 and same for ITS-2 were screened on SSCP gels. A number of gels have been done for that experiment and therefore, figures 4.4, 4.5, 4.6, 4.7, 4.8 and 3.9 just show examples of the ITS-1 and ITS-2 amplicons from each sample screened and figures, 8 and 9 show examples of the presented samples that were chosen from each of the same profile for sequencing and phylogenetic tree. As shown in figures 4.9 and 4.10, representative samples from each similar group band of ITS-1 and ITS-2 were selected for sequencing.

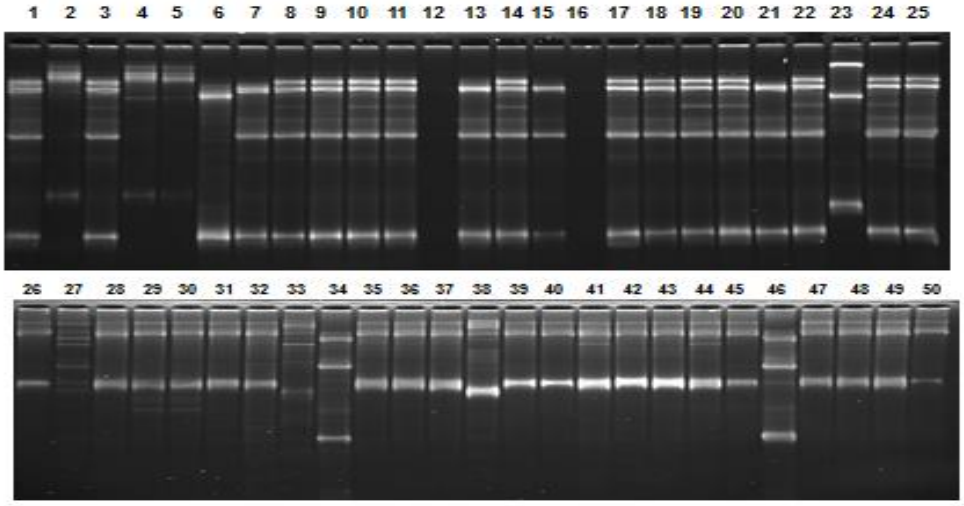


Figure 4.4 SSCP analysis of ITS1 regions amplified from different Anisakidae species.

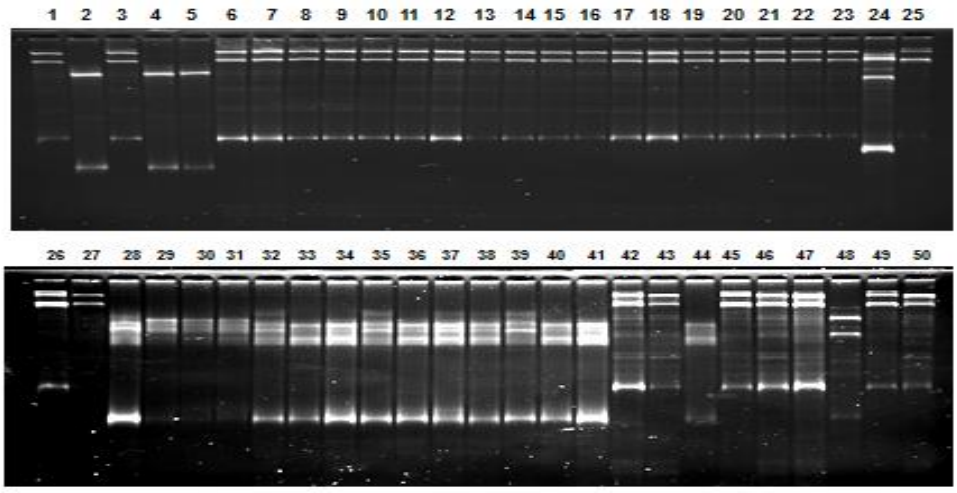


Figure 4.5 SSCP analysis of ITS1 regions amplified from different Anisakidae species.

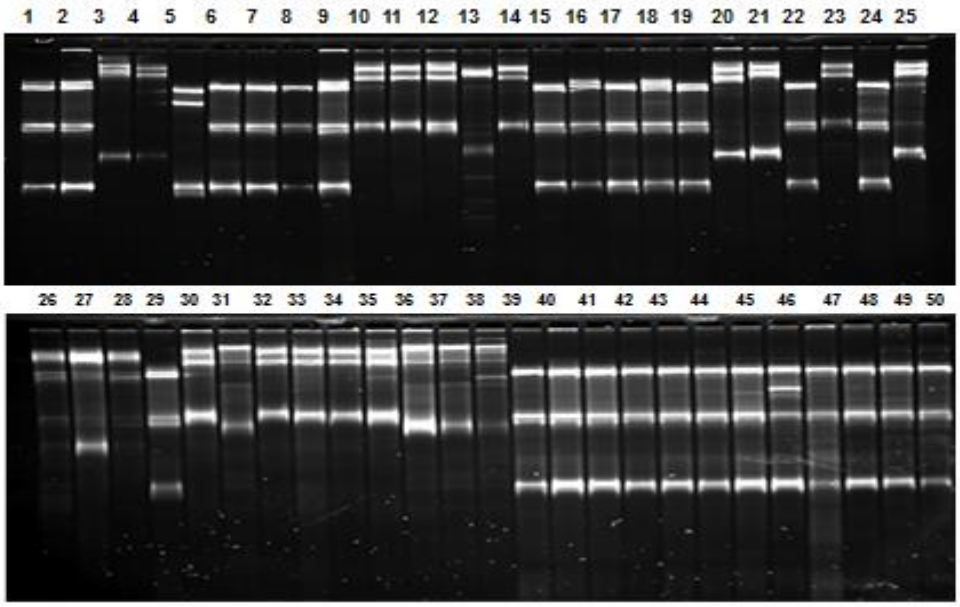


Figure 4.6 SSCP analysis of ITS2 regions amplified from different Anisakidae species.

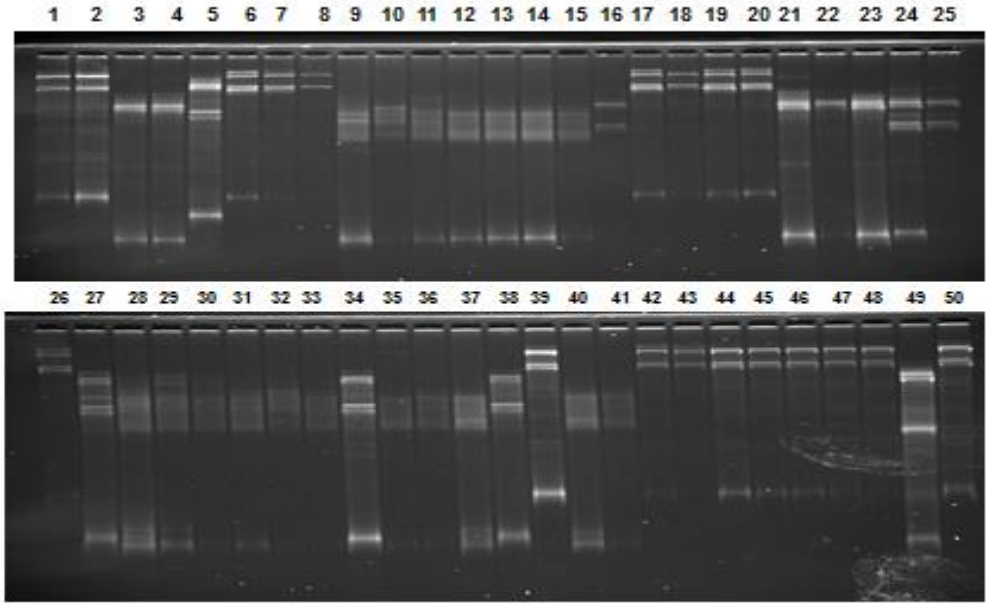


Figure 4.7 SSCP analysis of ITS2 regions amplified from different Anisakidae species.

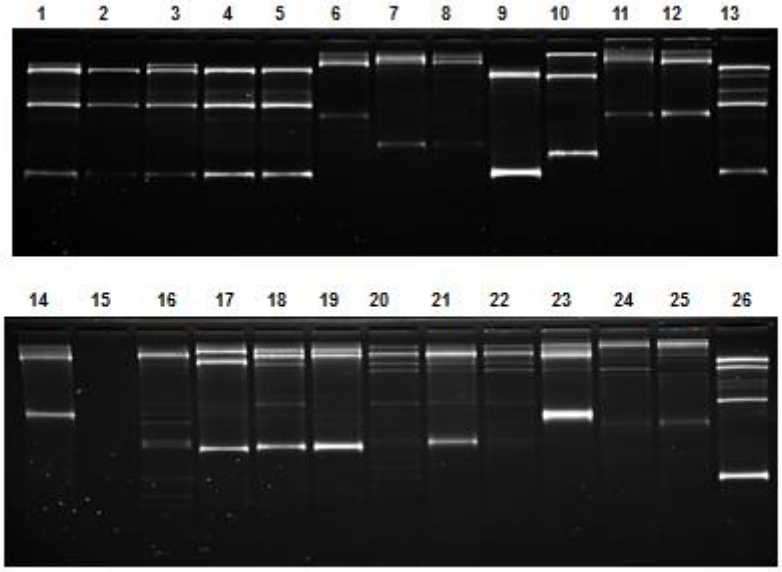


Figure 4.8 SSCP profile of ITS1 regions of different Anisakidae used for species identification by sequencing

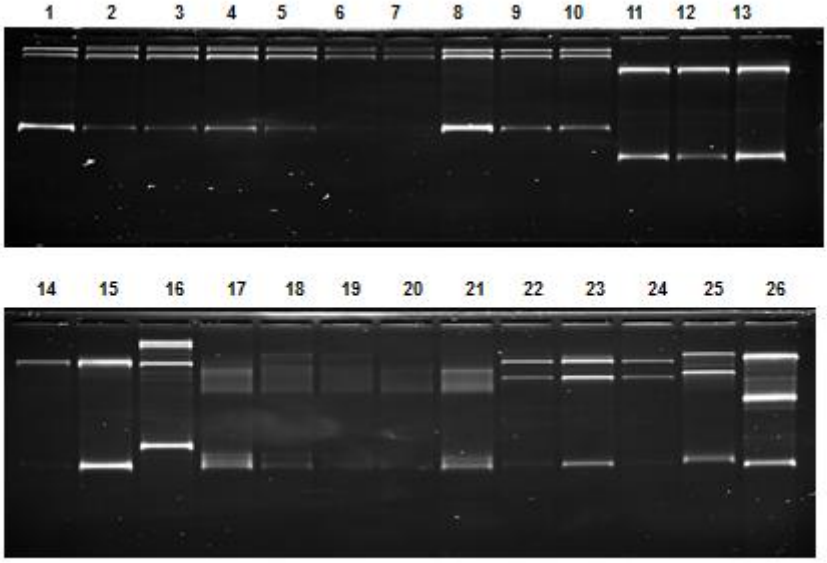


Figure 4.9 SSCP profile of ITS2 regions of different Anisakidae used for species identification by sequencing

4.3.5 Identification of parasite species by specific morphological and molecular methods

In total, 464 fish were examined from 32 families, 62 genera and 107 species. Anisakid nematodes were found in 46 (9.9%) of the fish examined (Table 4.2). Although sampling across fish families was very uneven, few or no anisakid nematode larvae were found in the families (only those families with five or more fish species examined were included): Acanthuridae, Apogonidae, Balistidae, Blenniidae, Chaetodontidae, Hyporhamphidae, Mugilidae, Mullidae, Nemipteridae, Siganidae and Tetraodontidae (Table 4.2). By contrast, nematodes were found commonly in members of the families Lethrinidae, Lutjanidae, Scombridae and Serranidae.

The nematodes examined were identified as *Anisakis typica*; two morphotypes of *Terranova*, corresponding closely with *Terranova* Types I and II of Cannon (1977b); and four morphotypes of *Hysterothylacium* corresponding with Type IV of Cannon (1977b) and Types V, VI and X of Shamsi (207).

Descriptions of larvae

***Anisakis typica* (= *Anisakis* Type I of Cannon, *1977b*, in part)**

Diagnosis ((Fig. 1, G-I))

Third stage larvae with prominent ventral tooth. Oesophagus 1.32-1.57 (1.47, n=10) long; nerve ring 0.22-0.28 (0.26, n=10) from anterior end; deirids 0.35 (n=1) from anterior end; ventriculus 0.48-0.88 (0.73, n=10) long; tail blunt, 0.09-0.12 (0.11, n=10) long with prominent mucro.

Remarks: The measurements presented here of larvae identified definitively using molecular methods as being those of *A. typica* correspond closely with larvae of *Anisakis* Type I of Cannon (1977b) which he described from various Queensland fishes. However, the description also potentially encompasses the features of the third stage larva of *A. pegreffii* (see (Shamsi *et al.* 2011a)).

In the larva of *A. pegreffii*, the oesophagus was longer 2.16 (1.67-2.66 mm) while the mean length of the ventriculus (0.84 mm) was similar to that of *A. typica* (see (Shamsi *et al.* 2011b) as was the mean length of the tail (0.12 mm). Consequently, the principal potential distinguishing feature of these larval stages appears to be the length of the oesophagus. However, as total lengths of larvae were not available, the ratios of these organs to total

lengths could not be compared, which needs to be done before using the data as differential criteria.

In all 155 larvae of this type were subjected to PCR-coupled SSCP analysis. Based on SSCP profiles, five and three representative specimens were selected for the sequencing of the ITS-1 and ITS-2, respectively. The consensus lengths of the ITS-1 and ITS-2 sequences for each sequence type, their mean nucleotide frequencies and positions for the polymorphisms (if any), G+C content and respective sequence accession numbers are given in Table 4.2. In the ITS-1, sequence polymorphism was detected (see Table 4.2; genotypes A-C) and upon pairwise comparison, the percentage difference among all of the different ITS-1 sequence types ranged from 0.3 to 1.2%. No sequence polymorphism was detected in the ITS-2.

***Terranova* Type I of Cannon (1977b)**

Diagnosis (Fig. 1, A-C)

Third stage larvae with prominent ventral tooth. Body with numerous prominent longitudinal striations. Oesophagus 1.08-1.65 (1.16, n=10) long; nerve ring 0.25-0.33 (0.30, n=10) from anterior extremity; deirids 0.30-0.37 (0.34, n=3) from anterior extremity; ventriculus 0.95-1.96 (1.32, n=10) long; intestinal diverticulum 0.73-2.08 (1.52, n=10) long, extending just anterior to oesophago-intestinal junction; tail with prominent transverse cuticular striations, 0.16-0.20 (0.18, n=10) long, blunt tipped.

Table 4.2 The classification of anisakid nematode specimens based on single-strand conformation polymorphism (SSCP) profiles for two loci (ITS-1 and ITS-2) used in the present study. The sequence linked to each unique SSCP profile is represented by its GenBank accession number, its length and polymorphism and G+C content. Mean nucleotide frequencies for the main anisakids are also provided.

Genotype	ITS-1									ITS-2							
	SSCP profile (no. of samples with unique profile)	Accession no.	Length (bp)	Polymorphism[c] (alignment position)	G+C Content (%)	Mean nucleotide frequencies A	C	G	T	SSCP profile (no. of samples with unique profile)	Accession no.	Length (bp)	Polymorphism[a] (alignment position)	G+C Content (%)	Mean nucleotide frequencies A	C	G	T
A (*Anisakis typica*)	AT-1.1 (153)	(JX848663)	348	---	50.86	0.23946	0.23705	0.27107	0.25241	AT-2 (155)	(JX848680)	355	---	47.32	0.25070	0.20282	0.27042	0.27606
B (*A. typica*)	AT-1.2 (1)	(JX848664)	348	Y(201)	50.57					-do-	-do-	-do-	---					
C (*A. typica*)	AT-1.3 (1)	(JX848665)	348	G (52) T (201) T (249) G (252)	50.86					-do-	-do-	-do-	---					
D (*Terranova I*)	TI-1.1 (37)	(JX848666)	437	---	46.68	0.25057	0.21510	0.25400	0.28032	TI-2.1 (35)	(JX848681)	266	T (43)	51.13	0.24812	0.19838	0.31517	0.23833
E (*Terranova I*)	-do-	-do-	-do-	---						TI-2.2 (1)	(JX848682)	266	S (94)	51.13				
F (*Terranova I*)	-do-	-do-	-do-	---						TI-2.3 (1)	(JX848683)	266	Y (43)	51.13				
G (*Terranova I*)	TI-1.2 (3)	(JX848667)	439	G (175) G (176)	46.92					TI-2.4 (2)	(JX848684)	266	C (43)	51.50				
H (*Terranova I*)	-do-	-do-	-do-	-do-						TI-2.5 (1)	(JX848685)	266	K (67)	51.13				
I (*Terranova II*)	TII-1.1 (184)	(JX848668)	437	---	47.60	0.24939	0.21440	0.26110	0.27511	TII_2.1 (187)	(JX848686)	252	---	46.43	0.27183	0.18651	0.27778	0.26389
J (*Terranova II*)	TII-1.2 (1)	(JX848669)	437	M (207)	47.37					-do-	-do-	-do-	---					
K (*Terranova II*)	TII-1.3 (1)	(JX848670)	437	R (243)	47.37					-do-	-do-	252	---					
L (*Terranova II*)	TII-1.4 (1)	(JX848671)	437	W (359)	47.60					-do-	-do-	-do-	---					
M (*Terranova II*)	TII-1.5 (1)	(JX848672)	437	28 (W) 29 (W) 399 (W)	47.60					TII_2.2 (1)	(JX848687)	253	A (152)	46.25				
N (*Hysterothylacium*	HIV-1 (1)	(JX848673)	438	---	51.14	0.21689	0.22374	0.28767	0.27169	HIV-2 (1)	(JX848688)	345	---	54.20	0.16812	0.23478	0.30725	0.28986

Type IV)																			
O (*Hysterothylacium*	HVb-1.1 (11)	(JX848674)	437	---	49.66	0.23717	0.22883	0.26855	0.26545	HVb-2 (13)	(JX848689)	272	---	50	0.19118	0.20588	0.29412	0.30882
Type VI)																			
P (*Hysterothylacium*	HVb-1.2 (1)	(JX848675)	437	R (64)	-do-					-do-	-do-	-do-	---						
Type VI)																			
Q (*Hysterothylacium*	HVb-1.3 (1)	(JX848676)	437	R (383)	-do-					-do-	-do-	-do-	---						
Type VI)																			
R (*Hysterothylacium*	HVc-1 (14)	(JX848677)	434	---	49.54	0.24424	0.22581	0.26959	0.26307	HVc-2 (14)	(JX848690)	270	---	49.26	0.21111	0.21111	0.28148	0.29630
Type V)																			
S (*Hysterothylacium*	HVI-1 (8)	(JX848678)	431	---	50.35	0.23898	0.22738	0.27610	0.25754	HVI-2 (8)	(JX848691)	267	---	49.06	0.19476	0.20225	0.28839	0.31461
Type V)																			
T (*Hysterothylacium*	HX-1 (5)	(JX848679)	436	---	51.15	0.22018	0.22706	0.28440	0.26835	HX-2 (5)	(JX848692)	276	---	54.35	0.15942	0.22101	0.32246	0.29710
Type X)																			
Total	424									424									

[a] Polymorphism for each sequence type was assessed by aligning these sequences with the reference sequences (see Table 2). R = A/G; K = G/T; S = C/G; Y = C

Remarks: The features of these larvae conform to the description of Cannon (1977b), being distinguished by an elongate ventriculus and the intestinal diverticulum reaching the oesophago-intestinal junction. Sequence data are provided for this larval type for the first time. Cannon (1977b) suggested that they were the larvae of *T. chyloscylii*. However, due to the absence of sequence data for the adults of this species, this suggestion cannot be confirmed.

Thirty-seven specimens of this larval type were subjected to PCR-coupled SSCP analysis. Based on the SSCP profiles, six and eight representative specimens were selected for the sequencing of the ITS-1 and ITS-2, respectively. The consensus lengths of the ITS-1 and ITS-2 sequences for each sequence type, their mean nucleotide frequencies and positions for the polymorphisms, G+C content and respective sequence accession numbers are given in Table 2. Sequence polymorphism was detected (see Table 4.2; genotypes D-H) both in ITS-1 and ITS-2 regions. Upon pairwise comparison, the percentage difference among all of the different sequence types of ITS-1 (n = 2) and those of ITS-2 (n = 5) was 0.5% and 0.4-0.8%, respectively.

***Terranova* Type II of Cannon (1977b)**

Diagnosis (Fig. 1, D-F).

Third-stage larvae with prominent ventral tooth. Body with numerous prominent longitudinal striations. Oesophagus 0.78-1.10 (0.94, n=10) long; nerve ring 0.26-0.36 (0.28, n=10) from anterior extremity; deirids 0.32-0.36 (0.34, n=7) from anterior extremity; ventriculus 0.35-0.45 (0.38, n=10) long; intestinal diverticulum 0.60-0.87 (0.72, n=10) long, extending well anterior to oesophago-intestinal junction; tail with prominent transverse cuticular striations, 0.12-0.18 (0.15, n=10) long, blunt tipped.

Remarks: The larvae corresponded closely to that described as *Terranova* Type II by Cannon (1977b), also from fishes in Queensland. Cannon (1977b) suggested that they may be the larvae of *T. galeocerdonis* or *T. scoliodontis*. However, the lack of sequence data from adults prevents the testing of this hypothesis.

One hundred and eighty larvae of this type were subjected to PCR-based SSCP analysis. Based on the SSCP profiles, seven and four representative specimens were selected for the sequencing of the ITS-1 and ITS-2, respectively. The consensus lengths of the ITS-1 and ITS-2 sequences for each sequence type, their mean nucleotide frequencies and positions for the polymorphisms, G+C content and respective sequence accession numbers are given in Table 2. Sequence polymorphism was detected (see Table 4.2; sequence types I-M) both in ITS-1 and ITS-2 regions. Upon pairwise comparison, the percentage difference among all of the different sequence types of the ITS-1 (n = 5) and those of the ITS-2 (n = 2) ranged from 0.3-1% and 0.5%, respectively.

***Hysterothylacium* Type IV**

(=*Thynnascaris* Type IV of Cannon, 1977b)

Diagnosis (Fig. 2, A-C)

Fourth stage larvae with three well developed lips and prominent interlabia. Oesophagus 0.85-1.25 (0.94, n=10) long; nerve ring 0.45-0.46 (0.46, n=2) from anterior end; ventriculus 0.10-0.15 (0.12, n=4) long; ventricular appendix 1.3 (n=1) long; intestinal diverticulum extending into posterior oesophageal region, 0.20 (n=1) long; tail 0.11-0.24 (0.18, n=6) long terminating in numerous spikes.

Remarks: The features of this larva agree well with the description of *Hysterothylacium* (=*Thynnascaris*) larval type IV of Cannon (1977b) and also with the description of similar larval stages by Shamsi (2007). The multiple spikes on the tail are characteristic of this larval form. Shamsi (2007) recognized two genotypes with similar morphological features, with Type A in fishes from the Great Barrier Reef and Type B from Victorian fishes.

As the morphological characteristics of this larval type are highly distinctive only a single larva from *Scomberomorus commerson* was subjected to PCR-based SSCP analysis. Based on the SSCP profiles, the ITS-1 and ITS-2 regions of one amplicon were sequenced. The consensus lengths of the ITS-1 and ITS-2 sequences, their mean nucleotide frequencies, G+C content and respective sequence accession numbers are given in Table 4.2 (sequence type N).

Hysterothylacium Type V of Shamsi *et al.* (2007)

Diagnosis (Fig. 2 F, G)

Third-stage larvae without prominent ventral tooth. Oesophagus 0.38-0.46 (0.42, n=6); nerve ring 0.22-0.29 (0.25, n=4) from anterior end; excretory pore 0.30 (n=1) from anterior end; ventriculus 0.04-0.06 (0.05, n=4) long; ventricular appendix 0.20-0.38 (0.31, n=4) long; intestinal diverticulum extending into posterior oesophageal region, 0.13-0.20 (0.15, n=3) long; tail 0.10-0.19 (0.13, n=6), conical with tiny mucro at tip.

Remarks: Morphologically these larvae resemble *Hysterothylacium* Type V of Shamsi (2007) by possessing a conical tail with a mucro at the tip and having the ventricular appendix longer than the intestinal caecum. Two different genotypes were recognised within this morphotype, both of which were closely related to but not identical to that described by Shamsi (2007). The three genotypes have been designated ‘a’, ‘b’ and ‘c’, with type ‘a’ being that described by Shamsi (2007).

Twenty-seven larvae of this type were subjected to PCR-coupled SSCP analysis. Based on the SSCP profiles, eight and six representative PCR amplicons were selected for the sequencing of the ITS-1 and ITS-2, respectively. Two different genotypes were identified and designated ‘HVb’ and ‘HVc’, as they differed slightly from Type V of Shamsi (2007), here considered to represent Type HVa. The consensus lengths of the ITS-1 and ITS-2 sequences for each sequence type, their mean nucleotide frequencies and positions for the

polymorphisms, G+C content and respective sequence accession numbers are given in Table 2 (see sequences O-R). For the sequence HVb, sequence polymorphism was detected in the ITS-1 region only (Table 4.2; genotypes O-Q); upon pairwise comparison, the percentage difference among all of the five sequence types of the ITS-1 ranged from 0.3 to 0.5%. No sequence polymorphism was detected in the ITS-2. For the sequence HVc, no sequence polymorphism was detected in the ITS-1 and ITS-2 regions.

Hysterothylacium Type VI of Shamsi (2007)

Diagnosis (Fig. 2 C, D)

Third-stage larvae without prominent ventral tooth. Oesophagus 0.43-0.65 (0.56, n=5); nerve ring 0.32-0.36 (0.34, n=3) from anterior end; excretory pore 0.40-0.48 (0.44, n=1) from anterior end; ventriculus 0.09-0.10 (0.10, n=2) long; ventricular appendix 0.60 (n=1) long; intestinal diverticulum extending into posterior oesophageal region, 0.31-0.33 (0.32, n=2) long; tail 0.12-0.21 (0.16, n=5), conical with enlarged blunt tip.

Remarks: This larva is similar to *Hysterothylacium* Type VI of Shamsi (2007) in that it possesses a broad, blunt tail and the ventricular appendix is longer than the intestinal diverticulum (see Shamsi 2007).

Eight larvae of this type were subjected to PCR-based SSCP analysis. Based on the SSCP profiles, the ITS-1 and ITS-2 regions of three PCR amplicons were sequenced. The consensus lengths of the ITS-1 and ITS-2 sequences, their mean nucleotide frequencies, G+C content and respective sequence accession numbers are given in Table 4.2 (genotype S). No sequence polymorphism was detected in the ITS-1 and ITS-2 regions.

***Hysterothylacium* Type X of Shamsi (2007)**

(Fig. 2 H)

Third-stage larvae without prominent ventral tooth. Oesophagus 0.40-0.60 (0.48, n=3); nerve ring 0.23 (n=1) from anterior end; tail 0.12-0.20 (0.16, n=2), terminal extremity directed posteriorly with prominent spikes.

Remarks: Few specimens of this larval type were available and those examined were in a poor state of preservation. The characteristic features of the tail with the posteriorly directed spines as well as the sequence data allowed the identification of *Hysterothylacium* Type X. However, other morphological features were not clearly discernible. Shamsi (2007) reported this type only from *Sphyraena novaehollandiae* from Victoria; based on the distinctive features of the tail, this type is now reported from reef fishes in northern Queensland. Shamsi (2007) reported that the ITS-1 and ITS-2 sequence of this larval type was identical to that of the adult of *H*. cf. *pelagicum*.

Five larvae of this type were subjected to PCR-coupled SSCP analysis. Based on the SSCP profiles, the ITS-1 and ITS-2 regions were sequenced from three amplicons. The consensus lengths of the ITS-1 and ITS-2 sequences, their mean nucleotide frequencies, G+C content and respective sequence accession numbers are given in Table 2 (genotype T). No sequence polymorphism was detected in the ITS-1 and ITS-2 regions.

4.3.5 Host associations

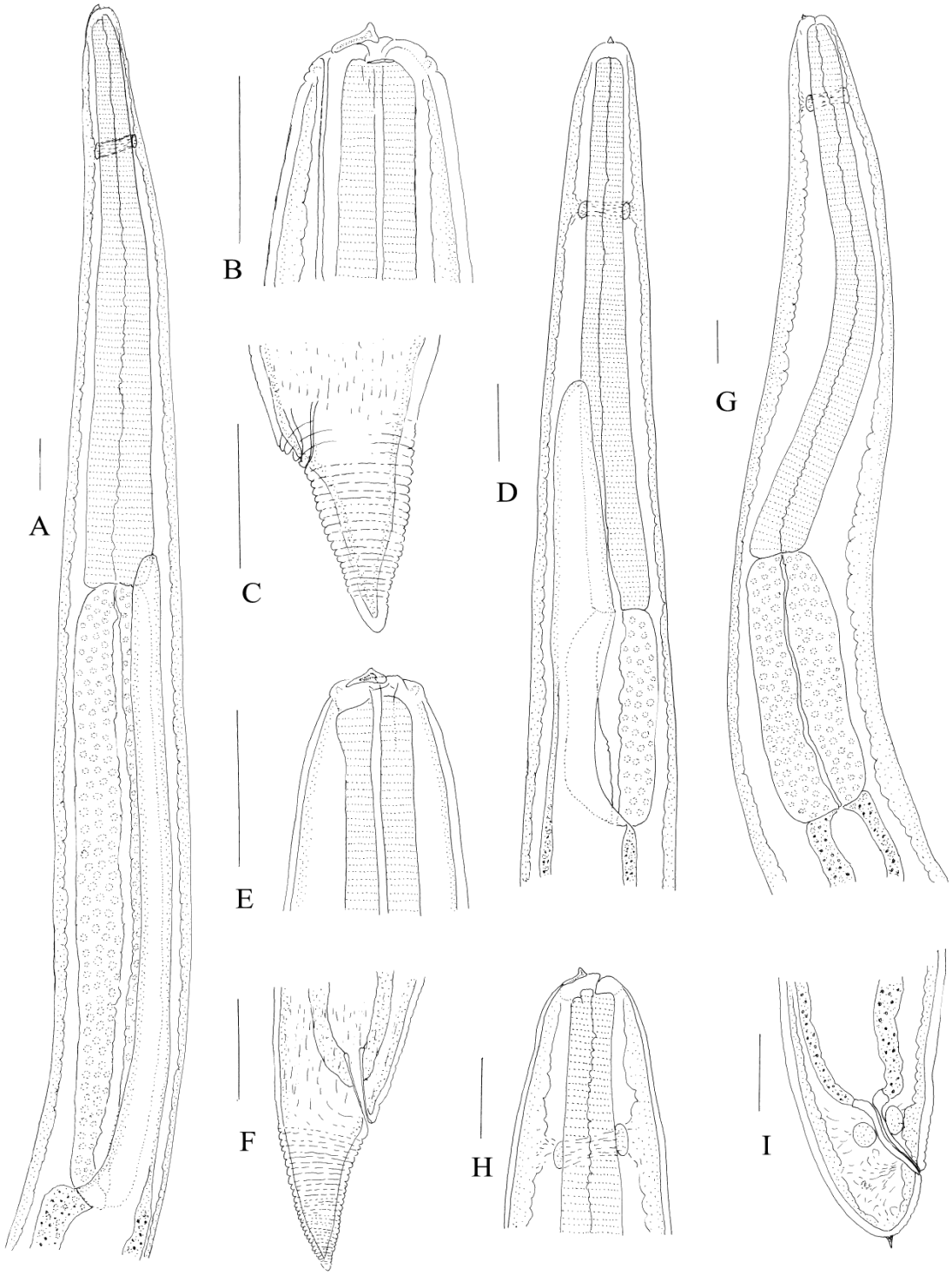
Infections in Atherinidae, Lethrinidae, Lutjanidae and Serranidae were more prevalent, with intensities of infection ranging from 1 to 80, whereas for the Sphyraenidae and Scombridae, the prevalence of infection was very high, with intensities of ranging from 1 to >375 (Table 4. 3). However, in the latter case, the number of nematodes recorded is only a subsample of those present, as the number of nematodes was too high to quantify accurately and only a proportion of the entire infection was collected.

Anisakis typica was found in a wide range of host species (Table 4.3), being most common in scombrids, serranids and sphyranids. Among the smaller fish examined, prevalence was highest in atherinids compared to mullids, pseudochromids, chaetodontids and cirhittids.

Terranova Type I was the least prevalent of the two species of this genus identified in this study and was found in scombrids, serranids, lutjanids, lethrinids and sphyraenids, all predatory fish of a medium to large size. *Terranova* Type II was very common and was

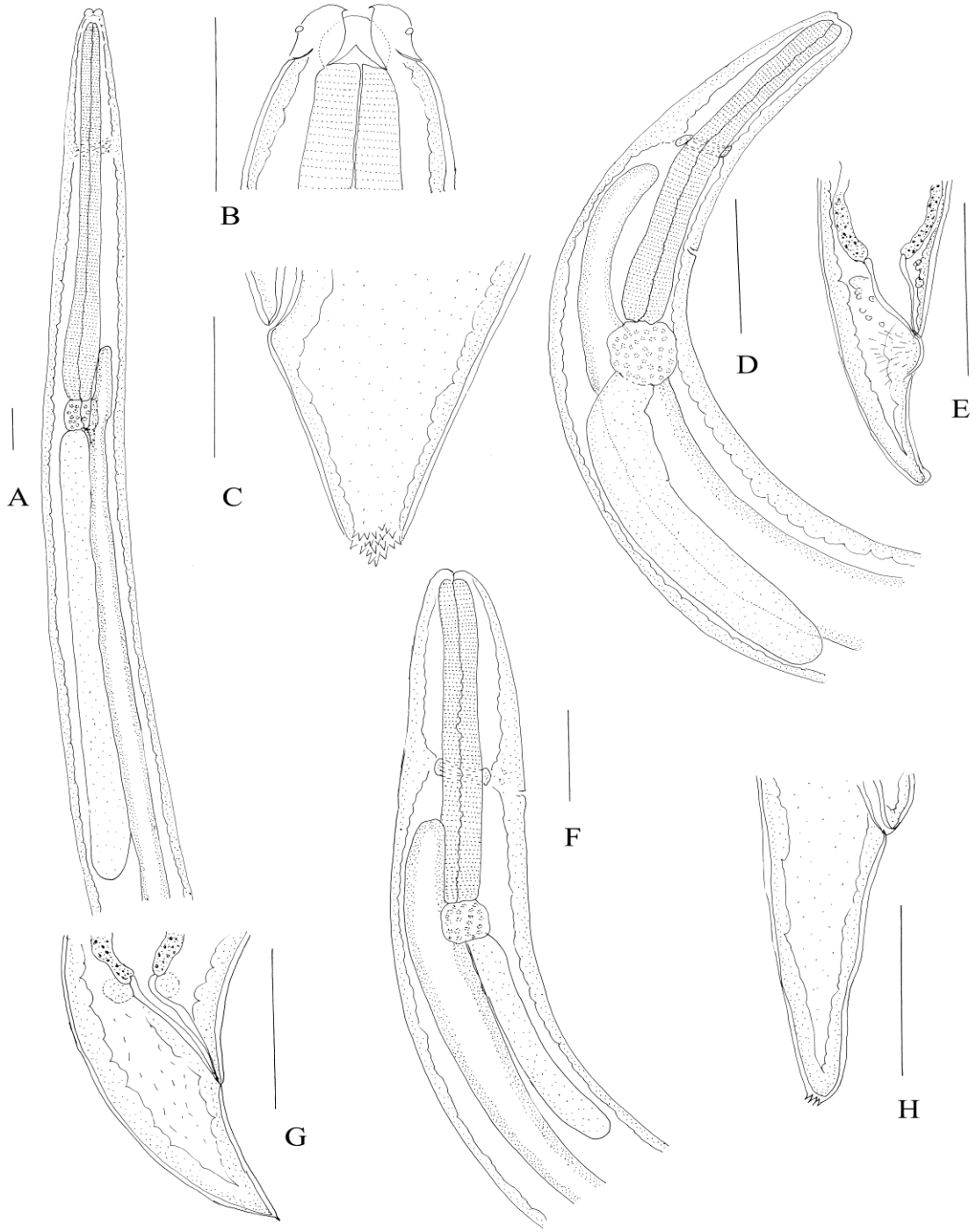
found in large numbers in sphyraenids, serranids and scombrids. Smaller numbers were found in lethrinids and lutjanids, with occasional infections in a wide range of families: Apogonidae, Atherinidae, Carangidae, Cirrhitidae, Chaetodontidae and Nemipteridae. The larger carnivorous species harboured more nematodes than the smaller fish species.

Only small numbers of *Hysterothylacium* Type IV were recovered from a disparate range of families: Carangidae, Cirrhitidae, Chaetodontidae, Pseudochromidae, Scombridae and Serranidae. *Hysterothylacium* Type V larvae were found in lethrinids and lutjanids in small numbers, with a single specimen collected from *Tylosurus* (Belonidae) (Table 4.3). The few specimens of *Hysterothylacium* Type VI larvae were found in Pomacentridae, Pseudochromidae, Chaetodontidae (small species) and, in one instance, in a scombrid. The least frequently encountered nematode, *Hysterothylacium* type X was found only in an atherinid, a serranid and a hyporhamphid (Table 4.2)



Keys:
A=Terranova Type I, anterior end; B, Terranova Type I, cephalic extremity; C, Terranova Type I, tail; D, Terranova Type II, anterior end; E, Terranova Type II, cephalic extremity; F, Terranova Type II, tail; G, Anisakis typica, anterior end; H. Anisakis typica, cephalic extremity; I, Anisakis typica, tail. Scale-bars: 0.1 mm.

Figure 4.10 Third stage larvae of Terranova and Anisakis.



Keys:

A= Fourth stage larva of Hysterothylacium Type IV, anterior end; B, Fourth stage larva of Hysterothylacium Type IV, cephalic extremity; C, Fourth stage larva of Hysterothylacium Type IV, tail; D, third stage larva of Hysterothylacium Type VI, anterior end; E, third stage larva of Hysterothylacium Type VI, tail; F, third stage larva of Hysterothylacium Type V, anterior end; G, third stage larva of Hysterothylacium Type V, tail; H, third stage larva of Hysterothylacium Type X, tail. Scale-bars: 0.1 mm.

Figure 4.11 Fourth and third stage larvae of Hysterothylacium spp.

4.3.6 Phylogenetic analysis

In order to determine the relationships of the anisakid larvae reported herein with other closely related anisakid nematodes, phylogenetic analyses was performed using concatenated ITS-1 and ITS-2 sequence data for all 20 genotypes detected in this study (GenBank accession nos. JX848663- JX848692), together with key reference data (comprising concatenated sequences from previous studies representing *Anisakis typica*, *Hysterothylacium* spp., *Pseudoterranova* spp., *Terranova* sp., and using *Raphidascaris trichiuri* as the outgroup; see Table 4.4).

The concatenated sequences (n = 59) were aligned over 877 positions and subjected to phylogenetic analysis. The analysis grouped three genotypes (designated as A-C) with the published sequences representing *A. typica* with strong nodal support (pp = 1.0) (Fig. 4.10).

Five genotypes (D-H) constituted a monophyletic group representing *Terranova* larval type I while five other genotypes (I-M) grouped with the previously reported sequence representing *Terranova* larval type II with a strong statistical support (Fig. 4.11). Genotype N clustered with *Hysterothylacium* type IV of Shamsi (2007), whereas three genotypes (O-Q) grouped with *Hysterothylacium* type VI of Shamsi (207) (Fig. 4.3). Genotypes R and S formed one clade and grouped with *Hysterothylacium* type V; whereas, genotype T clustered with *Hysterothylacium* type X with strong nodal support (Fig. 4.11). With the exception of genotypes R and S, separate analyses of ITS-1 (56 sequences aligned over ITS-1 = 459 bp) and ITS-2 (52 sequences aligned over 283 bp) sequence datasets produced trees with identical topologies to that generated for the concatenated ITS-1 and ITS-2 dataset (data not shown). Phylogenetic analyses revealed that genotypes R and S formed one clade based on the analyses of the ITS-1 as well as concatenated ITS-1 and ITS-2 datasets (Fig. 4.11), whereas on the basis of the ITS-2 dataset analysis, these form monophyletic groups (see inset Fig. 4.11).

Table 4.3 Intensity (no. of parasites per individual fish) of infection in the examined fish and the association of anisakid larvae with their hosts.

Family	Fish species	Intensity of infection (range (mean, n))	*Anisakis typica*	*Terranova* types I or II	*Hysterothylacium* Types IV, V, VI, X
Apogonidae	*Cheilodipterus intermedius*	6 (n=1)	-	II	-
Atherinidae	*Atherinomorus endrachtensis*	1-4 (2.2, n=5)	+	II	X
Cirrhitidae	*Paracirrhites forsteri*	3 (n=1)	+	II	IV
Lethrinidae	*Lethrinus nebulosus*	5-43 (26.7, n=3)	-	I, II	Vb
Lutjanidae	*Caesio cuning*	5-53 (26.0, n=3)	-	I, II	Vb, Vc
	Lutjanus carponotatus	4 (n=2)	-	II	-
	Lutjanus fulviflamma	1 (n=1)	-	II	-
Chaetodontidae	*Chaetodon ulietensis*	1 (n=1)	+	-	IV
	Chaetodon citrinellus	11(n=1)	-	II	VI
Pseudochromidae	*Stegastes apicalis*	7 (n=1)	+	-	IV, VI
Scombridae	*Grammatorcynus bicarinatus*	2-197 (88.8, n=6)	+	I, II	IV
	Scomberomorus commerson	19-254 (137, n=2)	+	I, II	IV, VI
Serranidae	*Cephalopholis boenak*	1 (n=1)	+	-	IV
	Cephalopholis cyanostigma	5-18 (11.5, n=2)	+	I, II	X
	Epinephelus ongus	1 (n=1)	+	-	IV
	Plectropomus leopardus	5-80 (42, n=6)	+	I, II	-
Belonidae	*Tylosurus crocodilus*	1 (n=1)	-	-	Vc

Mugilidae	*Liza vaigiensis*	1 (n=1)	+	-	-
Hyporhamphidae	*Hyporhamphus affinis*	2 (n=1)	-	-	X
Nemipteridae	*Scolopsis monogramma*	3 (n=1)	-	II	Vb
Carangidae	*Caranx papuensis*	2-4 (3, n=2)	+	II	IV
Sphyraenidae	*Sphyraena forsteri*	211-357 (284, n=2)	+	I, II	-
Pomacentrida	*Acanthochromis polyacanthus*	28 (n=1)	-	-	Vc, VI

(+) presence of a particular type of anisakid larva (e); (+) absence of a particular type of anisakid larva (e)

4.4 Discussion

Our understanding of the biodiversity and systematics of parasites has been revolutionised through the use of molecular approaches (Mattiucci and Nascetti 2008). These methods have been utilised not only for delimiting and identifying anisakid nematodes but also to reveal cryptic species, such as the members of *Anisakis simplex* complex, including *A. simplex C* and *A. pegreffii* (see Mattiucci and Nascetti 2006, 2008). In the present study, we used both molecular-phylogenetic and morphological approaches to characterise larval anisakid nematodes in fishes collected from a single location, Lizard Island. The genetic analysis of larvae has allowed the specific and genotypic identification and differentiation of larvae within single morphotypes based on matching their sequences with those available for morphologically defined specimens of species of *Anisakis*, *Terranova* and *Hysterothylacium*.

Although more than 450 individual teleost fishes and 107 species were sampled during this study, this represents a relatively limited portion of the teleost biodiversity occurring around Lizard Island and at other reefs on the GBR. Furthermore, many of the species (12 species) were represented by only a single individual. The following discussion is therefore presented within these acknowledged limitations in mind.

The present epidemiological survey revealed eight morphotypes representing *A. typica*, *Terranova* larval Types I and II, *Hysterothylacium* larval Types IV, Vb, Vc, VI and X; whereas, mutation scanning analysis revealed 20 profiles (see Table 4.3) representing 20 genotypes following sequencing. Based on the polymorphism in the ITS-1 region of *A. typica*, three genotypes were defined (designated as genotypes A-C) and the comparison of these sequences with the published ITS-1 sequences showed that genotypes A and B had almost identical sequences (except for a Y at position 201 in genotype B) as reported previously from China (GenBank accession number AM706345) and Indonesia (EU346093 and JN968932) (Zhang *et al*., 2007, Palm *et al*., 2008, Kuhn *et al*., 2011), whereas genotype C matched other *A. typica* ITS-1 sequences available on the GenBank database (see Figure 4.12). Two larval Types (I and II) of *Terranova* spp. were defined based on their morphological characteristics; molecular methods showed that each of these larval types was composed of five genotypes (see Figure 4.12). Comparison of the ITS-1 and ITS-2 sequences of both *Terranova* spp. with the published sequences revealed that those of *Terranova* larval Type I (genotypes D-H) reported herein are new sequences, while those of

Terranova larval Type II (genotypes I-M) matched *Terranova* larval Type II (301-1-1, 301-1-2) of Shamsi (2007).

Table 4.4 Anisakid internal transcribed spacers (ITS-1 and ITS-2) sequence data determined in the present study, together with reference sequences (see GenBank accession numbers) from previous studies used in the phylogenetic analysis (Fig. 3.12).

Parasite species/ genotype	Stage of parasite	Host species	Location	Accession nos. (ITS1)	Accession nos. (ITS2)	References
A (*Anisakis typica*)	Third stage larvae - L(3)	*Atherinomorus endrachtensis, Caranx papuensis, Cephalopholis boenak, C. cyanostigma, Chaetodon ulietensis, Epinephelus ongus, Grammatorcynus bicarinatus, Liza vaigiensis, Paracirrhites forsteri, Plectropomus leopardus, Scomberomorus commerson, Sphyraena forsteri, Stegastes apicalis*	Lizard Island, Australia	JX848663	JX848680	This study
B (*A. typica*)	L(3)	*S. forsteri*	Lizard Island, Australia	JX848664	-do-	This study
C (*A. typica*)	L(3)	*S. apicalis*	Lizard Island, Australia	JX848665	-do-	This study
D (*Terranova larval type I*)	L(3)	*Caesio cuning, C. cyanostigma, G. bicarinatus, Lethrinus nebulosus, P. leopardus, S. commerson, S. forsteri*	Lizard Island, Australia	JX848666	JX848681	This study
E (*Terranova I*)	L(3)	*G. bicarinatus*	Lizard Island, Australia	-do-	JX848682	This study
F (*Terranova I*)	L(3)	*G. bicarinatus*	Lizard Island, Australia	-do-	JX848683	This study
G (*Terranova I*)	L(3)	*G. bicarinatus*	Lizard Island, Australia	JX848667	JX848684	This study
H (*Terranova I*)	L(3)	*G. bicarinatus, S. forsteri*	Lizard Island, Australia	-do-	JX848685	This study
I (*Terranova larval type II*)	L(3)	*A. endrachtensis, C. cuning, C. papuensis, C. cyanostigma, Chaetodon citrinellus, Cheilodipterus intermedius, G. bicarinatus, L. nebulosus, Lutjanus carponotatus, P. forsteri, P. leopardus, S. commerson, S. forsteri*	Lizard Island, Australia	JX848668	JX848686	This study
J (*Terranova II*)	L(3)	*G. bicarinatus*	Lizard Island, Australia	JX848669	-do-	This study

K (*Terranova II*)	L(3)	*Scolopsis monogramma*	Lizard Island, Australia	JX848670	-do-	This study
L (*Terranova II*)	L(3)	*P. leopardus*	Lizard Island, Australia	JX848671	-do-	This study
M (*Terranova II*)	L(3)	*P. leopardus*	Lizard Island, Australia	JX848672	JX848687	This study
N (*Hysterothylacium larval type* IV)	L(3)	*C. papuensis, C. boenak, C. ulietensis, E. ongus, G. bicarinatus, P. forsteri, S. commerson, S. apicalis, Tylosurus crocodilus*	Lizard Island, Australia	JX848673	JX848688	This study
O (*Hysterothylacium larval type* VI)	L(3)	*A. polyacanthus, C. cuning, L. nebulosus, S. monogramma, T. crocodiles*	Lizard Island, Australia	JX848674	JX848689	This study
P (*Hysterothylacium* VI)	L(3)	*C. cuning*	Lizard Island, Australia	JX848675	-do-	This study
Q (*Hysterothylacium* VI)	L(3)	*C. cuning*	Lizard Island, Australia	JX848676	-do-	This study
R (*Hysterothylacium larval type* V)	L(3)	*Acanthochromis polyacanthus, C. cuning, T. crocodiles*	Lizard Island, Australia	JX848677	JX848690	This study
S (*Hysterothylacium* V)	L(3)	*A. polyacanthus, C. citrinellus, S. commerson, S. apicalis*	Lizard Island, Australia	JX848678	JX848691	This study
T (*Hysterothylacium larval type* X)	L(3)	*A. endrachtensis, C. cyanostigma, Hyporhamphus affinis*	Lizard Island, Australia	JX848679	JX848692	This study
Anisakis typica	Adult (A)	*Stenella longirostris*	Brazil	AY826724	AY826724	(Nadler *et al.* 2005)
A. typica	A	*Sotalia guianensis*	Brazil	EU327686	EU327686	(Iniguez *et al*. 2009)
A. typica	L(3)	*Auxis thazard*	Brazil	EU327689	EU327689	(Iniguez *et al*. 2009)
A. typica	L	*Auxis rochei rochei*	Indonesia	EU346092	EU346092	(Palm *et al.* 2008)
Anisakis cf. typica 1	L	*Auxis r. rochei*	Indonesia	EU346091	EU346091	(Palm *et al.* 2008)
Anisakis cf. typica 2	L	*Auxis r. rochei*	Indonesia	EU346093	EU346093	(Palm *et al.* 2008)
A. typica	L	*Scomber japonicas*	Japan	AB432908	AB432908	(Umehara *et al*. 2010)
A. typica	L	*Trichiurus* sp.	Japan	AB551660	AB551660	(Umehara *et al*. 2010)
A. typica	A	*Steno bredanensis*	Florida, USA	AB479120	AB479120	(Umehara *et al*. (2010)
A. typica	L	*Selar crumenophthalmus*	Guangdong province,	AM706345	AM706345	(Zhu *et al.* 2007)

			China			
A. typica	L	*C. cuning*	Indonesia	JN968932	JN968932	(Kuhn *et al.* 2011)
A. typica	L	*Trichiurus lepturus*	Indonesia	JN968944	JN968944	(Kuhn *et al.* 2011)
A. typica	L	*Selar crumenophthalmus*	Hawaii	JN968912	JN968912	(Kuhn *et al.* 2011)
A. typica	L	*Katsuwonus pelamis*	Hawaii	JN968906	JN968906	(Kuhn *et al.* 2011)
A. typica	L	*K. pelamis*	Moorea, Polynesia	JN968964	JN968964	(Kuhn *et al.* 2011)
A. typica	L(3)	*Merluccius polli*	Morocco	EU718476	EU718476	(Kijewska *et al*. 2009)
Hysterothylacium aduncum	L(3)	*Clupea pallasi, M. cephalus*	China	AM503955	AM503956	(Zhang *et al*. 2007)
H. aduncum	L(3)	*Platichthys flesus*	Poland	AJ225068	AJ225069	(Zhu *et al.* 1998)
H. aduncum	L(3)	*Hypomesus pretiosus japonicas*	Japan	AB277826	AB277826	(Umehara *et al*. 2008)
H. aduncum	L(3)	*P. flesus*	Poland	AJ937672	AJ937672	(Zhu *et al.* 2007)
H. aduncum	L(3)	*Zoarces viviparous*	Poland	AJ937673	AJ937673	(Zhu *et al.* 2007)
Hysterothylacium auctum	L(3), A	*Z. viviparous*	Baltic sea	AF115571	AF115571	(Szostakowska *et al.* 2001)
H. auctum	A	*Z. viviparous*	Finland	AF226591	AF226591	(Nadler *et al.* 2000)
H.pelagicum	A	*Coryphaena hippurus*	USA	AF226590	AF226590	(Nadler *et al.* 2000)
Hysterothylacium cf. pelagicum	A	*Seriolala lalandi*	Australia	175-5-18*	175-2-2*	(Shamsi 2007)
Hysterothylacium larval type III	L(3)	*Lutjanus argentimaculatus, Lutjanus fulviflammus*	Australia	336-8-43*	336-8-2*	(Shamsi 2007)
Hysterothylacium larval type IVA	L(4)	*C. cuning, L. argentimaculatus*	Australia	311-1-1*	311-1-2*	(Shamsi 2007)
*Hysterothylacium*larval type IVB	L(4)	*Sillago flindersi, S. australasicus*	Australia	11-2-1-4*	11-2-2-3*	(Shamsi 2007)
Hysterothylacium larval type V	L(3)	*Lutjanus carponotatus*	Australia	305-1-1*	305-1-2*	(Shamsi 2007)
Hysterothylacium larval type VI	L(3)	*Chaetodon lineolatus*	Australia	314-1-1*	314-1-2*	(Shamsi 2007)
Hysterothylacium larval type VII	L(3)	*C. cuning*	Australia	306-4*	306-4*	(Shamsi 2007)

Hysterothylacium larval type VIII	L(3)	*Engraulis australis, Sardinops neopilchardus*	Australia	141-3-1-4*	141-3-2-3*	(Shamsi 2007)
	L(3)	*Seriola hippos, S. lalandi*	Australia			
	L(3)	*S. flindersi*	Australia			
	L(3)	*S. australasicus*	Australia			
Pseudoterranova azarasi	A	*Eumetopias jubata*	Iwanai, Japan	AJ413973	AJ413974	(Zhu *et al.* 1998)
P. bulbosa	A	*Erignathus barbatus*	Newfoundland, Canada	AJ413969	AJ413971	(Zhu *et al.* 1998)
P. cattani	A	*Otaria byronia*	Central-southern, Chile	AJ413982	AJ413984	(Zhu *et al.* 1998)
P. decipiens	A	*Phoca vituline*	Newfoundland, Canada	AJ413968	AJ413967	(Zhu *et al.* 1998)
P. krabbei	A	*Halichoerus grypus*	Newfoundland, Canada	AJ413965	AJ413966	(Zhu *et al.* 1998)
Terranova II	L	*Abduefduf whitleyi, C. cuning. Carangoides fulvoguttatus, Caranax ignobilis, C. melampygus, Epinephelus cyanopodus, G. bicarinatus, L. argentimaculatus, L. buhar, L. fulviflammus, S. australasicus*	Australia	301-1-1*	301-1-2	(Shamsi 2007)
Raphidascaris trichiuri	A	*Muraenesox cinereus*	Taiwan	FJ009682	FJ009682	(Damin and Heqing 2001)

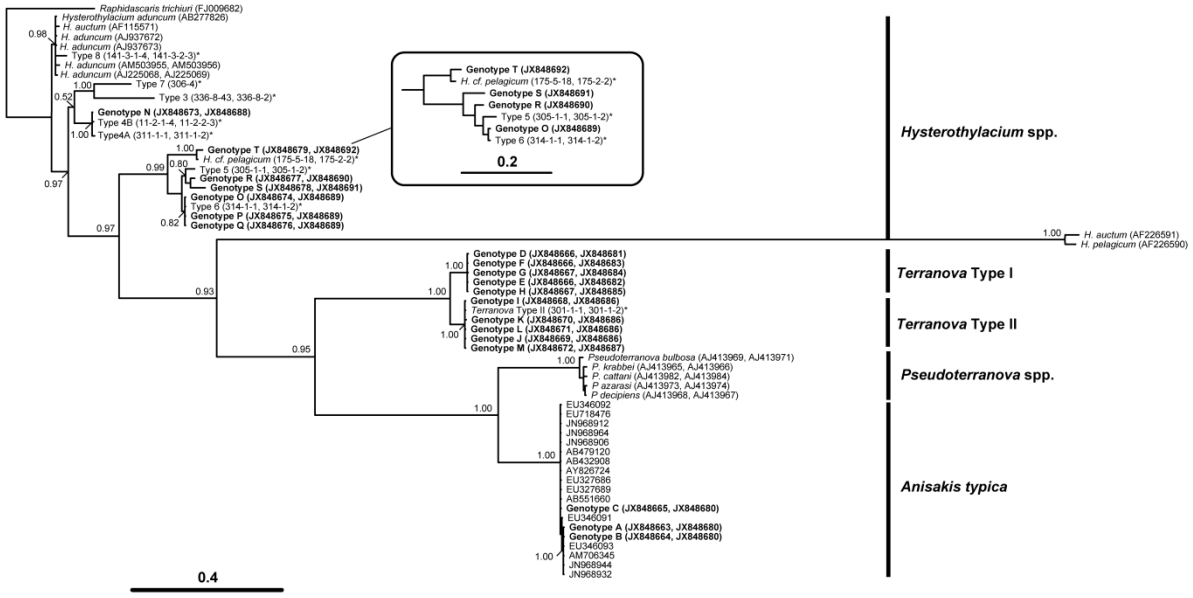


Figure 4.12: Phylogenetic relationships of anisakid nematodes representing genotypes A-T based on concatenated ITS-1 and ITS-2 sequence data determined herein, together with selected reference sequences for closely related anisakid nematodes. An inset box shows the difference in the topology of genotypes R and S based on the analysis of ITS-2 dataset. The sources and accession numbers of sequences are listed in Table 4. For each branch of the tree, a parasite species/genotype name is followed by GenBank accession or voucher (*) number. Relationships were inferred based on analyses employing Bayesian Inference method. Nodal support is given as a posterior probability for BI (below the line). Scale bars show the rate of substitution.

In the present study, five types of *Hysterothylacium* were identified based on their morphological characteristics; however, their molecular characterisation allowed the identification of seven genotypes (N-T). Sequence data for *Hysterothylacium* larval Type IV reported herein (genotype N) matched with those of *Hysterothylacium* larval Type IVB of Shamsi (2007). Although *Hysterothylacium* larval Types Vb and Vc reported herein resembled, based on morphological characteristics, *Hysterothylacium* larval Type V of Shamsi (2007), the comparison of ITS-1 and ITS-2 sequences of these two morphotypes revealed that the sequences of *Hysterothylacium* larval Type Vb (genotypes O-Q) matched with those of *Hysterothylacium* larval Type VI of Shamsi (2007) (see Fig 4.12). This discrepancy between morphological and molecular identification of the same larval type in two different studies cannot be readily explained, as we ensured that the sequence types being reported in this study corresponded to the correct morphotypes. In the present study,

we also found that there were at least three genotypes (O-Q) for *Hysterothylacium* larval Type Vb. Morphologically *Hysterothylacium* larval Type X reported herein (genotype T) resembled that reported by Shamsi (2007). Phylogenetic analysis revealed that this larval type grouped with *H. cf. pelagicum* of Shamsi (2007) (see Fig. 4.11). Based on morphological characteristics, *Hysterothylacium* larval Type Vc matched *Hysterothylacium* larval Type V of Shamsi (2007); however, phylogenetic analysis of the ITS-1 and concatenated ITS-1 and ITS-2 datasets revealed that genotype R formed a clade with *Hysterothylacium* Type VI (genotype S), with *Hysterothylacium* larval Type V of Shamsi (2007) as the sister group, whereas the phylogenetic analysis of the ITS-2 dataset revealed that all *Hysterothylacium* larval Type Vc (genotype R), *Hysterothylacium* Type VI (genotype S) and *Hysterothylacium* larval Type V of Shamsi (2007) formed monophyletic clades (see inset Fig. 3). This might be attributed to the percentage of sequence difference in the two ITS regions as pair-wise comparisons between *Hysterothylacium* larval Types Vb and Vc revealed difference for ITS-1 and ITS-2 of 2.6% and 3.0%, respectively; whereas comparison between *Hysterothylacium* larval Types Vc and VI revealed differences for the ITS-1 and the ITS-2 of 4.4 and 9.2%, respectively. It is possible that the groups currently recognized as *Hysterothylacium* Types V and VI each comprises two or more distinct species, but additional collections are required to confirm this hypothesis.

The data presented here suggest that anisakid infections are relatively uncommon in some families of small reef fishes which were extensively sampled; these fishes included the Apogonidae (cardinal fishes) and Chaetodontidae (butterfly fishes), while they occurred in very large numbers in members of the Scombridae (mackerels) and Sphyraenidae (barracudas), although the numbers of fish sampled in the latter families was small. In the case of the scombrids and sphyraenids, the infestations were too heavy to determine the precise numbers of nematodes present; the numerical data presented here therefore underestimate the abundance of larval nematodes present. Anisakid nematodes occurred at a moderate to high abundance in members of the Serranidae (gropers), although, again, only small numbers of fish in this family were examined for parasites. Prevalence in other predatory fish families such as the Lethrinidae (emperors) and Lutjanidae (snappers) were modest, although this may reflect limited sampling of the relevant families. Considering these limitations, anisakid nematodes do appear more prevalent and more abundant in higher order predator teleosts than in smaller teleosts occupying a lower position in the

food web, a pattern to be expected based on the known or presumed life cycles of these nematodes as summarised by Anderson (2000).

One particular obstacle with parasitic life cycles using trophic transmission is how parasites might avoid so-called "dead end" hosts that are not consumed by higher order predators. Such a group of fishes might be the Tetraodontiformes, which includes species of the Tetraodontidae (puffer fish, toad fish), which are highly toxic and, therefore, are unlikely to be consumed by higher order predators. In this study, none of the 38 tetraodontiform fishes examined was infected with anisakids. Although a relatively small sample size was used, the data do suggest not only that members of this order of fishes are unlikely to be significant contributors to anisakid transmission but that the 'avoidance' of these hosts, however achieved, removes or reduces the possibility of aniskaid larvae infecting "dead-end' hosts which are unlikely to contribute to the completion of the life cycle. The lack of infection in potential "dead-end" hosts belonging to the order Tetraodontiformes was intriguing but requires additional sampling to confirm this preliminary finding. Members of this order of fish are omnivores, algivores or feed on invertebrates. Given this spectrum of diets, the reasons for their lack of infection with anisakid larvae could prove to be of interest.

The scombrids and sphyraenids examined were infected with large numbers of *A. typica* and two species of *Terranova*. Adults of *A. typica* are found primarily in small, piscivorous cetaceans (Mattiucci and Nascetti 2006), while species of *Terranova* mature primarily in sharks (Bruce and Cannon 1990). Large scombrids and sphyraenids are likely to be suitable prey species for both of these groups of definitive hosts, thereby facilitating the completion of the life cycle. Serranids were infected with *A. typica* and species of *Terranova*, while lutjanids and lethrinids were also infected with species of *Terranova*, although the numbers recovered in all three families were smaller than those seen in scombrids and sphyraenids. All of these species represent potential prey for cetaceans and large elasmobranchs.

Of the smaller fishes examined, both *A. typica* and *Terranova* Type II were recovered from *Atherinomorus*. The extent to which definitive hosts might prey on this species is not known, but they may be preyed upon by the larger species of teleosts, such as the scombrids and sphyraenids, which act as effective 'accumulators' of larvae. The various types of *Hysterothylacium* encountered occurred in a wide range of species but predominated in medium sized to smaller sized fishes likely to be consumed as prey by

higher order teleost predators in which species of this genus mature (Deardorff and Overstreet 1980).

This preliminary study attempted to map the distribution of larval anisakids across a community of reef fishes and identified some broad features, which should guide future investigations to provide greater definition of this distribution. The scarcity of anisakid larvae in small fishes, such as apogonids and chaetodontids, was not surprising, given their position in the food chain with the apogonids feeding primarily on zooplankton (Marnane and Bellwood 2002), while chaetodontids are omnivorous or corallivores (Morand *et al.* 2000; Cole *et al.* 2008). By contrast, the higher order predators, such as the serranids, scombrids and sphyraenids, harboured large numbers of anisakid larvae, dominated by *A. typica* and species of *Terranova*. More extensive sampling of these families is clearly warranted to confirm their role in the transmission of anisakids. The species of *Hysterothylacium* detected occurred predominantly in smaller- or medium-sized fishes, but the numbers recovered were low and the range of families extremely broad, preventing any general conclusions from being drawn. However, given their apparent diversity, which was lower in this study than that reported by Shamsi (2007) from Heron Island in the southern Great Barrier Reef, a greater emphasis needs to be placed on the distribution of species of this genus in future studies of reef teleosts.

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Museum) for the identification of some of the species of fish encountered and Julian Caley (AIMS) for his organisation of one of the collecting trips. An Early Career Researcher (ECR) grant (to AJ) from The University of Melbourne is gratefully acknowledged. Fish were collected under the Queensland Museum's Great Barrier Reef Marine Park Authority's Memorandum of Understanding (GBRMPA MOU) granted to the Queensland Museum, covering staff Robert Adlard and Terrence Miller and the the GBRMPA Marine Parks Permit number G10/33400.1 issued to Robert Adlard. Fish were collected under animal ethics permit number 058 from the Queensland Museum, Brisbane.

Chapter five: Molecular and Immunological characterisation of allergenic proteins in *A. pegreffii* (tropomyosin) and comparison among the anisakid group.

Abstract

A. pegreffii can induce hypersensitivity IgE-mediated reactions with several clinical manifestations ranging from urticaria to angioedema to anaphylaxis and it has been suggested that tropomyosin could be involved in this IgE response (Arruda, 2005). During this study, the tropomyosin gene of *A. pegreffii* was amplified using primers, and shown to be 805bp. Recombinant tropomyosin protein was also produced and detected at 44kDa using anti-tropomyosin and anti-crustacean polyclonal antibodies raised in rabbits. The allergenicity of the nematode recombinant tropomyosin was evaluated using human serum of an atopic patient with shellfish allergy.

Background:

The larvae third stage (L3) of the nematode *A. pegreffii* parasitizes marine organisms, such as fish and squid. The *Anisakis* protein extract from L3 frequently causes allergic reactions in sensitized individuals. The dominant allergen protein in different crustaceans is tropomyosin, which has been identified in many species and is responsible for about 80 % of cases related to allergic incidents. Based on the results presented in chapter three, the immunoblotting suggested that both tested native and recombinant *A. pegreffii* tropomyosins share one or more epitopes with crustacean tropomyosin. Therefore, it has been thought that tropomyosin from *A. pegreffii* may cause allergic reactions.

Aim:

To produce recombinant tropomyosin by cloning, compare its activities with native tropomyosin and use it to identify antibody responses in crustacean -allergic patients.

Methods:

Double-stranded cDNA encoding *A. pegreffii* L3 tropomyosin isoform (*As*-TPM) was synthesised from poly $(A)^+$ mRNA and amplified by PCR using primers designed according to described nucleotide sequences for *A. simplex* tropomyosin. The tropomyosin gene was cloned into PCR2.1 for DNA sequencing and then pRSET-A and transformed into BL21 *E. coli* to produce recombinant tropomyosin protein. The protein was expressed and purified using IMAC and SDS-PAGE analysis was performed. The characterisation of recombinant tropomyosin was carried out by immunoblotting tests.

Results

The PCR product was shown as a single band 805 bp in size. After expression and purification methods, SDS PAGE gel analysis indicated purified recombinant tropomyosin protein at 44kDa in size and this was confirmed by immunoblotting.

Conclusion

In the present work, *A. pegreffii* recombinant tropomyosin cDNA was cloned, sequenced, characterized and amplified using the PCR method. Its predicted amino acid sequence was compared with those of other species and the native tropmyosin. Result of this work shows that recombinant tropomyosin of *A. pegreffii* can be used to identify immune responses in allergic patient.

5.1 Introduction

Although an extensive range of helminths have been reported to parasitize seafood, only a few species are capable of infecting humans (Adams *et al*., 1997). Humans acquire larval anisakid nematodes (particularly *A. simplex, A. pegreffii and Pseudoterranova decipiens*) by eating raw, inadequately cooked, poorly salted, pickled or smoked seafood (Adams *et al*., 1997). Larvae, in a bid to survive and establish themselves in the host (human), continuously secrete or excrete proteins to evade/confront the hosts' immune system (Hewitson *et al*., 2008, Nagaraj *et al*., 2008, Smith *et al*., 2009). Tropomyosin protein is one of the biomolecules found responsible for allergic reactions in sensitized individuals. Tropomyosins are a family of highly conserved actin-binding muscle proteins (Sereda et al., 2008). It has been known that tropomyosins from different invertebrate species can induce potent IgE antibodies that recognize and cross-react to tropomyosins from other invertebrate species, meaning that invertebrate tropomyosins have a high degree of similarity and shared epitopes that can explain specificity and affinity of IgE antibody binding (Subba Rao *et al*., 1998). Therefore, invertebrate tropomyosin is described as a pan-allergen. Cross-reactivity among tropomyosins in invertebrate species was discussed in chapter 2.

5.2 Materials and methods

5.2.1 RNA extraction

5.2.1.1 Total RNA extraction from tissue cells (Kit)

Total RNA was extracted using an RNA Mini Kit (Bioline) following the manufacturer's instructions. In brief, *Anisakis* larvae (n=35) were ground using a mortar and pestle in liquid nitrogen. The powder was transferred to a 1.5 ml tube and 450 µl Lysis Buffer R was added and homogenized. After that, the sample was spun at maximum speed for 1 minute in a microfuge and the supernatant was transferred to spin column R1 placed in a collection tube. The supernatant was centrifuged at 10,000g for 2 minutes and the filtrate collected. Afterwards 1 volume of 70% ethanol was added and mixed well with filtrate to clear the lysate, then transferred to a spin column R2 placed in a collection tube. After, the sample was centrifuged at 10,000g for 2 minutes, then the collection tube was discarded, and the spin column R2 was placed in a new collection tube. 500µl Wash Buffer AR was applied to spin column R2 and spun at 10,000g for 1 minute, then the collection tube was discarded, and spin column R2 was placed in a new collection tube. 700µl Wash Buffer BR was added to spin column R2 and centrifuged at 10,000g for 1 minute, then the collection tube was discarded, and spin column R2 was placed in a new collection tube. After that, spin column R2 was centrifuged at 10,000g for 2 minutes to remove traces of ethanol, then the collection tube was removed, and spin column R2 was placed in a 1.5ml Elution Tube. 30µl of RNase-free water was added to spin column R2 membrane and incubated at room temperature for 1 minute. Finally, spin column R2 was centrifuged at 10,000g for 1 minute to elute RNA.

5.2.2 Complimentary DNA synthesis

Once confirmed that the samples have RNA by running on a gel, complimentary deoxyribonucleic acid (cDNA) was synthesized from isolated RNA using the Reverse Transcription protocol (Bioline) following the manufacturer's instructions. All reactions were prepared on ice, where priming premix was prepared as following; 1.8µl of RNA was mixed with 1µl Oligo (dT)18, 1µl of 10mM dNTP mix and 6.2µl DEPC-treated water up to 10µl. The samples were incubated at 70°C for 5 minutes, then chilled on ice for at least 1 minute. After that, the reaction premix was prepared as following; 4µl of 5x RT Buffer was mixed with 1µl Ribosafe RNase inhibitor, 0.25µl Reverse Transcriptase (200µ/µl) and 3.75µl DEPC-treated water up to 10µl. 10µl of the reaction premix was added to 10µl of

priming premix and mixed gently by pipetting. After that, samples were incubated at 42°C for 30 minutes to activate Reverse Transcription and increase the yield of cDNA synthesized. After, cDNA synthesized was incubated at 85°C for 5 minutes to terminate the reaction, and then the samples were chilled on ice. Finally, cDNA was stored at 20°C for long-term storage.

5.2.3 Measurement of DNA and RNA concentration and quality by Nanodrop spectrophotometer

DNA and RNA concentration and quality were determined by Nanodrop spectrophotometer (Thermo Scientific™ 2000) following the manufacturer's protocol. The absorbance at wavelengths 260 nm (nucleic acid) was compared to wavelengths at 280 nm (protein) and 230 nm (organics) to determine protein or organic contamination. Samples with 260/280 and 260/230 ratios between 1.8 and 2.0 were deemed as good quality DNA/RNA and used for further analysis.

5.2.4 Predict signal peptides for tropomyosin gene

It is usual that most secretory proteins are initially synthesized in the cytoplasm as precursors containing 15-30 residues of extra peptide extensions at the beginning of their sequence (N-terminus). These so-called signal peptides play an important role in the translocation of newly synthesized proteins across the membrane of the endoplasmic reticulum (ER) in eukaryotic cells (Tsuchiya et al., 2003). To improve the expression level of recombinant protein expressed in *E. coli*, signal peptides are excluded from designed recombinant genes. Therefore, the tropomyosin protein sequence was subjected to SignalP 4.1 Server – CBS analysis to identify any signal sequence. For the tropomyosin gene no signal peptide was identified, as expected.

5.2.5 Designing the primers

5.2.5.1 Design primer for Tropomyosin gene

The primers to amplify the whole gene coding for tropomyosin (Ani p 3) protein were designed manually as well as designing the orientation of the genes within pCR2.1 and pRSETA vectors by SE Central (Clone Manger) software. The first and last 20 digits corresponding to the forward and reverse primers of the genes were selected and to which restriction enzyme sites were added. The restriction enzymes selected were *Bam*HI (forward) and *Xho*I (reverse), which recognise (GGATCC) and (GAGCTC), respectively. Those restriction enzymes were chosen because they both do not have recognition sites

within the gene but do have in pCR2.1 and pRSETA vectors. CGG nucleotides were added to increase the stability of the primers. The primers were ordered from GeneWorks Custom Oligo Service.

Table 5.1 The primers used in this study (5'-3')

Amplification of cDNA gene tropomyosin (*Ani p* 3)

Gene	Primer sequences
Ani p 3	*Ani p 3* f CGGGATCCATAGACGCAATCAAGAAGAAGATG *Ani p 3* r CGGAGCTCTATTAATATCCAGACAGCTCTTG

5.2.5.2 Preparation of the primers

The ordered two primers were supplied in a dried form, and of which 100 µmol stock solutions were prepared as shown below:

For-Fila (forward primer),

58.4nmol/tube

Concentration= Mass/volume

$$\text{Volume} = \frac{58.4\times10^{-3}}{100\ \mu\text{mol/L}}$$

=584µl of MG water was added to reconstitute.

2) Rev-Fila (reverse primer),

52.4nmol/tube

Concentration= Mass/volume

$$\text{Volume} = \frac{52.4\times10^{-3}}{100\ \mu\text{mol/L}}$$

=524µl of MG water was added to reconstitute.

To prepare 10 µmol working, the stock solutions were diluted 1:10 (50 µl of each vial mixed with 450 µl of MG water), and they were all stored at -20°C.

5.2.5.3 PCR reaction

The PCR reaction was performed to amplify the gene coding for the Ani p 3 protein from the extracted Ani p 3 cDNA.

Table 5.2 PCR cycling conditions for amplification of the tropomyosin gene of *A. pegreffii.*

PCR Components			PCR cycling conditions			
Reagent	Volume		Step	Temperature	Time	number of cycles
	Ani p 3 gene	Negative control	Heated lid	110°C	--	30
cDNA	1.5µl	-	Hot start	94°C	00:02:00	
Forward primer	0.5µl	0.5µl	Denaturation	95°C	00:00:20	
Reverse primer	0.5µl	0.5µl	Annealing	55°C	00:00:15	
MyTaq DNA Polymerase	12.5	12.5	Elongation	72°C	00:00:50	
MG water	10µl	11.5µl	Final Elongation	-	-	
Total volume	25µl	25µl	Hold	4°C	Infinite	

5.2.5.4 Purification of DNA from PCR amplification

Purification of DNA from the PCR amplification was performed by using a Wizard PCR prep kit. One hundred µl of purification buffer was added to each PCR reaction and mixed briefly with a pipette. One ml of resin was added to the mixture and vortexed briefly. The entire mixture was then transferred to a column and was gently passed through it. Two mls of isopropanol was added and the solution centrifuged for 1 minute. The DNA was then eluted in 20 µl of elution buffer and stored in a -20°C freezer.

5.2.5.5 Gel electrophoresis

A 1% agarose (0.5 agarose dissolved in 50 ml TAE buffer) gel was performed to confirm that the right gene size gene had been amplified. The size of the bands was compared to the λ *Pst1* Marker, of which 20µl was loaded in the first well, and 5µl of each sample was mixed with 1µl of the Orange loading dye and then loaded in the gel. The gel was run for an hour at 100 volts and then stained using ethidium bromide for 15 minutes followed by destining for 30 minutes in running water.

5.2.5.6 Amplification of native/recombinant tropomyosin of Anisakis pegreffii by PCR

The gDNA was amplified using designed primers (section 5.2.5.1) and compared with cDNA to determine the length size of tropomyosin gene, and infer if any introns were present in the gene.

5.2.6 First cloning stage.

5.2.6.1 Preparing electro-competent DH5α E. Coli

Firstly, DH5α *E. coli* was grown overnight in 5ml LB broth, then on the following day the whole volume was inoculated into 200ml LB broth and grown at 37°C on the shaker (220 rpm) until an OD 600 nm 0.35 to 0.4 (Early-Mid-Log-Phase). Secondly, the broth was chilled on ice for about 15-30 minutes and divided into 4 Falcon tubes (50ml each), which were then centrifuged at 3500g for 15 minutes at 4°C. Thirdly, the supernatant was removed and the pellets were resuspended in 50ml ice-cold sterile Milli-Q water with slow flat shaker until the pellets disappeared, then centrifuged at 3500g for 15 minutes at 4°C. Fourthly, the pellets were resuspended in 25ml ice-cold sterile Milli-Q water and centrifuged again using the same conditions. Fifthly, the pellets were resuspended in 25ml of 10% ice cold glycerol, and the tubes were combined and centrifuged at 3500g for 15 minutes at 4°C. Finally, the cells were resuspended into a double pellet volume (around 300 µl) of 10% ice-cold sterile glycerol, and cell aliquots of 50µl in each eppendorf tube were stored at 80°C.

5.2.6.2 Assessing the efficiency of the electro-competent cells

To ensure the efficiency of the prepared electro-competent cells, 0.5µl of PUC18 plasmid (0.1ng/ µl), which has shown to be effectively transformed into electro-competent cells, was mixed with 50µl of prepared electro-competent cells and then transferred to a cuvette. The transformation was done by placing the cuvette in a chamber attached to the BIORAD electroporation system set at 2.5 volt, 25µF, 200 ohms and then giving the cells an electrical shock. Following the addition of 1ml of LB broth to the cells, they were transferred to a micro-centrifuge tube and incubated at 37°C on a shaker at 225 rpm for an hour. The culture (100 µl) was plated on LB agar containing 100µg/ml of ampicillin and incubated overnight at 37°C, and afterwards the colony forming units (CFU) were counted

and used in the transformation efficiency calculation. The calculation was done as follows;

1) Total DNA transformed (ng) = plasmid concentration × the added volume
= 0.1ng/ µl × 0.5 µl= 0.05ng.

2) Transformation efficiency (CFU/µg) $= \frac{\text{Number of CFU}}{\text{Total DNA transformed (ng)}} \times 10^3 \times \frac{\text{Total volume transformed}}{\text{The plated volume}}$

$= \frac{540\ \text{CFU}}{0.05\text{ng}} \times 10^3 \times \frac{(250+50)\ \mu\text{l}}{100\mu\text{l}}$

$= 3.24 \times 10^7\ \text{CFU}/\mu\text{g}$

Hence, it can be concluded that prepared DH5α *E. coli* cells are efficiently electro-competent.

5.2.6.3 Cloning the Ani p 3 gene into the pCR2.1 vector

In order to ligate the Ani p 3 gene into the pCR2.1 vector, the PCR reaction to amplify the required gene was repeated to obtain a fresh product using the optimal annealing temperature (55°C). Then, the ligation constituents shown in Table 1 were mixed and incubated overnight at 14°C. Afterwards, 2µl of the ligation product was transformed into electro-competent DH5α *E. coli* using the method applied for the positive control plasmid (Section 5.2.6.2), and then the transformed cells were plated on LB agar containing 100µg/ml of ampicillin, 40 µg/ml X-gal, and 0.1Mm IPTG for blue/white colony selection.

Table 5.3 Cloning conditions of the Ani p 3 gene into the pCR2.1 vector

Components	Volume
Fresh PCR product	2µl
10x Ligation buffer	1µl
pCR2.1 vector	2µl
MG water	4µl
T4 DNA Ligase	1µl
Total volume	10µl

5.2.6.4 Restriction analysis

To confirm the success of ligation of the PCR product into pCR2.1 vector, each purified plasmid was digested with both *Bam*HI and *Xho*I restriction enzymes. The components shown in Table 5.4 were mixed in a microfuge and then incubated in a 37°C water bath for 4 hours. Afterwards the restriction products were viewed by running a 1% gel electrophoresis, which was previously described (5.2.5.5).

Table 5.4 Restriction analysis of the Ani p 3 gene into pCR2.1 vector

Components	Volume
MG water	10µl
10x Buffer H	1µl
*Bam*HI enzyme	2µl
*Xho*I enzyme	2µl
Plasmid DNA	5µl
Total volume	10µl

5.2.7 Second cloning step

5.2.7.1 Isolation of pRSET-A vector

pRSET-A vector was purified from DH5α *E. coli* using the Plasmid Mini Kit (BIOLINE), by applying the isolation of high copy plasmid DNA protocol described in the kit. Firstly, the bacteria stored at -80°C was inoculated into 5ml LB broth containing 100µg/ml of ampicillin and incubated overnight at 37°C with vigorous shaking. On the following day, the culture was centrifuged at maximum speed for one minute. Secondly, the pellets were resuspended in 250µl of Resuspension Buffer by thorough vortexing, followed by adding 250µl Lysis Buffer P and mixing by inverting the tube carefully 4 to 6 times. Thirdly, 350µl of Neutralization Buffer was added to the sample and mixed by inverting the tube 4 to 6 times and then the sample was centrifuged for 10 minutes at maximum speed. Fourthly, the sample was transferred to Spin Column P placed in a 2ml collection tube and then centrifuged at 12,000 g for 1 minute. After discarding the filtrate, 500 µl of Wash Buffer AP was added to the column and centrifuged at 12,000 g for 1 minute. Fifthly, after discarding the filtrate, 700 µl of Wash Buffer BP was added to the column and centrifuged at 12,000 g for 1 minute. After that, the filtrate was discarded and the column was centrifuged again at maximum speed for 1 minute to remove all trace of ethanol. Finally, the column was placed

into 1.5ml Elution Tube and then 30µl of MG water was directly added to the membrane. Following the incubation of the column for 1 minute at room temperature, it was centrifuged at 12,000 g for 1.5 minutes to elute the plasmid DNA. The elution step was repeated to increase the DNA yield and then it was stored at -20°C.

5.2.7.2 Restriction analysis

In order to clone the Ani p 3 gene into pRSET-A vector, both the insert and the dedicated cloning site of the pRSET-A vector should be digested with both *Bam*HI and *Xho*I restriction enzymes before running on the gel. The components shown in Table 5.5 were mixed in a microfuge and then incubated in a 37°C water bath for 4 hours. Afterwards the restriction products are viewed by running a 1% gel electrophoresis for the next steps.

Table 5.5 Conditions for double digestion of Ani p 3 gene and pRSET-A vector

Components	Volume
MG water	10µl
10x Buffer H	1µl
*Bam*HI enzyme	2µl
*Xho*I enzyme	2µl
Plasmid DNA	5µl
Total volume	10µl

5.2.7.3 Gel extraction of DNA

In order to clone the Ani p 3 gene into pRSET-A vector, both the insert and the digested pRSET-A vector should be isolated from the gel. Consequently, in order to increase the DNA yield, restriction reactions were prepared for both the clone pCR2.1 and the pRSET-A in 50 µl total volume separately. After that, 1.5% agarose gel (1.5 g of agarose dissolved into 100ml TAE buffer) electrophoresis was run for all the reactions. The DNA extraction was performed using the QIAquick gel extraction kit microcentrifuge protocol. First, the bands corresponding to the Ani p 3 insert and the pRSET-A backbone were excised from

the gel using a sharp scalpel, and then weighed in 2 ml colourless tubes. Second, 3 volumes of Buffer QG were added to one volume of the gel, and thus approximately 600µl of the Buffer was added to each tube containing around 200 mg of the gel. Third, the tubes were incubated in a heating block set at 50°C for 10 minutes to dissolve the gel, and then one volume of isopropanol was added to each tube and mixed. Fourth, each sample was applied to a QIAquick column placed in a 2 ml collection tube, and then all tubes centrifuged at a maximum speed for one minute. After discarding the flow through, 0.5 ml of Buffer QG was added to each column and centrifuged for one minute to remove traces of agarose. Fifth, 0.75 ml of Buffer PE was added to wash each column and was centrifuged for one minute. Subsequently, the flow through was discarded and the columns were centrifuged again at 13,000 g for one minute to remove residual ethanol. Last, each QIAquick column was placed in a clean 1.5ml microcentrifuge tube, and the DNA was eluted by adding 30 µl of MG water directly to the centre of the QIAquick membrane. The columns were incubated for one minute at room temperature and then centrifuged for one minute at maximum speed. In order to calculate the concentration of the purified DNA, 3µl of each sample was mixed with 1µl of Orange loading dye and run on a 1% agarose gel electrophoresis. The rest of the samples were stored at -20°C.

5.2.7.4 Ligation of Ani p 3 gene into pRSET-A vector

The ligation reaction was performed using 9ng of pRSET-A vector and 3.5ng of Ani p 3 insert by applying 1:1 and 1:3 ratios of insert to vector, and the calculations were done using the formula below. The ligation reactions were prepared according to the components listed in Table 5.6, and then the tubes were incubated overnight at 14°C.

$$\text{Ng of gene} = \frac{\text{Gene size X ng of vector X molar ration}}{\text{Size of the vector}}$$

Subsequently, 2µl of the ligation product was transformed into electro-competent DH5α *E. coli* using the method previously described (Section 5.2.6.3), and then the transformed cells were plated on an LB agar plate containing 100µg/ml of ampicillin and grown overnight at 37°C.

Table 5.6 pRSET-A vector and Ani p 3 gene Ligation Conditions.

Components	Volume
Gel extracted pRSET-A backbone	1µl
Gel extracted *Ani p 3* insert	3µl
20 mM ATP	1µl
T4 DNA Ligase	1µl
10x T4 DNA Ligase Reaction Buffer containing 10mM ATP	2µl
MG water	11µl
Total volume	20µl

5.2.7.5 Sequencing Ani p 3 gene

One successfully cloned the Ani p 3 gene in the pRSET-A vector was purified from DH5α *E. coli* cells using the Plasmid Mini Kit (BIOLINE) protocol described before (Section 5.2.7.1). The purified plasmid was prepared to be sequenced at the Australian Genome Research Facility LTD (AGRF), which recommends sending 12 µl containing 600 to 1500ng double stranded plasmid and 0.8 pmol/1 µl primer. A 1% agarose gel electrophoresis was performed to determine the DNA concentration in the purified sample. The calculation below was done to determine how much of the 10µm T7 primers should be added to the sample. Hence, 1µl of the 10 µm T7 forward primer was added to 11 µl of the purified plasmid, and the same step was done with the T7 reverse primer. Then, the two 12 µl samples were sent to AGRF for sequencing.

$$C1 \times V1 = C2 \times V2$$

$$10\text{pmol}/\mu\text{l} \times V1 = 0.8\text{pmol}/\mu\text{l} \times 12\mu\text{l}$$

$$V1 = 1\mu\text{l}$$

5.2.8 Protein Expression and Purification

5.2.8.1 Preparation of electro-competent DH5α E. coli strain BL21 (DE3) pLysS

Electro-competent DH5α *E. coli* strain BL21 (DE3) pLysS cells were prepared in the same method described previously (Section 5.2.6.1) using LB broth containing 35 µg/ml chloramphenicol. To assess the efficiency of the prepared electro-competent cells, 0.5 µl of pUC18 plasmid (0.1ng/µl) was transformed using the method previously described (Section 5.2.6.2) and inoculated onto LB agar containing 35µg/ml chloramphenicol and 50µg/ml ampicillin. After overnight incubation at 37°C, the same calculation described was done as follows:

1) Total DNA transformed (ng) = plasmid concentration x the added volume

= 0.1ng/ µl x 1µl= 0.1ng.

2) Transformation efficiency (CFU/µg) = $\frac{\text{Number of CFU}}{\text{Total DNA transformed (ng)}} \times 10^3 \times \frac{\text{Total volume transformed}}{\text{The plated volume}}$

$= \frac{60 \text{ CFU}}{0.05\text{ng}} \times 10^3 \times \frac{(1000+52)\ \mu\text{l}}{100\mu\text{l}}$

$= 6.3 \times 10^6$ CFU/µg

Hence, it can be concluded that the prepared *E. coli* strain BL21 (DE3) pLysS are efficiently electro-competent.

5.2.8.2 Pilot expression

Firstly, the purified pRSET-A with the cloned Ani p 3 gene was transformed in 50µl electro-competent DH5α *E. coli* strain BL21 (DE3) pLysS cells, and then plated onto LB agar containing 35 µg/ml chloramphenicol and 50 µg/ml ampicillin. Secondly, after overnight incubation at 37°C, one colony was inoculated into 5 ml LB broth containing the required antibiotics and incubated with vigorous shaking at 37°C until an OD 600nm 0.6 to 1.0. Afterwards, 0.5 ml of the culture was stored in 40% glycerol at -80°C and 0.5ml was stored at 4°C to be used on the following day. Thirdly, the 0.5 ml culture was centrifuged for 30 seconds at maximum speed, and the pellet was resuspended in 250 µl fresh LB broth.

This amount was inoculated into 25 ml LB broth containing 35 µg/ml chloramphenicol and 50 µg/ml ampicillin and grown at 37°C until OD 600nm was between 0.4 to 0.6. Fourthly, 1 ml of the culture was collected (the zero hour concentration) and stored at 4°C, and then 240 µl of 100 mM IPTG stock was added to the rest of the culture to get a final concentration of 1mM IPTG. Fifthly, the culture was incubated for 5 hours at 37°C with vigorous shaking, and 1ml aliquots were collected every hour and stored 4°C. Sixthly, the whole cell lysate process was performed on the 6 collected aliquots by centrifugation for one minute at maximum speed and then performing two washing steps. The pellets were washed by being resuspended in 1ml 10mM Tris-HCL (pH8.0) and centrifuged for 2 minutes at maximum speed. Finally, the cells were lysed resuspending the pellets in 500µl whole cell lysis buffer and boiling them in a heating block (100°C) for 5 minutes. The samples were centrifuged at maximum speed for 5 minutes and the supernatants were collected and stored at -20°C for later analysis by SDS-PAGE.

5.2.8.3 First protein expression approach (freezer and 37°C water bath)

The BL21 (DE3) pLysS cells containing pRSET-A with the cloned Ani p 3 gene was plated onto agar containing 35µg/ml chloramphenicol and 50 µg/ml ampicillin. After overnight incubation at 37°C, one colony was inoculated into 5 ml LB broth containing the required antibiotics and incubated with vigorous shaking at 37°C until getting an OD 600 nm 0.6 to 1.0 and then stored at 4°C. On the following day, the 2 ml culture was centrifuged for 30 seconds at maximum speed, and the pellet was resuspended in 1ml fresh LB broth and added to 200 ml LB broth containing required antibiotics. The culture was grown at 37°C with vigorous shaking until the OD 600 nm 0.3 to 0.6 (pre-induction), then 2 ml of 100 mM ITPG stock was added to culture to get a final concentration of 1 mM IPTG. To induce protein production, the culture was incubated for 5 hours at 37°C with vigorous shaking, which is the time determined to be optimal for protein production during the pilot expression. Afterwards, the culture was harvested by centrifugation at 4700g for 15 minutes at 4°C, and the pellet was resuspended in 8 ml lysis buffer containing 1mg/ml lysozyme and 20 mM imidazole. Cells then underwent 4 freeze/thaw cycles between the -80°C freezer and 37°C water bath, and 30 units of DNAse was added to the cells after the second cycle. Following the fourth cycle, the sample was centrifuged at 4700g for 15 minutes at 4°C and the supernatant was filter sterilized using an 0.2µM filter.

5.2.8.4 Second protein expression approach (Sonication)

The second approach was performed to maximise lysis which could have contributed to the failure of purifying the Ani p 3 protein by IMAC. The same processes performed with the first approach were undertaken until pelleting the 200 ml culture that was washed twice with 50 ml and then 25 ml of 10 mM Tris-HCL (pH7.4). After, the pellet was resuspended into 10 ml lysis buffer containing 2mg/ml lysozyme and 1mM imidazole, and then sonicated using the Branson Digital Sonifier at 35% (15 seconds on and 30 seconds off for 3 cycles) and of which 200 µl was collected representing the total fraction. Next, the sample was centrifuged at 4700g for 15 minutes at 4°C to remove cell debris. The supernatant was transferred to a new tube as the soluble fraction and filtered using a 0.2 µM filter and stored at -20°C to be used for purification while the debris pellet was discarded.

5.2.8.5 Preparation of immobilized metal affinity chromatography (IMAC) column

A 1ml polypropylene column (Qiagen) was prepared for IMAC purification of Ani p 3 protein by washing with 10ml Milli-Q water, with both taps being open, until getting a steady speed of water flow and removing air bubbles. Next, chelating sepharose TM fast flow through (resin) was added and left to settle to the pre-marked 1ml column level. After, the column was washed with 10ml MilliQ water (10 column volumes (CV)) to remove the ethanol in which the resin is stored. Next, the column was charged with 0.5ml (0.5 CV) of 0.2M $NiSO_4$ and washed with 10ml (10 CV) of MilliQ water to remove unbound metal ions. The flow through was collected in a disposable tube and discarded. Finally, the column was equilibrated with 5 ml (5 CV) of the equilibration wash buffer.

5.2.8.6 Optimization of IMAC protein purification

The 5ml of sample prepared in the previous step was added to the column and mixed with the resin by slowly flipping the column up and down. Consequently, the column was incubated for one hour at room temperature with horizontal shaking, and then it was returned to the vertical position and left to settle for 15 minutes. Subsequently, a 2 ml crude eluent was collected and the column was washed with 10 ml (10 CV) post sample wash buffer, and then the flow through eluent containing unbound protein was collected. Successively, 1ml of elution buffer containing increasing concentrations (1 mM, 50 mM, 100 mM, 150 mM, 200 mM and 300 mM) of imidazole was added to the column and then eluates were collected in labelled tubes. Next, the collected samples underwent SDS-PAGE and were then stained with Coomassie blue. Finally, the presence of the protein of interest was confirmed by performing western blot analysis.

5.2.9 Protein electrophoresis and analysis

5.2.9.1 Tris-glycine sodium dodecyl sulphate- polyAcrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is a common technique used to separate proteins based on their molecular weight. The negatively charged detergent, sodium dodecyl sulphate (SDS), with a reducing agent such as (mercaptoethanol), binds to the proteins, denatures and breaks down the disulfide bonds of the protein.

The negatively charged SDS-protein complex is then applied into an acrylamide gel with different concentrations and pH, the low acrylamide percentage-stacking gel-with lower pH (6.8) and the high pH (8.8). When this gel is placed in an electric field, the proteins move towards the anode with smaller proteins moving faster than the larger ones (Smith, 2003).
The Laemmli protocol was used to prepare all the stock solutions of stacking gel, resolving gel, running and loading buffer. According to the manufacturer's instructions (BIORAD), two glass plates were assembled using 0.75mm spacer glass and checked for the absence of leakage with distilled water. The size of protein is predicted between 10-60 kDa, and thus a resolving gel with 12% polyacrylamide was prepared (Table 5.7) just prior the experiment.

Table 5.7 As per one resolving gel (12% polyacrylamide) the recipe is:

Components	Volume
30% Acrylamide stock	3.13ml
1.5M Tris-HCL pH 8.8	1.88ml
10%SDS	0.08ml
MG water	2.38ml
10% w/v APS	37.5µl
TEMED	2.5µl

The gel is then poured gently between the glasses leaving a 1.5 cm space for the stacking gel. To obtain a sharp surface and prevent the diffusion of oxygen, bubble formation and

drying of the gel, a solution of saturated butanol was added. After one hour the resolving gel was polymerized, and liquid on the surface was poured off and washed several times with running buffer, and dried with blotting paper. Then, around 1ml of 4% stacking gel was prepared (Table 5.8), and layered over the top, and immediately a plastic Teflon comb was inserted into the stacking gel to form 10 or 15 wells between the plates, and allowed to polymerize for 40 minutes.

Table 5.8 As per one stacking gel (4% polyacrylamide) the recipe is:

Components	Volume
30% Acrylamide stock	0.33ml
0.5M Tris-HCL pH 8.8	0.63ml
10%SDS	0.03ml
MG water	1.50ml
10% w/v APS	12.5µl
TEMED	1.25µl

The gel was then removed from the casting stand and clamped to the electrode assembly placed in the tank of Mini-PROTEIN Terta Cell electrophoresis system (Bio-Rad, USA) and filled with 1x electrophoresis running buffer.

5.2.9.2 Sample preparation for SDS-PAGE.

Depending on the result of the Bradford assay, different protein amounts of each extract were prepared by diluting them in PBS to obtain a final volume of 20 µl, then 5 µl of 5x sample buffer was added and heated in a water bath or heater at 100°C for 5 minutes. After, a volume of 25 µl of the sample was loaded into the wells using special loading tips. 5 µl of protein molecular marker, Precision Plus Protein Dual Color Standards (Bio-Rad, USA) was loaded in the first well of the gel.

5.2.9.3 SDS-PAGE: Running conditions and Staining

The electrophoresis apparatus was connected to the power supply and the voltage set in two stages, the first stage at 80 volts for 10 minutes until the protein reached the top of the resolving gel and the second stage at 160 volts for 60 minutes until the dye front reached the bottom of the gel glasses.

5.2.9.4 AcquaStain Blue dye

When the electrophoresis was complete, the stacking gel region was cut away by a scalpel blade and the gel was removed from the glass plates. The gel was stained with around 50ml AcquaStain Blue dye for 2 hours on a rotating shaker at room temperature. Afterwards, the stain was removed and the gel was washed with distilled water. To visualize the protein bands, the gel was placed on a transparency paper and scanned in Gel Doc EQ system (Bio-Rad, USA).

5.2.9.5 Western blotting

The SDS-PAGE electrophoresis was performed again for the protein samples using the Kaleidoscope pre-stained marker. After that, the proteins were transferred from SDS-PAGE gel to a nitrocellulose membrane using the iBlot TM Dry Blotting System (Invitrogen). First, the anode stack was placed on the tray, and the SDS-PAGE gel was overlaid on the top. Second, a pre-soaked iBlot TM filter paper in deionized water was placed on the top, and air bubbles were removed using the blotting roller. Third, the cathode was placed with the electrode side facing up, and the disposable sponge was positioned on the top with the metal contact on the upper side. Finally, the lid was closed on the assembled stacks and the blotting process was started for 7 minutes. After that, the nitrocellulose membrane was ready for further western immunoblotting analysis.

5.2.10 Immunoblotting analysis

5.2.10.1 Immunoblotting with anti- Histidine polyclonal antibody

After removing the nitrocellulose membrane from the device, it was placed in a plastic container bag and blocked with 50 ml in 5% (w/v) skim milk in TBS for 1 hour on the shaker at room temperature, followed by washing three times with TBS for 5 minutes each time. Then, the membrane was placed in plastic bag and incubated at 4°C for overnight or 2 hours at room temperature with 10 ml of the rabbit anti- Histidine polyclonal antibody diluted 1:10,000 with 1% (w/v) skim milk in TBS 0.5 Tween20 (primary antibody). Subsequently, the membrane was placed in a plastic container bag and washed three times

with 20 ml TBS-T for 5 minutes each time, then incubated for 2 hours at room temperature with 10 ml of the rabbit anti mouse antibody conjugated to biotin (Abcam, USA) and diluted 1:10,000 with 1% (w/v) skim milk in TBS 0.5 Tween (secondary antibody). The membrane was washed 3 times with TBS containing 0.05% Tween20, and then immersed in the detection buffer for 10 minutes. The colour was developed by adding equal proportions of the detection buffer (3 ml) and BCIP/NBT solution (3 ml) and rinsing it with distilled water after a few minutes.

5.2.10.2 Immunoblotting with anti-tropomyosin monoclonal antibody

After removing the nitrocellulose membrane from the device, it was placed in a plastic container bag and blocked with 50 ml of 5% (w/v) skim milk in TBS for 1 hour on the shaker at room temperature, followed by washing three times with TBS for 5 minutes each time. Then, the membrane was placed in a plastic bag and incubated at 4°C for overnight or 2 hours at room temperature with 10 ml of the rat monoclonal anti-tropomyosin antibody diluted 1:6000 with 1% (w/v) skim milk in TBS 0.5 Tween20 (primary antibody). Subsequently, the membrane was placed in plastic container bag and washed three times with 20 ml TBS-T for 5 minutes each time, then incubated for 2 hours at room temperature with 10ml of the rabbit anti mouse antibody conjugated to biotin (Abcam, USA) and diluted 1:8000 with 1% (w/v) skim milk in TBS 0.5 Tween (secondary antibody). The membrane was washed 3 times with TBS containing 0.05% Tween20, and then immersed in the detection buffer for 10 minutes. The colour was developed by adding equal proportions of the detection buffer (3 ml) and BCIP/NBT solution (3 ml) and rinsing it with distilled water after a few minutes.

5.2.10.3 Immunoblotting with anti-tropomyosin polyclonal antibody

After removing the nitrocellulose membrane from the device, it was placed in a plastic container bag and blocked with 50 ml of 5% (w/v) skim milk in TBS for 1 hour on the shaker at room temperature, followed by washing three times with TBS for 5 minutes each time. Then, the membrane was placed in a plastic bag and incubated at 4°C for overnight or 2 hours at room temperature with 10 ml of the rabbit polyclonal anti-tropomyosin antibody produced in mouse diluted 1:40,000 with 1% (w/v) skim milk in TBS 0.5 Tween20 (primary antibody). Subsequently, the membrane was placed in a plastic container bag and washed three times with 20 ml TBS-T for 5 minutes each time, then incubated for 2 hours at room temperature with 10 ml of the goat anti rabbit IgG, AP conjugate polyclonal antibody, (DAKO, USA) and diluted 1:40,000 with 1% (w/v) skim milk in TBS 0.5 Tween

(secondary antibody). The membrane was washed 3 times with TBS containing 0.05% Tween20, and then immersed in the detection buffer for 10 minutes. The colour was developed by adding equal proportions of the detection buffer (3 ml) and BCIP/NBT solution (3 ml) and rinsing it with distilled water after a few minutes.

5.2.10.4 Immunoblotting with anti-crustacean polyclonal antibody

After removing the nitrocellulose membrane from the device, it was placed in a plastic container bag and blocked with 50 ml of 5% (w/v) skim milk in TBS for 1 hour on the shaker at room temperature, followed by washing three times with TBS for 5 minutes each time. Then, the membrane was placed in a plastic bag and incubated at 4°C for overnight or 2 hours at room temperature with 10 ml of the rabbit polyclonal anti-crustacean antibody produced in mouse diluted 1:40,000 with 1% (w/v) skim milk in TBS 0.5 Tween20 (primary antibody). Subsequently, the membrane was placed in a plastic container bag and washed three times with 20 ml TBS-T for 5 minutes each time, then incubated for 2 hours at room temperature with 10 ml of the Goat anti rabbit IgG, AP conjugate polyclonal antibody, (DAKO, USA) and diluted 1:40,000 with 1% (w/v) skim milk in TBS 0.5 Tween (secondary antibody). The membrane was washed 3 times with TBS containing 0.05% Tween20, and then immersed in the detection buffer for 10 minutes. The colour was developed by adding equal proportions of the detection buffer (3 ml) and BCIP/NBT solution (3 ml) and rinsing it with distilled water after a few minutes.

5.2.11 Immunoblotting with human serum

The human serum obtained from Alfred Hospital, Melbourne, Australia from many patients with shellfish allergy was stored at -20°C at Monash Medical Centre. For immunoblotting 5 µl and 10 µl of each protein sample was loaded into SDS gel and electrotransfered to PVDF membrane.

The blocking solution of 1% skim milk was prepared in phosphate buffered saline containing 0.5% Tween, pH= 7.2 (PBS-T). After blocking for 1 hour, this membrane was incubated overnight at 4°C with human serum diluted 1:20 in 1% skim milk PBS-T. Then, it was washed three times with 20-25 ml PBS-T for 15 minutes (5 minutes each). This serum contains IgE, which is considered the primary antibody in this experiment.

After, the membrane was placed in a plastic bag and incubated at 4°C for overnight or 2 hours at room temperature with 10ml of the rabbit anti-human IgE antibody (DAKO, USA) diluted 1:8,000 with 1% (w/v) skim milk in TBS 0.5 Tween 20 (secondary antibody). Subsequently, the membrane was placed in a plastic container bag and washed three times with 20ml TBS-T for 5 minutes each time, then incubated for 2 hours at room temperature with 10ml of the Goat anti rabbit IgG, HRP conjugate polyclonal antibody, (DAKO, USA) and diluted 1:8,000 with 1% (w/v) skim milk in TBS 0.5 Tween (tertiary antibody). The membrane was washed 3 times with TBS containing 0.05% Tween20, and then the immunoblot was developed using the enhanced chemiluminescent (ECL) method (*3.2.4.4.1*).

5.2.12 Phylogenetic analyses

It was performed as mentioned in chapter three (Section 3.2.2.6.2). The tropomyosin protein of *Anisakis pegreffii* was compared with twelve tropomyosin proteins of some parasitic nematodes and living organism as follows; *Anisakis simplex* tropomyosin (JF902160), *O, volvulus* tropomyosin (L41633.1), *C. elegans* tropomyosin (D38540.1), *A. lumbricoides* tropomyosin (FJ655903), *D. melanogaster* tropomyosin (NM_169633), *P. monodon tropomyosin* (AY827100), *H. americanus* tropomyosin (AF034954.1), *H. diversicolor* tropomyosin (AF216518.1), *P. camtschaticus* tropomyosin (AB270632.1), *P. americana* tropomyosin (Y14854.1), Human tropomyosin (M19267.1) and Chicken tropomyosin (J00910.1). The aim of this comparison is to examine and analyse polymorphisms and the sequence of the tropomyosins of parasitic species and other living organisms and their phylogenetic realtionships.

For the analysis, NCBI was used to obtain the tropomyosin genes of *Anisakis pegreffii* and amino acid sequences of the various species. Expasy was used to translate nucleotide sequence to amino acids (Artimo et al. 2012). The Clone manager software suite was used for alignment of the sequences. Molecular evolutionary genetics analysis (MEGA) software was used for the generation of phylogenetic trees by the neighbour-joining dendrogram (Felsenstein 1989).

5.2.13 Immunofluorescence staining and microscopy

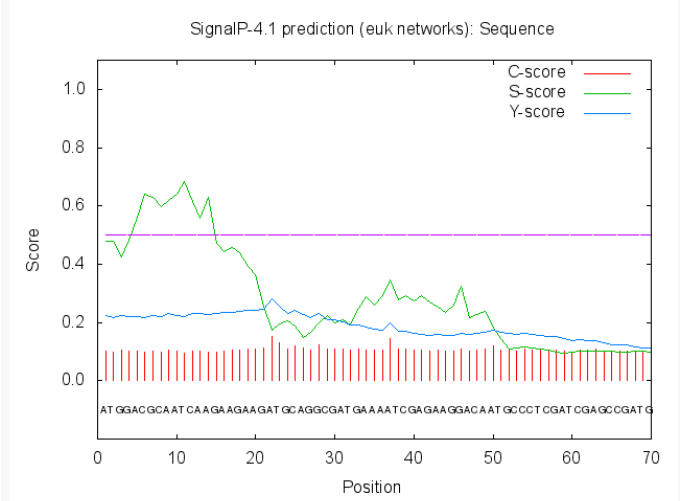
One larvae was put in a small plastic cassette and embedded in optimal cutting temperature (OCT) compound medium. After the samples became solid (indicated by white color) they were frozen for 3 minutes, then cut into many sections (7-10 um thick) by a microtome

cryostat machine. Every two sections were melted onto Super Frost slides and labeled with pencil. Slides with sections were put into blocks and left at 4°C overnight to allow the sections to dry. The sections were fixed by placing slides into cold acetone (100%) for 10 minutes and laid flat on paper towel to allow complete drying for at least 10 minutes. To dissolve the OCT medium, the slides were put in staining chamber containing PBS for 10 minutes. During that time the primary and secondary antibodies were prepared and diluted in PBS-T and placed on ice until required. For the labelling step, 200µl of rabbit polyclonal anti-tropomyosin antibody solution (primary antibody) was added directly to slides to cover whole the sections and incubated at 4°C overnight or 2 to 3 hours at room temperature. After, the slides were washed with PBS for 10 minutes. Subsequently, 200 µl of HRP mouse anti-rabbit IgG secondary antibodies conjugated to FITC was added directly to slides as before then incubated at room temperature for 30 minutes and washed with PBS for 10 minutes. For development, both the small and large sections were incubated with 20ul of DAB substrate solution for 10 minutes. Then the slides were washed with PBS for 10 minutes to remove the excess DAB solution. After, the slides were kept in a chamber with PBS overnight. Next day the slides were shaken, removed from PBS and dried. 5-10µl of 50% glycerol solution was pipetted onto the section and a cover slip placed over. Images were then taken with microscopy (Nikon) and analyzed.

5.3 Results

5.3.1 Signal peptide prediction for tropomyosin gene.

The complete amino acid sequence of the tropomyosin protein of *A. simplex* was submitted to the SignalP 4.1 server, revealing no predicted signal peptide. Therefore, it is not predicted to be a secreted protein (Figure 5.1).

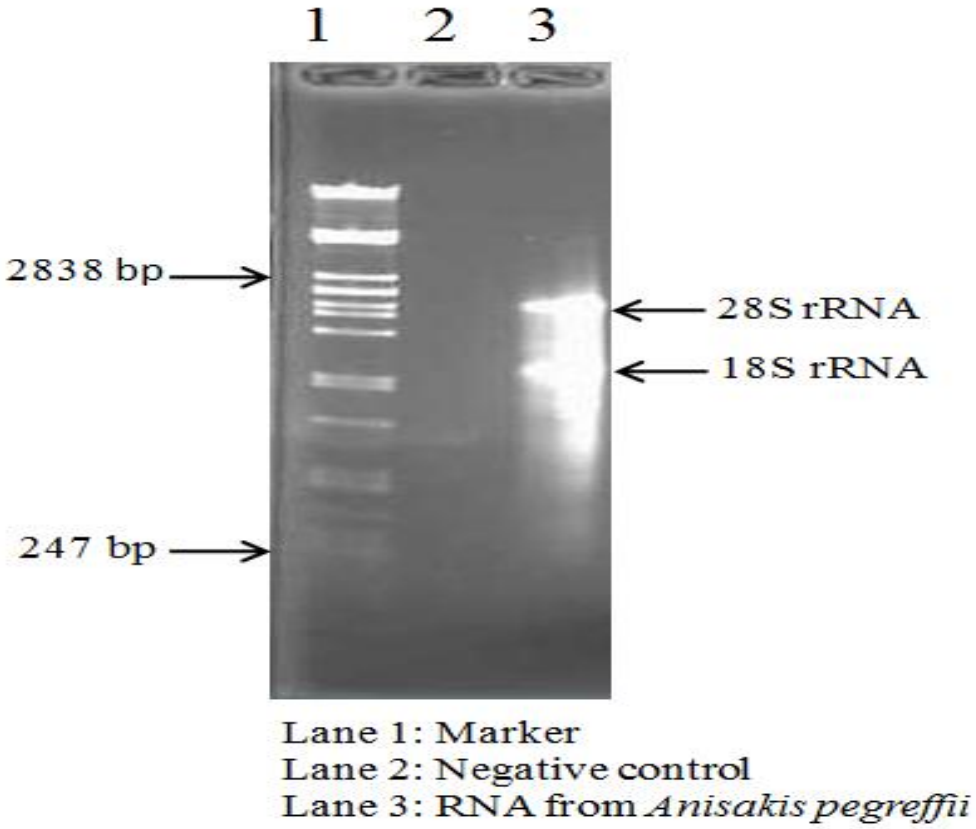



```
# Measure  Position  Value    Cutoff   signal peptide?
  max. C    22        0.153
  max. Y    22        0.281
  max. S    11        0.685
  mean S     1-21     0.517
       D     1-21     0.409   0.450   NO
Name=Sequence   SP='NO' D=0.409 D-cutoff=0.450 Networks=SignalP-noTM
```

Figure 5.1 Signal peptide prediction for the Tropomyosin protein of *A. simplex* using the SignalP 4.1 server.

5.3.2 Isolation of RNA from *A. pegraffii* using the kit.

Total RNA was isolated from whole tissue of *A. pegreffii* (35 larvae) and then eluted using 30 µl of RNase-free water. Using a 1% electrophoresis gel, 10 µl of the sample was loaded, and by ethidium bromide staining, there were indeed two major rRNA subunit bands (small 18S and the large 28S) detected in the total RNAs of *A. pegreffii* (Figure 5.2).

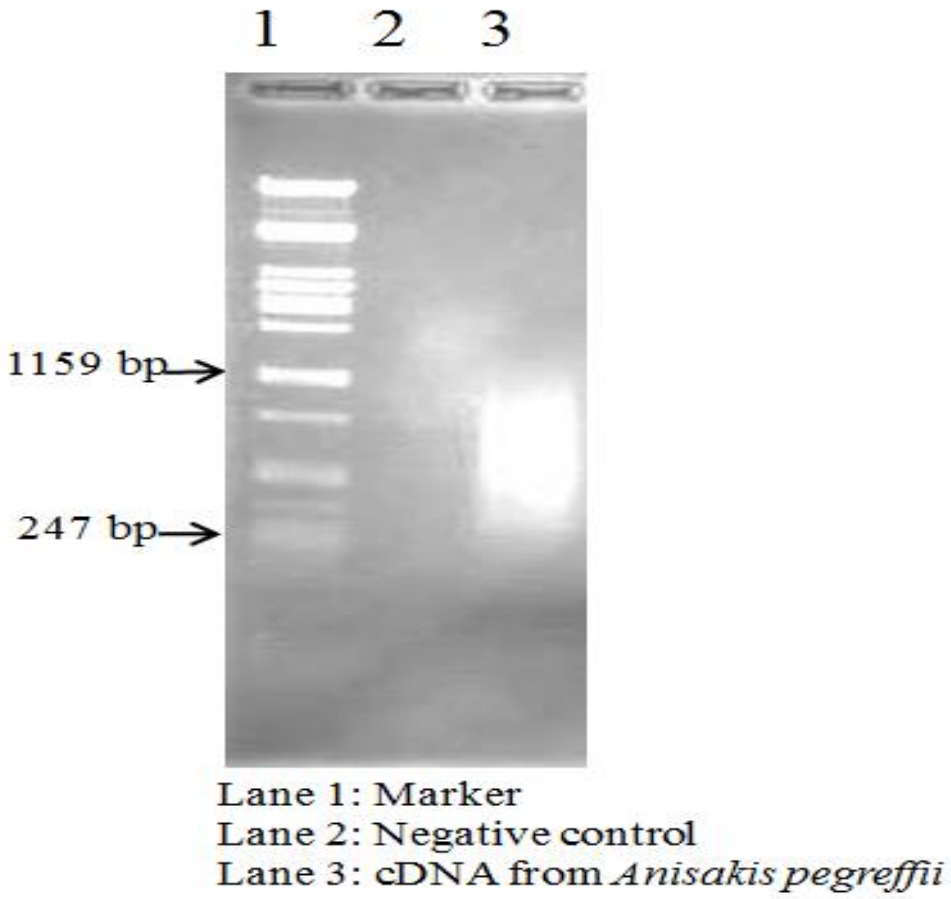


Keys:
L=Lane
L1=Marker, L2= Negative control and L3= RNA of *A. pegreffii*.

Figure 5.2 Isolation of RNA from *A. pegreffii* using ISOLATE RNA Mini Kit.

5.3.3 Double stranded cDNA synthesized from messenger RNA (mRNA) from *A. pegreffii.*

10 µl of PCR product was electrophoresed in a 1 % agarose gel and visualised by ethidium bromide staining (Figure 5.3). The cDNA sequences obtained from *A. pegreffii* were estimated to be within 247 and 1159 bp.

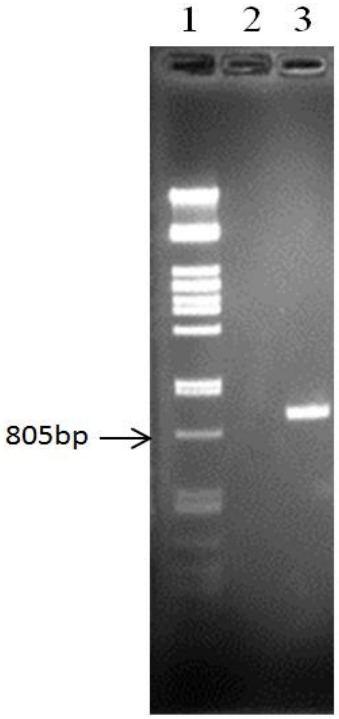


Keys:
L=Lane
L1=Marker, L2=Negative control and L3=cDNA of *A. pegreffii*.

Figure 5.3 Double stranded cDNA synthesized from messenger RNA (mRNA) using cDNA Synthesis Kit.

5.3.4 Amplification of tropomyosin gene of *A. pegreffii* DNA by specific design primer.

Here the designed primers were tested with DNA to amplify the tropomyosin gene to determine the length of the tropomyosin gene (Figure 5.4).



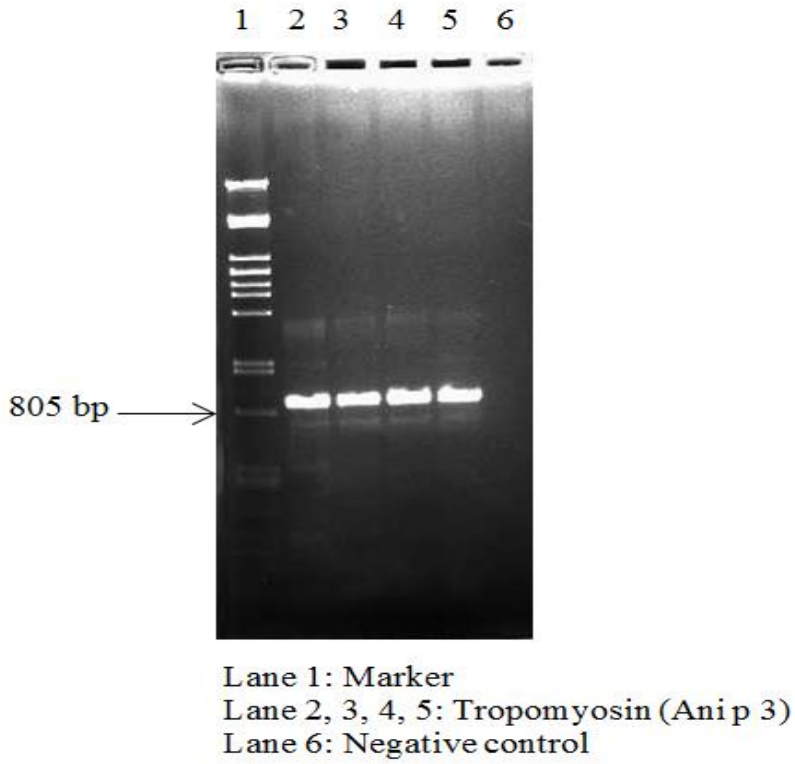
Lane 1: Marker
Lane 2: Negative control
Lanes 3: tropomyosin gene (Ani p 3)

Keys:
L=Lane
L1=Marker, L2=Negative control and L3=Tropomyosin gene (Ani p 3).

Figure 5.4 Amplification of tropomyosin gene of *A. pegreffii* by specific design primer.

5.3.5 Amplification of tropomyosin gene of *A. pegreffii* from cDNA by specific design primer.

The designed primers were tested with cDNA to amplify the tropomyosin gene (Figure 5.5). The observation that the amplified product was the same length from cDNA as from genomic DNA indicates that there are no introns present in the gene.



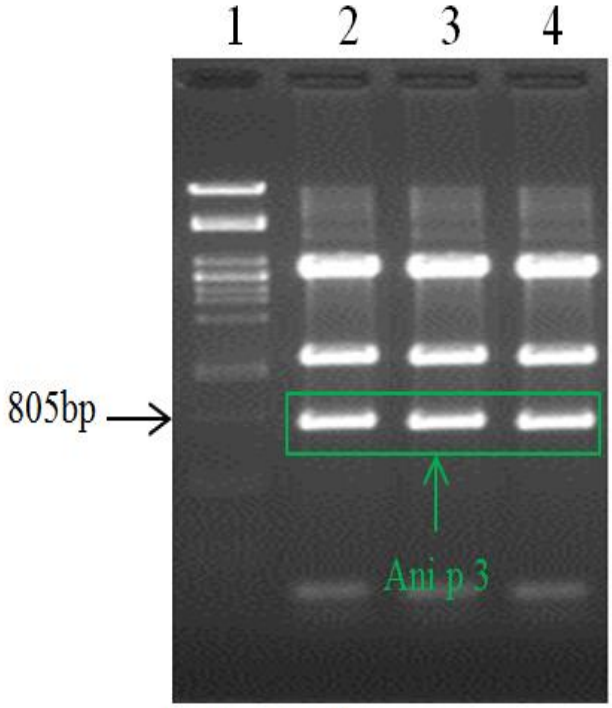
Keys:
L=Lane
L1=Marker, L2, 3, 4, 5=Tropomyosin gene (Ani p 3), and L6=Negative control.

Figure 5.5 Amplification of Ani p 3 genes by designed primers.

5.3.6 Digestion of PCR2.1 including Anip3 gene by both *Bam*HI and *Xho*I restriction enzyme respectively.

DH5α competent cells transformed with pCR2.1 vector ligated with Ani p 3 (tropomyosin) inserts were randomly selected and the vector pCR2.1 was isolated, then digested to confirm the presence and correct orientation of the Ani p 3 gene. Agarose gel electrophoresis (1%) revealed the positive recombinant PCR2.1/Ani p 3 clones.

Both the positive recombinant PCR2.1/Ani p 3 were double digested with the restriction enzymes *Bam*HI and *Xho*I which released the same three fragments bands at different sizes in each lane (Figure 5.6). Ani p 3 gene fragments from the pCR 2.1 vector were confirmed in lane 2, 3 and 4 at size 805 bp while others two fragments from each lane are from the PCR2.1 vector. Lane 1 represents the DNA marker ladder.

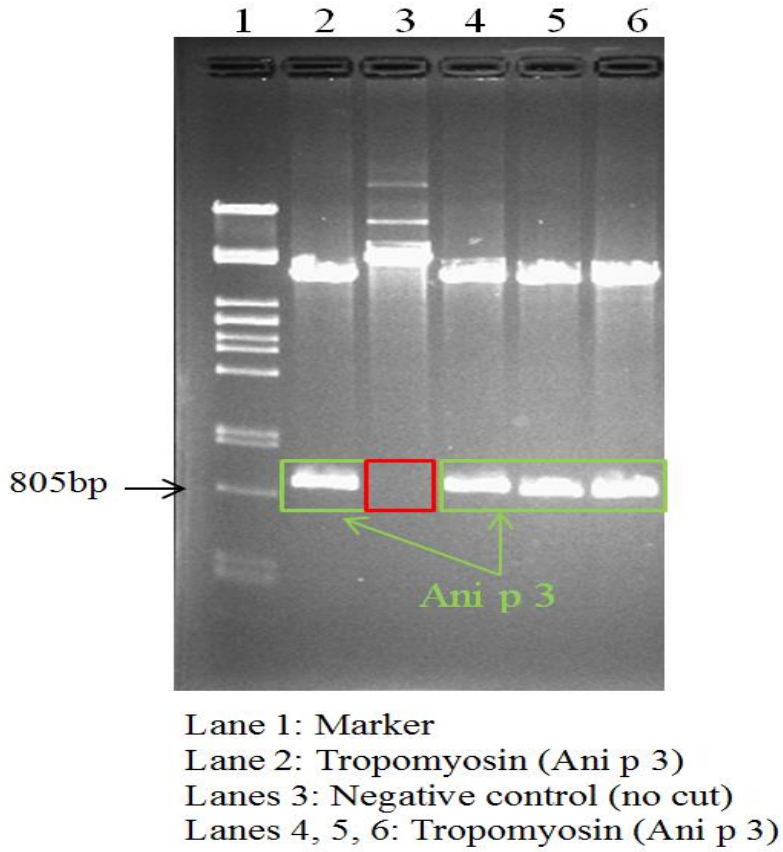


Keys:
L=Lane
L1=Marker, L2, 3, 4=Tropomyosin gene (Ani p 3) labelled inside green box.

Figure 5.6 Digestion of PCR2.1 including Ani p 3 gene by restriction enzyme.

5.3.7 Digestion of pRSET-A including Anip3 gene by both *Bam*HI and *Xho*I restriction enzyme, respectively.

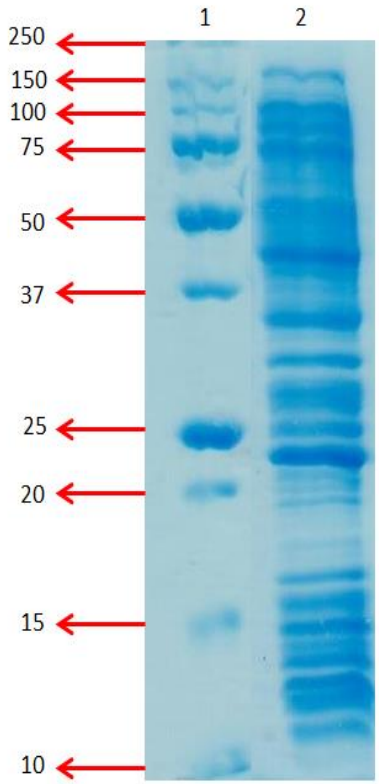
The presence of Ani p 3 excised from pCR2.1 vector and which was subcloned into pRSET- A plasmid was also confirmed in pRSET-A as described for pRC2.1. In the case of pRSET-A, two bands were obtained from this digestion. The presence of the Ani p 3 gene fragment from the pRSET-A vector was observed at size 805 bp while the other band was the rest of the pRSET-A vector (Figure 5.7).



Keys:
L=Lane
L1=Marker, Lane 2, 4, 5, 6=Tropomyosin gene (Ani p 3) labelled inside green box. L3=Undigested digestion of pRSET-A including Anip3 gene by restriction enzyme.

Figure 5.7 Digestion of pRSET-A including Anip3 gene by restriction enzyme.

5.3.8 Extraction of raw protein from *A. pegreffii* (0.1M Tris 0.5M Glycine buffer (pH=8.7).



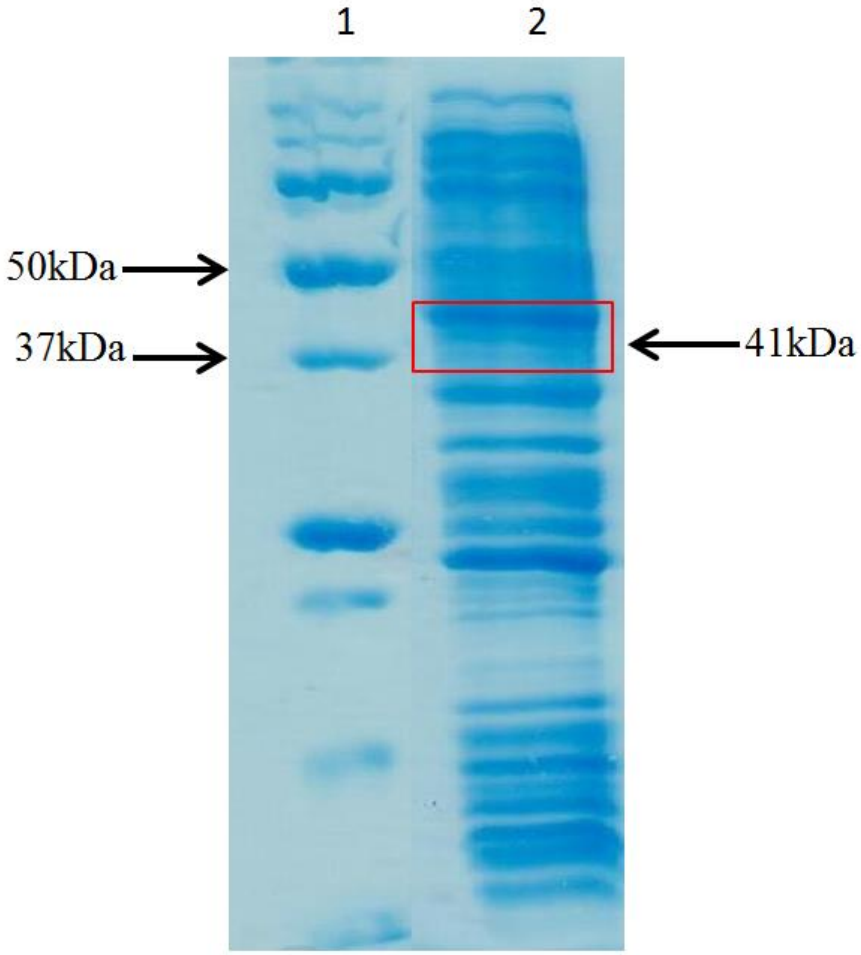
1-Marker
2- Whole protein from *Anisakis pegreffii*

Keys:
L=Lane

From left to right: 1 =protein ladder (marker), 2= whole protein extract.

Figure 5.8 Identification of the presence of protein from *A. pegreffii*, using SDS PAGE gel stained with AcquaStain Blue dye.

5.3.9 Identification of the presence of native tropomyosin protein from *A. pegreffii* using SDS PAGE gel.



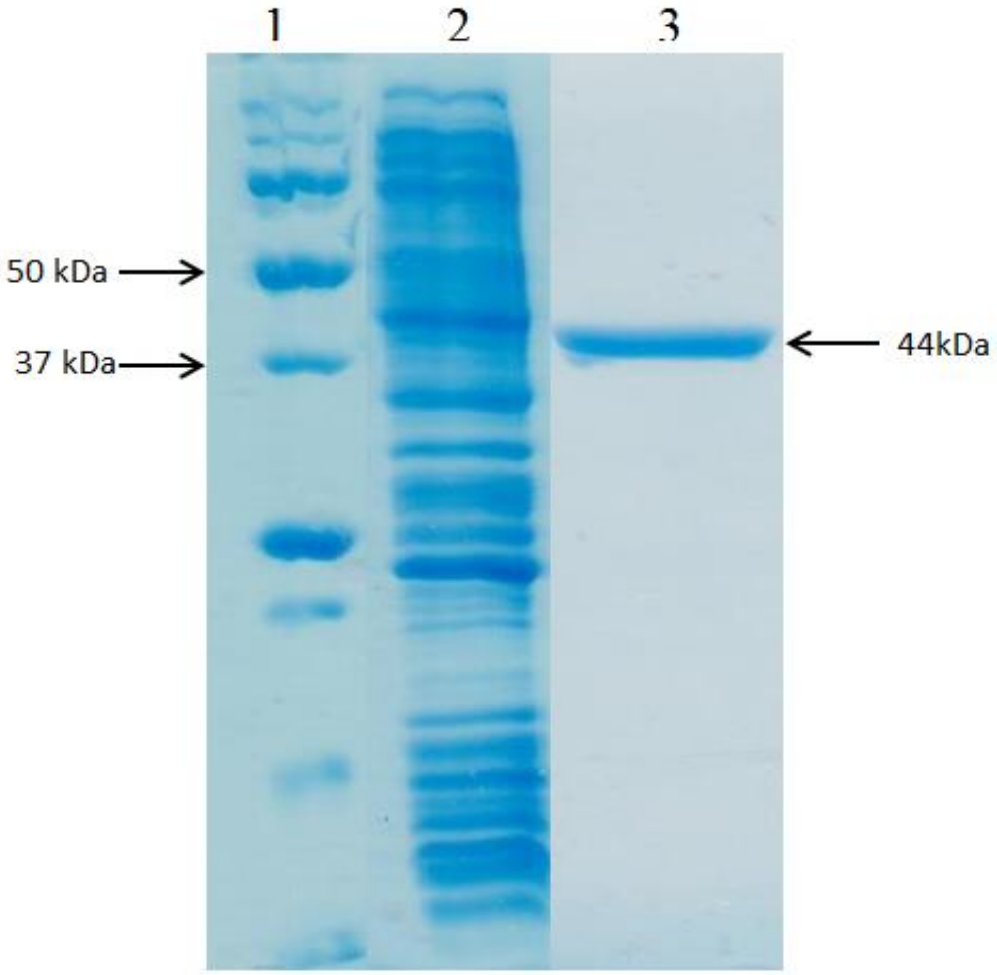
1-Marker
2- Whole protein from *Anisakis pegreffii*

Keys:
L=Lane
From left to right: Lane 1= protein ladder (marker), Lane 2= the expected size of native tropomyosin protein in raw extract labelled in red box.

Figure 5.9 Identification of the presence of tropomyosin protein from *A. pegreffii*, using SDS PAGE gel stained with Acqua Stain Blue dye.

5.3.10 Identification of the size of recombinant tropomyosin protein from *A. pegreffii* and comparison with tropomyosin of whole protein from *A. pegreffii*.

Confirmation of the presence of recombinant tropomyosin protein from *A. pegreffii* using SDS PAGE gel stained in Acqua Stain Blue dye in the first stage of the Western Blot (Figure 5.10).

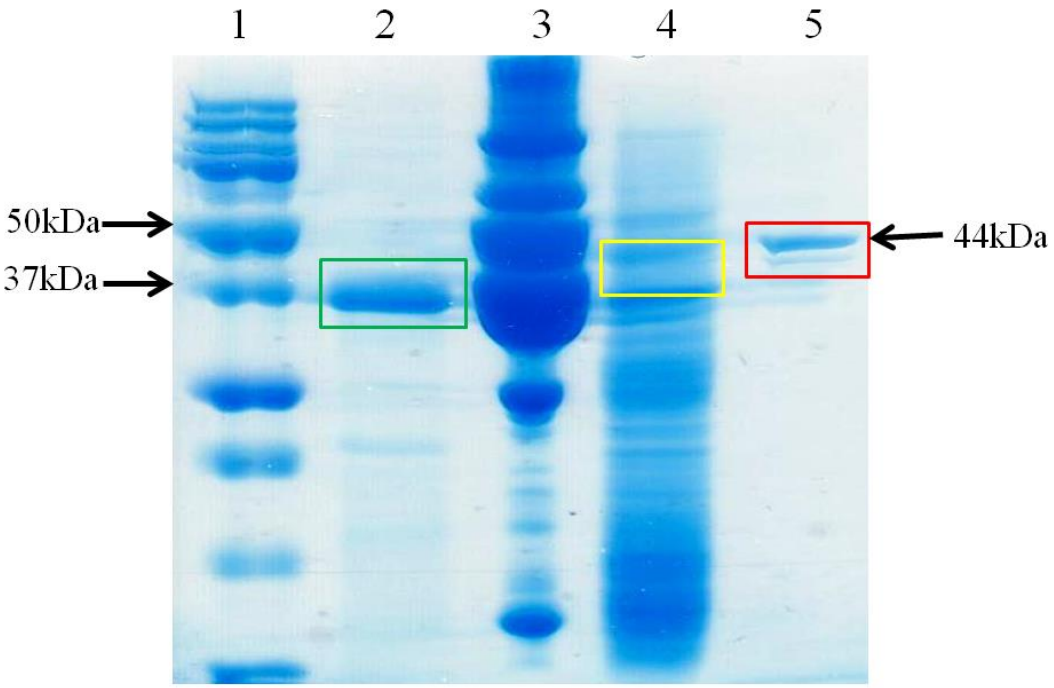


Keys:
L=Lane
From left to right: L1= protein ladder (marker), L 2= the expected size of native tropomyosin protein in raw extract, L3= the purified recombinant tropomyosin protein from *A. pegreffii* at 44kDa.

Figure 5.10 SDS-PAGE electrophoresis of the raw and recombinant tropomyosin protein from *A. pegreffii*.

5.3.11 Comparison of tropomyosin protein in Crustaceans and *A. pegreffii* using SDS PAGE gel.

The comparison of tropomyosin protein size was identified using SDS PAGE gel stained with Aqustaining dye.



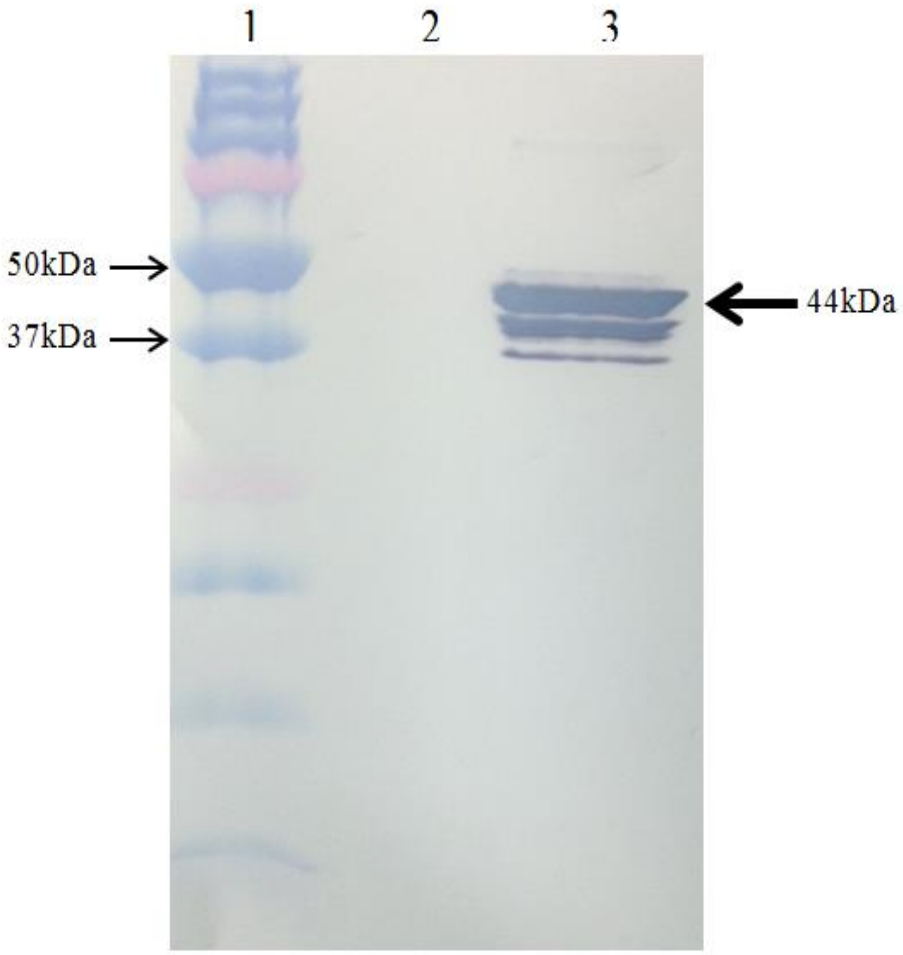
1-Marker
2- Positive control (recombinant tropomyosin Black tiger 37 KDa)
3- Negative control (Fish)
4- Crude *Anisakis pegreffii* protein (native tropomyosin 41KDa)
5- Recombinant tropomyosin protein (*Anisakis pegreffii* 44KDa)

Keys:
L=Lane
From left to right: (L1) a protein ladder of markers, (L2) purified recombinant tropomyosin protein from black tiger as positive control at 37 kDa, (L3) raw fish protein as negative control, (L4) the expected size of native tropomyosin protein in raw extract at 41kDa, (L5) the purified recombinant tropomyosin protein from *A. pegreffii* at 44kDa.

Figure 5.11 SDS-PAGE electrophoresis of the raw tropomyosin protein from *A. pegreffii*, recombinant tropomyosin protein from *A. pegreffii* and black tiger prawn (positive control) and fish (negative control).

5.3.12 Immunoblotting profile using anti-polyHistidine monoclonal antibody showing the recombinant tropomyosin.

Immunoblotting profile using polyHistidine polyclonal antibody showing the blots developed recombinant tropomyosin band (44kDa) within 5 min of exposure (Figure 5.12). This shows reactivity with several bands, with the strongest activity a band of 44 kDa. The other bands may be either prematurely terminated protein or degradation products.

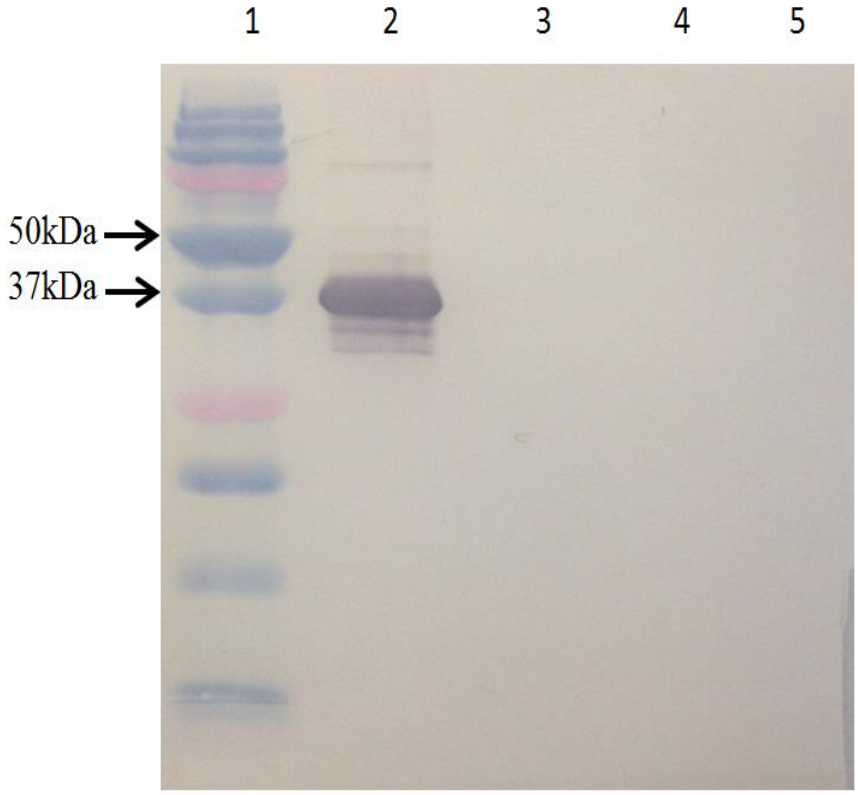


Keys:
L=Lane
From left to right: (L1) a protein ladder of markers, (L2) is showing negative control (PBS), (L3) is showing the developed tropomyosin band (44kDa) within 5 min of exposure.

Figure 5.12 Immunoblotting profile using anti-polyHistidine polyclonal antibody.

5.3.13 Immunoblotting with anti-tropomyosin monoclonal antibody.

Immunoblotting profile using mouse anti crustean tropomyosin monoclonal antibody against recombinant tropomyosin from black tiger, extract fish protein, extract *Anisakis* protein and recombinant tropomyosin protein samples (Figure 5.13). This shows that the anti-tropomyosin antibody does not react with the anisakis and fish proteins.



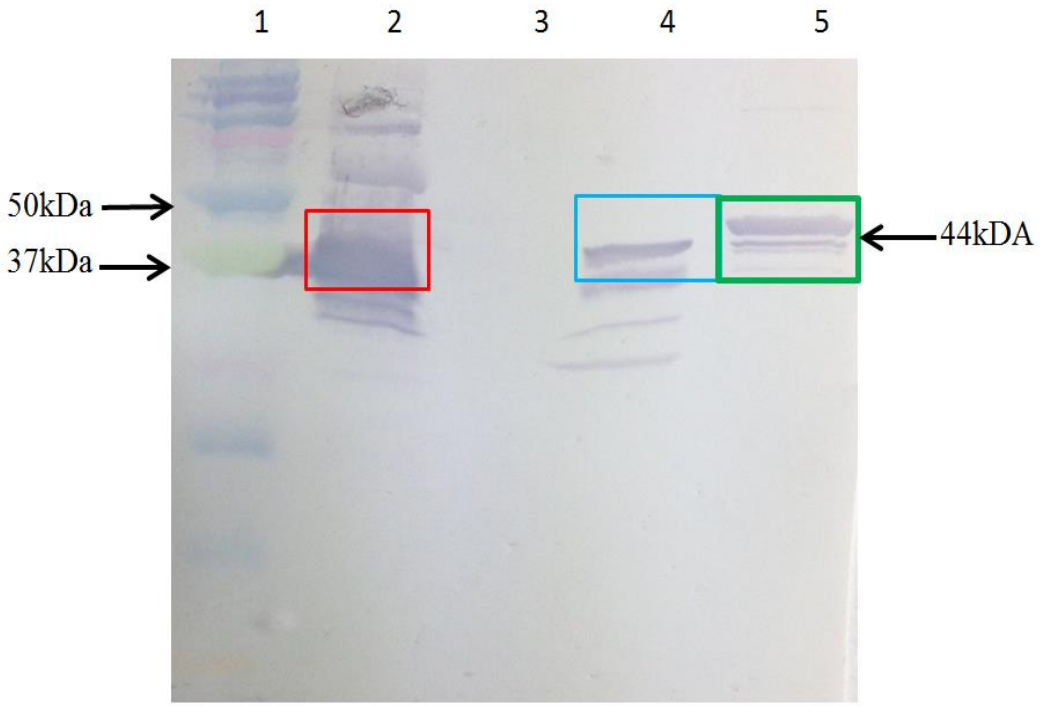
1-Marker
2- Positive control (recombinant tropomyosin Black tiger 37 KDa)
3- Negative control (Fish)
4- Crude *Anisakis pegreffii* protein (native tropomyosin 41KDa)
5- Recombinant tropomyosin protein from *Anisakis pegreffii* (44kDa)

Keys:
L=Lane
This figure shows the blots developed tropomyosin band (37kDa) within 5 min of exposure with recombinant tropomyosin from black tiger prawn in red box (L2), while all other extracts including fish protein (L2), raw extract *A. pegreffii* protein (L4) and recombinant tropomyosin protein from *A. pegreffii* (L5) samples did not respond to the tropomyosin monoclonal antibody. The positive band developed on the blot within 5 min of exposure to 1ml of developer and identified with marker size (L1).

Figure 5.13 Immunoblotting profile using mouse anti prawn tropomyosin monoclonal antibody.

5.3.14 Immunoblotting with anti-tropomyosin polyclonal antibody.

Immunoblotting profile using anti- tropomyosin polyclonal antibody against tropomyosin from black tiger prawn, fish extract fish protein, extract *Anisakis* protein and recombinant tropomyosin protein samples (Figure 5.14). This antibody reacts with the Anisakis protein. Recombinant tropomyosin protein is bigger than the native tropomyosin, as it has a N – terminal extension.



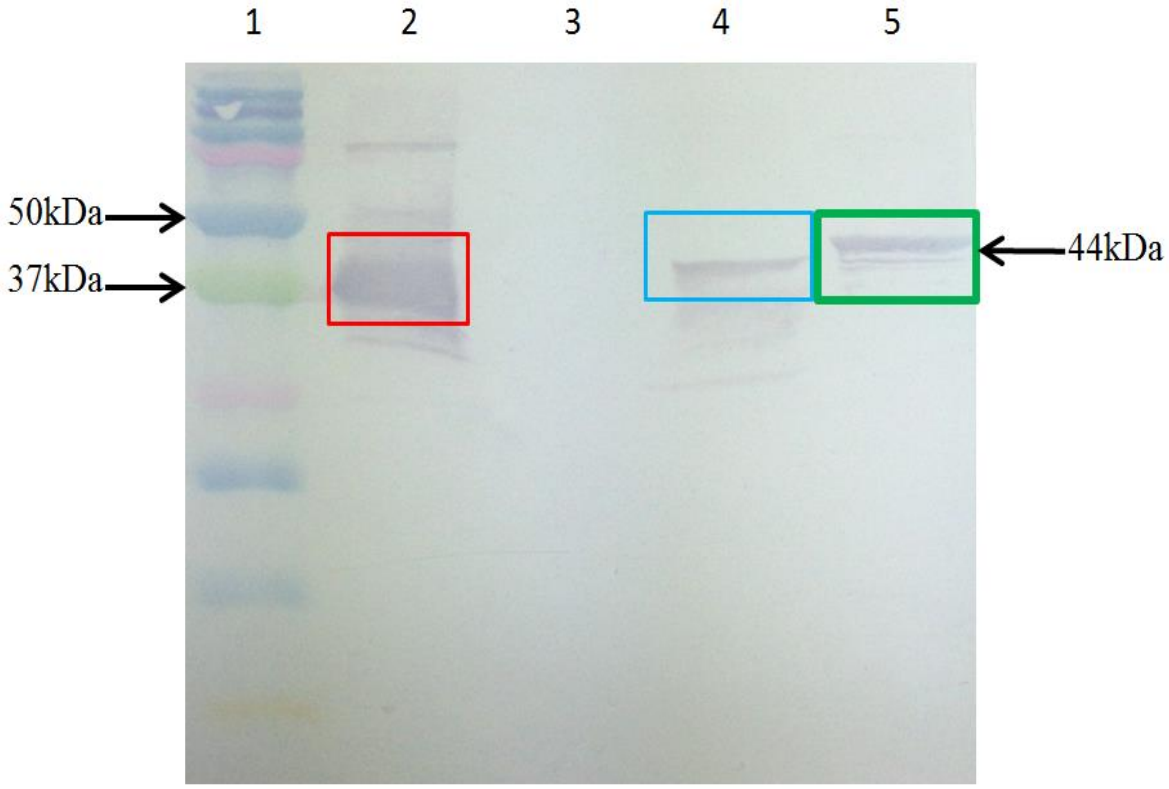
1-Marker
2- Positive control (recombinant tropomyosin Black tiger 37 KDa)
3- Negative control (Fish)
4- Crude *Anisakis pegreffii* protein (native tropomyosin 41KDa)
5- Recombinant tropomyosin protein from *Anisakis pegreffii* (44kDa)

Keys:
L=Lane
This figure shows the tropomyosin band of the positive control (recombinant tropomyosin from black tiger prawn) at 37kDa molecular weight- highlighted inside the red box (L2)- tropomyosin band (41 kDa) of Tris-glycine extract of *A. pegreffii*-highlighted inside the blue box (L4)- and recombinant tropomyosin band (44 kDa) highlighted inside the green box (L5), while the extract fish protein sample did not respond, as negative control (L2). All positive bands developed on the blot within 5 min of exposure to 1ml of developer and identified with marker size (L1).

Figure 5.14 The immunoblotting profile using anti- tropomyosin polyclonal antibody

5.3.15 Immunoblotting with anti-polycrustacean antibody for recombinant protein.

Immunoblotting profile using anti-crustacean polyclonal antibody against tropomyosin from black tiger, extract fish protein, extract *Anisakis* protein and recombinant tropomyosin protein samples (Figure 5.15). This antibody reacts with Anisakis tropomyosin.



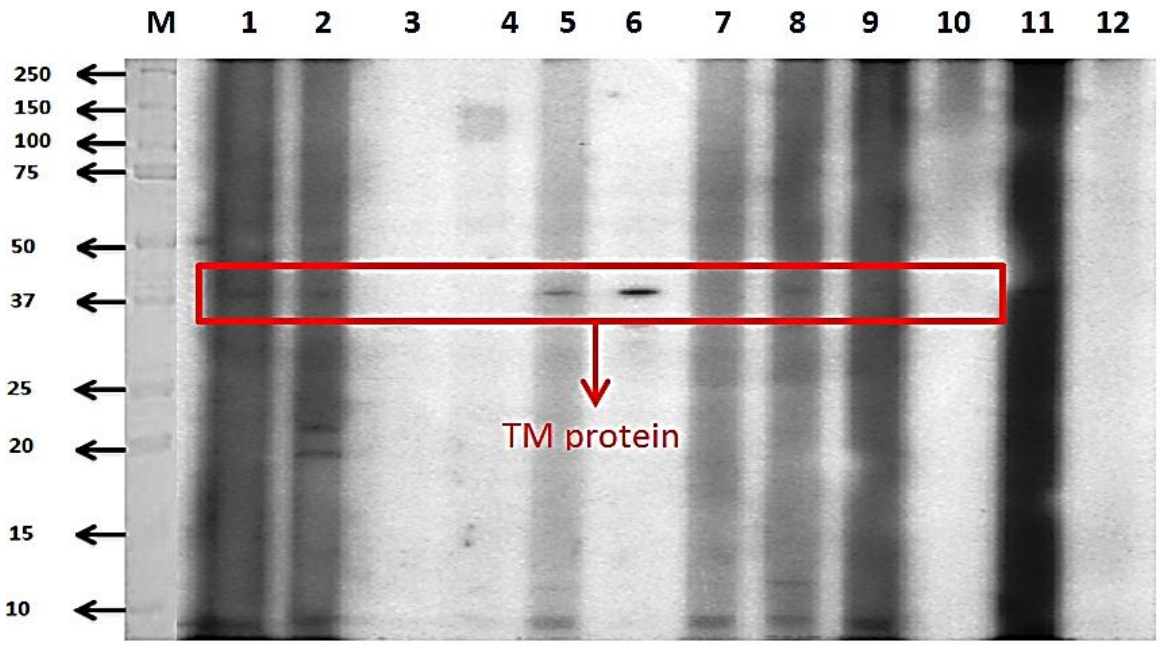
1-Marker
2- Positive control (recombinant tropomyosin Black tiger 37 KDa)
3- Negative control (Fish)
4- Crude *Anisakis pegreffii* protein (native tropomyosin 41KDa)
5- Recombinant tropomyosin protein from *Anisakis pegreffii* (44kDa)

Keys:
L=Lane
This figure shows the tropomyosin band of the positive control at 37kDa molecular weight- highlighted inside the red box (L2)- tropomyosin band (41 kDa) of Tris-glycine extract of *A. pegraffii*- highlighted inside the blue box (L4)- and recombinant tropomyosin band (44 kDa) highlighted inside the green box (L5), while extract fish protein did not response as negative control (L2). All positive bands developed on the blot within 5 min of exposure to 1ml of developer and identified with marker size (L1).

Figure 5.15 Immunoblotting profile using anti- crustacean polyclonal antibody.

5.3.16: Response of allergic human serum of shellfish to recombinant tropomyosin of *A. pegreffii.*

Immunoblotting profile using human serum of shellfish atopic patient showing that the crustacean specific IgE antibody from some patients reacted strongly with the tested recombinant tropomyosin of *A. pegreffii* as shown in Figure 5.16. A single dark band of tropomyosin of *A. pegreffii* was highlighted inside the red box at 44kDa molecular weight.



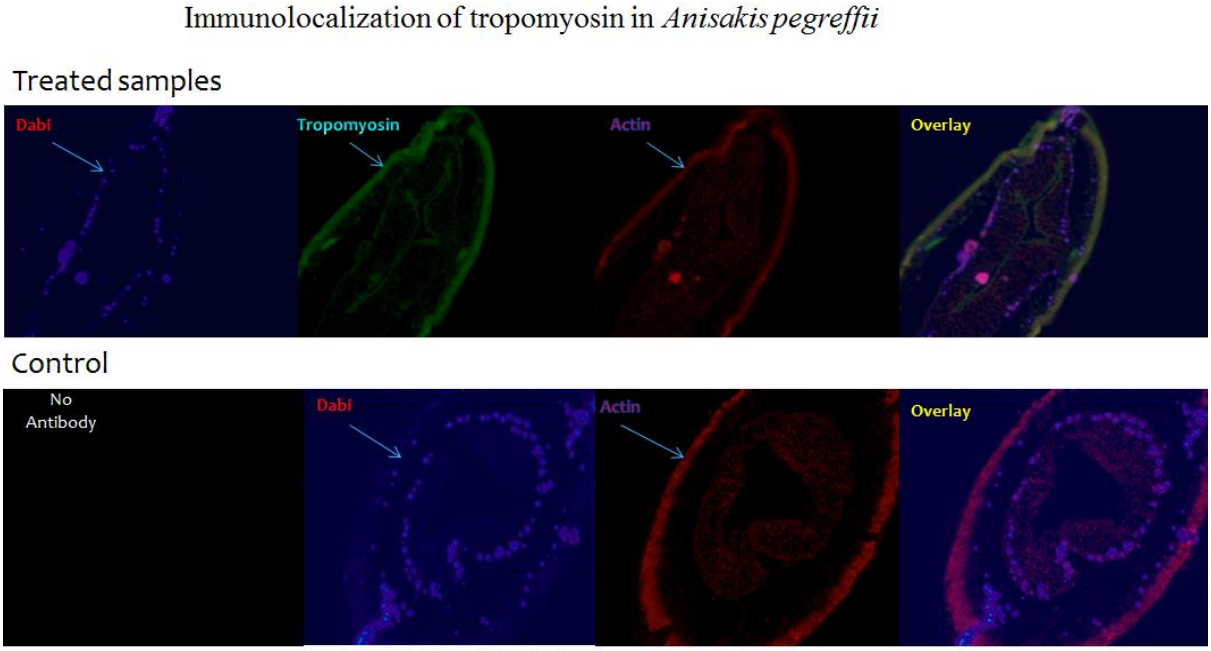
Lane 1: Marker
Lane 2: Patient serum (DM32)
Lane 3: Patient serum (GC10)
Lane 4: Patient serum (AN01)
Lane 5: Patient serum (JP47)
Lane 6: Patient serum (CF31)
Lane 7: Patient serum (DG46)
Lane 8: Patient serum (ME34)
Lane 9: Patient serum (KF41)
Lane 10: Patient serum (EV09)
Lane 11: EJ FISH
Lane 12: Negative control

Keys:
L=Lane
This figure shows the nematodes recombinant tropomyosin allergen at 44 kDa in L1, 2, 5, 6, 7, 8 and 9. The size of positive bands were identified with marker size (L1) and labelled inside red box**.**

Figure 5.16 Immunoblot profile of *A. pegreffii* with serum of shellfish–allergic patients.

5.3.17 Immunolocalization of tropomyosin in body sections of *A. pegreffii* L3.

Immunolocalization of DAPI, tropomyosin protein, actin and overlay in body tissue sections of *A. pegreffii* L3 tests using anti- tropomyosin polyclonal antibody against native tropomyosin.. The overlay results were showed that intense labelling of native tropomyosin in the skin and basal layer of the cuticle in L3 (Figure 5.17).



Immunolocalization of DAPI, tropomyosin protein, actin and overlay in body tissue sections of A. pegreffii L3 tests using anti- tropomyosin polyclonal antibody against native tropomyosin. Control, primary antibody only. The overlay results showed intense labelling of native tropomyosin in the skin and basal layer of the cuticle in L3.

Figure 5.17 Immunolocalization of somatic tropomyosin protein in *A. pegreffii*.

5.3.18 Nucleic acid sequence and protein sequence of recombinant tropomyosin of *A. pegreffii* (Anip3).

Nucleic acid sequence

ATGGACGCAATCAAGAAGAAGATGCGCAAGGCAATGGAAATCGAGAAGGACAATGCC
CTCGATCGAGCCGATGCCGCCGAAGAGAAAGTCCGACAGATGACCGACAAGTTGGAG
AGAATCGAAGAGGAACTCCGAGACACACAAAAGAAGATGATGCAAACTGAGAATGAT
TTGGACAAAGCTCAAGAAGACCTTTCAACGGCCAACTCGAATCTGGAAGAGAAGGAA
AAGAAAGTGCAAGAAGCTGAGGCAGAAGTAGCTGCTTTGAATCGTCGTATGACACTGC
TCGAAGAGGAACTCGAAAGGGCTGAAGAACGTCTGAAATTGGCAACTGACAAACTTG
AAGAGGCGACACATACAGCTGATGAATCTGAGCGTGTGCGCAAGGTGATGGAGAACC
GCTCATTCCAAGATGAAGAGCGTGCGAATACGGTAGAATCACAACTCAAGGAAGCGCA
AATGCTTGCCGAAGAGGCTGATCGCAAATACGATGAGGTTGCCCGTAAATTGGCCATG
GTTGAAGCGGATTTAGAAAGAGCTGAAGAGCGTGCAGAGGCCGGAGAAAATAAGATC
GTTGAATTGGAAGAGGAATTGCGAGTCGATGGTAACAACTTGAAATCGTTGGAGGTTT
CTGAAGAGAAGGCTTTGCAACGAGAGGATTCATACGAAGAACAGATCCGTACCGTTTC
TGCACGTCTCAAGGAGGCCGAAACTCGTGCCGAATTTGCAGAAAGATCCGTTCAGAAA
CTGCAGAAGGAAGTTGACAGACTCGAAGACGAACTCGTCCACGAGAAAGAGCGATAC
AAGAGCATTTCTGAGGAACTCGATCTACTCCAAGAGCTGTCTGGATATTAATA

Protein sequence

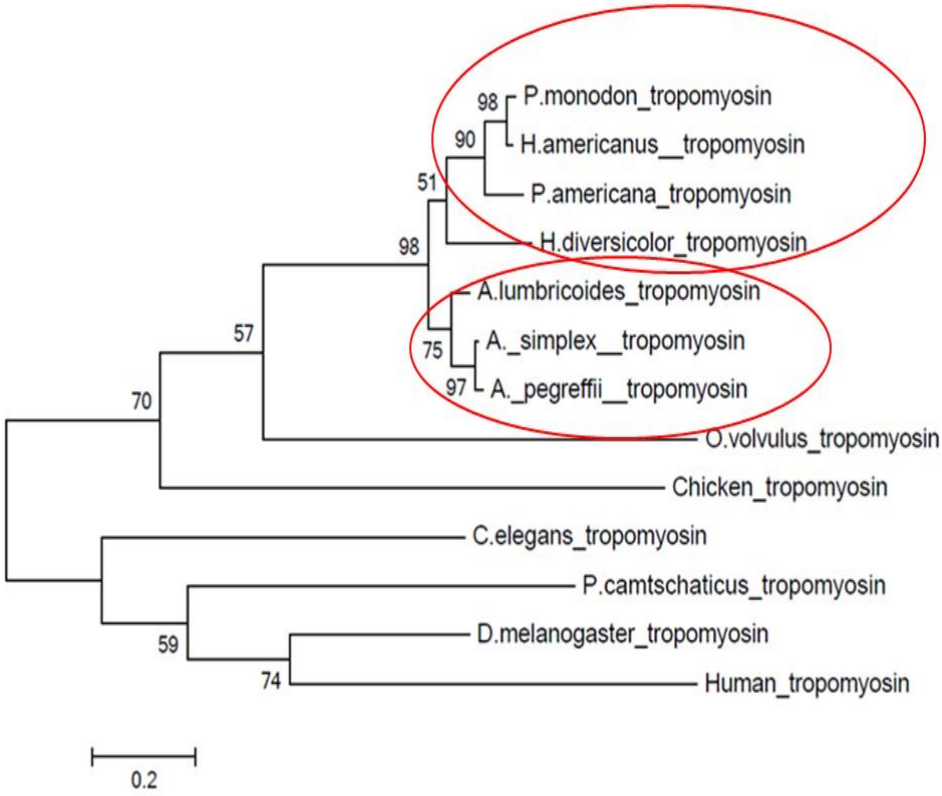
M D A I K K K M R K A M E I E K D N A L D R A D A A E E K V R Q M T D K L E R
I E E E L R D T Q K K M M Q T E N D L D K A Q E D L S T A N S N L E E K E K K V
Q E A E A E V A A L N R R M T L L E E E L E R A E E R L K L A T D K L E E A T H
T A D E S E R V R K V M E N R S F Q D E E R A N T V E S Q L K E A Q M L A E E A
D R K Y D E V A R K L A M V E A D L E R A E E R A E A G E N K I V E L E E E L R
V D G N N L K S L E V S E E K A L Q R E D S Y E E Q I R T V S A R L K E A E T R A
E F A E R S V Q K L Q K E V D R L E D E L V H E K E R Y K S I S E L D L L Q E L S
G Y Stop

The estimated molecular mass of *A. pegreffii* recombinant tropomyosin is 44kDa. The initiation codon ATG is translated in M, and the stop codon TAA is indicated with Stop. The signature pattern for tropomyosins L-K-E-A-E-x-R-A-E, indicated in red underlined, is conserved in the C-terminal section of *A. pegreffii* tropomyosin, and the highly conserved N-terminal motif DAIKKK, which contributes to binding to actin, is yellow underlined. Highly conserved IgE-binding B-cell epitopes regions is identified with green underlined and conserved IgE-binding T-cell epitopes regions is identified with red and black underlined. One potential N-linked glycosylation site N R S was identified with blue underlined.

Figure 5.18 Nucleotide and deduced amino acid sequence of *A. pegreffii* cDNA clone, encoding *A. pegreffii* tropomyosin allergen.

5.3.19 Phylogenetic analysis using Clustal W programme showing the relationship of *A. pegreffii* tropomyosin with tropomyosin of some parasitic nematodes and living organisms.

Phylogenetic analysis using Clustal W programme showing the relationship of *A. pegreffii* tropomyosin with tropomyosin of some parasitic nematodes and living organisms (Figure 5.19). The results of phylogenetic analysis showed that *A. pegreffii* tropomyosin is closely related to that of *A. simplex* tropomyosin. Among the other nematodes, the tropomyosin of *A. pegreffii* clusters closest to *A. lumbricoides*. It is interesting to also note that the tropomyosin protein of shellfish are more closely related to *A. pegreffii*'s tropomyosin compared to other nematodes such as *C. elegans* and *O. volvulus*. This may explain the cross-reactivity observed with shellfish tropomyosin.



This shows the relationship of *A. pegreffii* tropomyosin with tropomyosins of some parasitic nematodes and other living organisms, *A. simplex, A. lumbricoides, O. volvulus, C. elegans, D. melanogaster, P. monodon, H. americanus, P. Americana, H. diversicolor, P. camtschaticus*, Human, Chicken. Uniprot Accession IDs, JF902160, FJ655903, L41633.1, CELTMY1B, NM_169633, AY827100, AF034954.1, Y14854.1, AF216518.1, AB270632.1, J00910.1, M19267.1, respectively.

Figure 5.19 Phylogenetic analysis using Clustal W programme

5.4 Discussion

Ingestion of raw or undercooked contaminated seafood with *Anisakis* L3 can be lead to infection of human subjects and causes diseases known as anisakiasis and also an allergic reaction. The allergic response is characterised by production of high levels of IgE specific antibodies to both somatic and/ or E/S proteins from L3 of *Anisakis* species. Diagnostic testing is usually based on the detection of IgE antibodies, which bind to crude parasite extract and recombinant proteins.

Tropomyosin is the major allergic protein of invertebrates and is considered a pan allergen due to its wide distribution (Reese *et al*., 1999). It is taken via the skin, eating or by inhalation. Tropomyosin has several epitopes that feature high cross-reactivity among invertebrate tropomyosins of different species and may elicit hypersensitivity reactions in atopic individuals (Acevedo and Caraballo, 2011). Therefore, detection of specific IgE against parasite tropomyosin protein has been proposed as a more precise diagnostic method for anisakiasis and *Anisakis* allergy.

Using molecular markers *A. pegreffii* larvae has been confirmed to cause infections in humans (Fumarola *et al*., 2009), after consuming raw or undercooked seafoods that harbor the *L3* in their flesh and muscle (Fumarola *et al*., 2009). Therefore, the aim of this present work was to clone, sequence, and obtain recombinant tropomyosin from *A. pegreffii L3* that is harbored inside the body of tiger flathead. In addition, it was used in diagnostic screening of crustacean allergic patients for immunological cross-reactivity.

During this study, total RNA was initially extracted from the entire bodies of the *L3 A. pegreffii* specimens using RNA Extraction mini kit. Electrophoresis of RNA under denaturating condition showed two major rRNA subunit bands (small 18S and the large 28S) which means good quality of purified total RNA from *A. pegreffii L3* and cDNA synthesis of the selected poly $(A)^+$ mRNA using cDNA Synthesis Kit. The cDNA encoding Ani p 3 was amplified by normal PCR with the specific primer derived from conserved amino acid sequences and nucleotide sequences of the tropomyosin from *A. simplex* L3 (Ani s 3) in database (Asturias *et al*., 2000).

The purified PCR products (insert) of *Ani p 3* was directly cloned into pCR2.1 TOPO as intermediate vector and transformed into *Escherichia coli* DH5α. The resulting plasmids were analysed by *Bam*HI and *Xho*I to establish the positive clones. The amplification of the

DNA encoding mature Ani p 3 give rise to a DNA fragment of the expected size. Once successfully cloned Ani p 3 into intermediate vector, the insert was cloned in pRSET-A expression vector and transformed into *Escherichia coli* DH5α. Also, the resulting plasmids were analysed by *Bam*HI and *Xho*I to establish the positive clones. Finally, the purified pRSET-A expression vector containing Ani p 3 gene was sequenced using designed primers and also transformed into *E. coli* BL21 for expression of target protein and purification.

The gel electrophoresis results showed that the recombinant tropomyosin protein from *A. pegreffii* was not recognized by immunoblotting with the anti-tropomyosin monoclonal antibodies (MAbs), this indicates that the specific eptitope to which the monoclonal antibody reacts is not conserved in Anisakis tropomyosin. In contrast, immunoblotting with the anti-tropomyosin and anti-crustacean polyclonal antibodies (PAbs) showed a reaction and a strong band at 44kDa, higher than crustacean tropomyosins bands at the 37 kDa mark. PAbs frequently have broader specificity and are not affected by small changes in the structure of single or small number of epitopes. Thus, this fact confirmed that subjects with crustacean tropomyosin hypersensitivity are clinically shown to react to nematode tropomyosins and are the panallergen responsible for cross-reactivity between nematodes and different classes of shellfish (crustacean and mollusks).

Localization of tropomyosin (Ani p 3) has been investigated using immunohistochemistry to L3 of *A. pegreffii*. It was detected in the muscles and the under the basal layer of the cuticle of L3 using anti-tropomyosin polyclonal antibodies (PAbs) and mouse anti rabbit polyclonal antibodies conjugate with fluorescence. This is in agreement with several studies that reported that tropomyosin has been detected in the muscles of parasites (MacGregor and Shore, 1990, Jenkins *et al*., 1998).

To confirm rAni p 3 retained the allergenic characteristics as of nAni p 3, IgE sera of patients with allergic shellfish food were reacted with rAni p 3 and showed a strong band at 44kDa. The same tests have been performed with native tropomyosin (data not included) and the result showed a strong band at 41kDa with sera of patients having allergic shellfish food reaction. Hance, the result of the study confirmed that rAni p 3 retained the allergenic characteristics as of nAni p 3. This is agreement with several studies that reported the reaction of serum from allergic patients with tropomyosin of *A. simplex* (Acevedo and Caraballo, 2011). Therefore, although Ani p 3 allergen did not respond to all IgE patient's

sera, this validated cross-reactivity between Ani p 3 and allergic patient sera. The closeness of some of these species can be seen in the phylogenetic tree.

Sequence similarity searches using the BLAST program revealed that tropomyosin of *A. pegreffii L3* has the highest identity to α tropomyosins isoform from *A. simplex L3* (98%) and *A. lumbricoides* (84%), respectively (Asturias *et al*., 2000). The insert has a length of 855 bp, encoding 284 amino acids with a predicted mass of 44 kDa. Recombinant tropomyosin protein is bigger than the native one, as it has a N –terminal extension. This is in agreement with previous results that confirmed a similar length and size of tropomyosins (Johansson *et al*., 2001, Guarneri *et al*., 2007). The deduced amino acid sequences of tropomyosin of *A. pegreffii L3* has revealed that the tropomyosin signature, L-K-E-A-E-x-R-A-E, is conserved in the C-terminal region at position 231–239, as is the highly conserved N-terminal motif DAIKKK, which contributes to binding to actin. Taken together, the findings from this study are in agreement with several studies that reported similar results (Asturias *et al*., 2000, Santos *et al*., 1999b, Santos *et al*., 1999a) (Asturias *et al*., 2000; Santos *et al*., 1999; Santos *et al*., 2008).

The data obtained from this study also indicated that the one potential N-linked glycosylation site N R S was identified in tropomyosin of *A. pegreffii*. This observation is in agreement with the results published by Asturias *et al*. (2000).

Phylogenetic analysis using Clustal W program revealed that tropomyosin of *A. pegreffii* has the closest relationship to tropomyosins from other nematodes and crustaceans (figure 22), and showed less similarity to tropomyosins from vertebrates (humans and chickens). These results support the idea of tropomyosin as a pan-allergen. However, the clinical and allergological implications of the high similarity found among tropomyosins remains to be investigated.

Allergenic cross-reactivity is probably due to the presence of highly conserved IgE-binding epitopes on invertebrate tropomyosins and may have significant clinical implications. Shrimp tropomyosin has been identified as an important allergic protein (Ayuso *et al*., 2002). It has been identified as having two IgE-binding B-cell epitopes and six T-cell epitopes of the allergenic protein (Subba Rao *et al*., 1998). When the recombinant tropomyosin of *A. pegreffii* L3 (Ani p 3) was compared with the allergenic tropomyosin from parasites, crustaceans and living organisms, it showed almost identical sequence with

one IgE-binding B-cell epitope region and two T-cell epitopes regions as shown in Table 5.9, 5.10 and 5.11.

Table 5.9 Comparison of IgE-binding B-cell epitope between *A. pegreffii*, other parasite Crustaceans and living organism

Tropomyosin species	B-cell epitope shared between species	References
Shrimp tropomyosin	A R F L A E E A D R K Y D	Shanti *et al*., 1993
A. pegreffii tropomyosin	A Q M L A E E A D R K Y D	This study
A. simplex tropomyosin	A Q M L A E E A D R K Y D	Asturias *et al*., 2000
A. lumbricoides	V Q M L A E E A D R K Y D	Santos *et al*., 2008
T. colubriformis tropomyosin	A Q M L A E E A D R K Y D	Jenkins *et al*., 1998
C. elegans tropomyosin I	A Q L L A E E A D R K Y D	Jenkins *et al*., 1998
O. volvulus tropomyosin	A Q L L A E E A D R K Y D	Jenkins *et al*., 1998
D. melanogaster tropomyosin II	A R F L A E E A D K K Y D	Jenkins *et al*., 1998
Human muscle α tropomyosin	A K H I A E D A D R K Y E	Jenkins *et al*., 1998
Chicken muscle β tropomyosin	A K H I A E E A D R K Y E	Jenkins *et al*., 1998

Table 5.10 Comparison of binding T-cell epitope between *A. pegreffii*, other parasite Crustaceans and living organism

Tropomyosin species	T-cell epitope shared between species	References
Shrimp tropomyosin	L A E E A D R K Y D E V A R K L A M V E A	Shanti *et al*., 1993
A. pegreffii tropomyosin	L A E E A D R K Y D E V A R K L A M V E A	This study
A. simplex tropomyosin	L A E E A D R K Y D E V A R K L A M V E A	Asturias *et al*., 2000
A. lumbricoides	L A E E A D R K Y D E V A R K L A M V E A	Santos *et al*., 2008
T. colubriformis tropomyosin	L A E E A D R K Y D E V A R K L A M V E A	Jenkins *et al*., 1998
C. elegans tropomyosin I	L A E E A D R K Y D E V A R K L A M V E A	Jenkins *et al*., 1998
O. volvulus tropomyosin	L A E E A D R K Y D E V A R K L A M V E A	Jenkins *et al*., 1998
D. melanogaster tropomyosin II	L A E E A D K K Y D E V A R K L A M V E A	Jenkins *et al*., 1998
Human muscle α tropomyosin	I A E D A D R K Y E E V A R K L V I I E S	Jenkins *et al*., 1998
Chicken muscle β tropomyosin	I A E E A D R K Y E E V A R K L V I I E G	Jenkins *et al*., 1998

Table 5.11 Comparison of binding T-cell epitope between *A. pegreffii*, other parasite Crustaceans and living organism

Tropomyosin species	T-cell epitope shared between species	References
Shrimp tropomyosin	A E F A E R S V Q K L Q K E V D R L E D E L V N E K	Shanti *et al*., 1993
A. pegreffii tropomyosin	A E F A E R S V Q K L Q K E V D R L E D E L E N E K	This study
A. simplex tropomyosin	A E F A E R S V Q K L Q K E V D R L E D E L V H E K	Asturias *et al*., 2000
A. lumbricoides	A E F A E R S V Q K L Q K E V D R L E D E L V H E K	Santos *et al*., 2008
T. colubriformis tropomyosin	A E F A E R S V Q K l Q K E V O R l E D E k V M E K	Jenkins *et al*., 1998
C. elegans tropomyosin I	A E F A E R S V Q K L Q K E V D R L E D E L V H E K	Jenkins *et al*., 1998
O. volvulus tropomyosin	A E F A E R S V Q K L Q K K V D R L E D E L V H E K	Jenkins *et al*., 1998
D. melanogaster tropomyosin II	A E F A E R S V Q K L Q K E V D R L E D D L V L E K	Jenkins *et al*., 1998
Human muscle α tropomyosin	A E F A E R S V T K L E K S I D D L E E K V A H A K	Jenkins *et al*., 1998
Mouse muscle β tropomyosin	A E F A E R S V T K L E K S I D D L E D E L Y A Q K	Jenkins *et al*., 1998

The recombinant tropomyosin of *A. pegreffii* L3 (Ani p 3) shows a sequence of seven heptad repeating amino acids residues that are usually labelled *abcdefg*. Similar results were obtained by Asturias *et al*. (2000) who investigated the recombinant tropomyosin of *A. simplex L3 and* found that in the sequence (*abcdefg*) in tropomyosin of *A. simplex L3*, *a* and *d* amino acids are the hydrophobic positions of each chain. These amino acids interact

with each other to result in a parallel α-helical coiled coil chain for tropomyosin (Mason and Arndt, 2004). This report provides strong indications of α-helical coiled-coil structures of recombinant tropomyosin of *A. pegreffii.*

Chapter six: Structural, immunological and functional properties of the *Anisakis pegreffii* allergen Ani p 4 as a cysteine-protease inhibitor and comparison among the anisakid group.

Abstract

A. pegreffii larval stage can cause gastro-allergic anisakiasis and allergic disease in humans when infected marine fishes and cephalopods are consumed raw. Patients sensitized to infected fish with *L3* exhibit high levels of total and specific IgE against a potent allergen produced from the excretory gland of *A. simplex*. During this study, the cysteine-protease inhibitor gene of *A. pegreffii* was amplified using designed primers at 339 bp. Recombinant cysteine-protease inhibitor protein was produced and detected using anti-polyHistidine antibodies raised in rabbit at 12.8kDa.

Background:

The larvae third stage (L3) of the nematode *A. pegreffii* parasitizes marine organisms, such as fishes and cephalopods. The *Anisakis* protein extract from L3 infrequently causes not only Human anisakiasis but also allergic reactions in sensitized individuals. Cysteine-protease inhibitor has been identified in different adult and larvae helminth species. It is a major stable allergic protein and responsible for 80 % of cases related to allergic incidents.

Aim

In the present work, we cloned, sequenced, and characterized the *A. pegreffii* recombinant cysteine-protease inhibitor cDNA amplified by PCR, and compared its predicted amino acid sequence with other species.

Methods

Double-stranded cDNA encoding for an *A. pegreffii* L3 cysteine-protease inhibitor isoform (*As*-TPM) was synthsised from poly $(A)^+$ mRNA and amplified by PCR using primers designed according to described nucleotide sequences for *A. simplex* cysteine-protease inhibitor. The cysteine-protease inhibitor gene was cloned in PCR2.1 then pRSET-A for DNA sequencing and transformed into BL21 *E. coli* to produce recombinant cysteine-protease inhibitor protein. The characterisation of recombinant cysteine-protease inhibitor was confirmed by immunoblotting tests.

Results

The PCR product showed a single band of 339 bp in size. After expression and purification methods, SDS PAGE indicated purified recombinant cysteine-protease inhibitor protein at 12.8 kDa in size and this was confirmed by polyHistidine antibody immunoblotting. In the

case of *A. pegreffii* L3, the size of recombinant cysteine-protease inhibitor was compared to the native cysteine-protease inhibitor produced in the environment and showed the same size.

Conclusions

The data presented in this chapter suggest that a cysteine-protease inhibitor have been identified in *A. pegreffii* L3. Furthermore, it is 12.8 kDa in size, the same as the *A. simplex* L3 cysteine-protease inhibitor isoform. The characterization of recombinant cysteine-protease inhibitor from *A. pegreffii* L3 needs more investigation and testing.

6.1 Introduction

Adult and larvae helminths species follow extremely diverse and complicated routes to avoid the host immune system and have adapted to host tissues to survive and/or complete part of their life cycle. The infective stage of the helminths release a variety of proteinases and their inhibitors help them to penetrate the defensive barriers and inhibit or avoid host immune responses (Dzik, 2006). Cysteine protease inhibitors (CPI) or cystatins are widely distributed across the helminths and inhibit their own proteinases and proteinases of the host tissue during invasion and might cause pathological disease in the host. Hence, recombinant cysteine protease inhibitors have shown to be useful for inhibition of cysteine proteases such as recombinant onchocystatin (rOv17, Ov-CPI-2) cystatins from *filaria Onchocerca volvulus* (Schonemeyer *et al*., 2001), Av - cystatin (Av17, from *Acanthocheilonema viteae*), Bm-CPI-2 (from *Brugia malayi*), recombinant nippocystatin (rNbCys) and recombinant *H. contortus cystatin* of gastrointestinal nematodes (Dainichi et al., 2001, Newlands et al., 2001). These proteases showed that all recombinant cystatins inhibit the cysteine proteinases cathepsin B, L and S that are involved in the proteolytic processing of polypeptides and suppression of specific immune responses (Schierack *et al*., 2003). Ani s 4 as a cysteine protease inhibitor was identified from *A. simplex* as a 12.8 kDa allergen that causes allergy in humans. It was also shown to be heat-resistant and inhibits the papain family of cysteine proteases (Rodriguez-Mahillo et al., 2007). Therefore, it has been thought that cysteine protease inhibitors from *A. pegreffii* may cause anisakiasis and allergic reactions in humans.

6.2 Materials and methods

6.2.1 RNA extraction

6.2.1.1 Total RNA extraction from tissue cells (Kit)

Total RNA was extracted using RNA Mini Kit (Bioline) following manufacturer's instructions, which were previously described (Section 5.2.1.1).

6.2.2 Complimentary DNA synthesis

Once confirmed that samples have RNA by running on the gel, the complimentary deoxyribonucleic acid (cDNA) was synthesized from isolated RNA using Reverse Transcription protocol (Bioline) following manufacturer's instructions, which were previously described (Section 5.2.2). .

6.2.3 Measurement of DNA and RNA concentration and quality by Nanodrop spectrophotometer

DNA and RNA concentration and quality were determined by Nanodrop spectrophotometer (Thermo Scientific™ 2000) flowing the manufactory protocol. The absorbance at wavelengths 260 nm (nucleic acid) was compared to wavelengths at 280 nm (protein) and 230 nm (organics) to determine protein or organic contamination. Samples with 260/280 and 260/230 ratios between 1.8 and 2.0 were deemed as good quality DNA/RNA and used for further analysis.

6.2.4 Predict signal peptides for cysteine-protease inhibitor gene

It is usual that most secretory proteins are initially synthesized in the cytoplasm as precursors containing 15-30 residues of extra peptide extensions at the beginning of their sequencing (N-terminals). These so-called signal peptides play an important role in the translocation of newly synthesized proteins across the membrane of the endoplasmic reticulum (ER) in eukaryotic cells (Tsuchiya *et al*., 2003). To improve the expression level of recombinant proteins, signal peptides must be excluded from the genes, because the encoded proteins are not designed to be secreted. Therefore, the protein sequence for the cysteine-protease inhibitor was subjected to SignalP 4.1 Server – CBS to confirm the presence and location of signal peptide cleavage sites in amino acid sequences. For the cysteine-protease inhibitor protein cleavage was predicted between position 25 and 26.

6.2.5 Designing the primers

6.2.5.1 Design primer for Cysteine-protease inhibitor gene

The primers to amplify the whole gene coding for cysteine-protease inhibitor (Ani p 4) protein was designed manually as well as designing the orientation of the genes within pCR2.1 and pRSETA vectors by SE Central (Clone Manger) software. The first and last 20 nucleotides, corresponding to the forward and reverse primers, of the genes were selected and to which restriction enzyme sites were added. The restriction enzymes selected were *Bam*HI (forward) and *Pst*I (reverse), which cut at (GGATCC) and (GAGCTC), respectively. Those restriction enzymes were chosen because they both do not have restriction sites within the gene but do have in pRSET-A vector. CGG bases were added to the primers to increase their stability. The primers were then evaluated for the ligation success within pCR2.1 and pRSETA downstream of the T7 promoter. The primers were ordered from GeneWorks Custom Oligo Service.

Table 6.1 The primers used in this study (5'-3') Amplification of cDNA gene tropomyosin (*Ani p* 3)

Gene	Sequencing
Ani p 4	*Ani p 4* f CGGGATCCATCCAGAATCGTCGTAGCG *Ani p 4* r CGCTGCAGTTACTGATGATCGCATT

6.2.5.2 Preparation of the primers

The ordered two primers were supplied in a dried form, and of which 100µmol stock solutions were prepared as shown below:

For-Fila (forward primer),

41.6nmol/tube

Concentration= Mass/volume

$$\text{Volume} = \frac{41.6\times10^{-3}}{100\ \mu\text{mol/L}}$$

=416µl of MG water was added to reconstitute.

2) Rev-Fila (reverse primer),

41.3nmol/tube

Concentration= Mass/volume

$$\text{Volume} = \frac{41.3\times10^{-3}}{100\ \mu\text{mol/L}}$$

=413µl of MG water was added to reconstitute.

To prepare 10 µmol working, the stock solutions were diluted 1:10 (50 µl of each vial mixed with 450 µl of MG water), and they were all stored at -20°C.

6.2.5.3 PCR reaction

The PCR reaction was performed to amplify the gene coding for the Ani p 3 proteins from the extracted Ani p 3 DNA. The PCR Components for both the Ani p 3 gene and the negative control were used for annealing step in order to determine the optimal temperature for amplifying a pure gene.

Table 6.2 PCR cycling conditions for cysteine-protease inhibitor gene of *A. pegreffii*

PCR Components			PCR cycling conditions			
Reagent	Volume		Step	Temperature	Time	number of cycles
	Ani p 4 gene	Negative control	Heated lid	110°C	--	30
cDNA	1.5µl	-	Hot start	94°C	00:02:00	
Forward primer	0.5µl	0.5µl	Denaturation	95°C	00:00:20	
Reverse primer	0.5µl	0.5µl	Annealing	55°C	00:00:15	
MyTaq DNA Polymerase	12.5	12.5	Elongation	72°C	00:00:50	
MG water	10µl	11.5µl	Final Elongation	-	-	
Total volume	25µl	25µl	Hold	4°C	Infinite	

6.2.5.4 Purification of DNA from PCR amplification

Purification of DNA from PCR amplification was performed by using a Wizard PCR prep kit. One hundred µl of purification buffer was added to each PCR reaction and mixed briefly with a pipette. One ml of resin was added to the mixture and vortexed briefly. The entire mixture was then transferred to a column and was gently passed through it. Two mls of isopropanol was added and centrifuged again for 1 minute. The DNA was then eluted in 20 µl of elution buffer and stored in a -20°C freezer.

6.2.5.5 Gel electrophoresis

A 1% agrose (0.5 agarose dissolved in 50 ml TAE buffer) gel electrophoresis was performed to confirm that the right gene size has been amplified. The size of bands was compared to the λ *Pst1* Marker, of which 20µl was loaded in the first well, and 5µl of each sample was mixed with 1µl of the Orange loading dye and then loaded in the gel. The gel was run for an hour at 100 volt and then stained using ethidium bromide for 15 minutes followed by destining for 30 minutes in running water.

6.2.5.6 Amplification of native/recombinant cysteine-protease inhibitor of *A. pegreffii* by PCR

The gDNA was amplified using design primer (section 5.2.5.1) and compared with cDNA to determine if the cysteine-protease inhibitor gene contains introns and also to determine the length of the cysteine-protease inhibitor gene.

6.2.6 First cloning stage.

6.2.6.1 Preparing electro-competent DH5α E. Coli

The electro-competent cells DH5α were prepared for both the clone pCR2.1 and pRSET-A using the method previously described (Section 5.2.6.1).

6.2.6.2 Assessing the efficiency of the electro-competent cells

To ensure the efficiency of the prepared electro-competent cells, 0.5µl of PUC18 plasmid (0.1ng/ µl), which has shown to be effectively transformed into electro-competent cells, was mixed with 50µl of prepared electro-competent cells and then transferred to a cuvette. The transformation was done by placing the cuvette in a chamber attached to the BIORAD electroporation system set at 2.5 volt, 25µF, 200 ohms and then giving the cells an electrical shock. Following the addition of 1ml of LB broth to the cells, they were transferred to a micro-centrifuge tube and incubated at 37°C on shaker with 225 revolutions per minute (rpm) for an hour. The culture (100 µl) was plated on LB agar containing 100µg/ml of ampicillin and incubated overnight at 37°C, and afterwards the colony forming units (CFU) were counted and used in the transformation efficiency calculation. The calculation was done as follows:

1) Total DNA transformed (ng) = plasmid concentration × the added volume
= 0.1ng/ µl × 0.5 µl= 0.05ng.

2) Transformation efficiency (CFU/µg) $= \frac{\text{Number of CFU}}{\text{Total DNA transformed (ng)}} \times 10^3 \times \frac{\text{Total volume transformed}}{\text{The plated volume}}$

$= \frac{540\ \text{CFU}}{0.05\text{ng}} \times 10^3 \times \frac{(250+50)\ \mu\text{l}}{100\mu\text{l}}$

$= 3.24 \times 10^7$ CFU/µg

Hence, it can be concluded that prepared DH5α *E. coli* cells are efficiently electro-competent.

Hence, it can be concluded that prepared DH5α *E. coli* cells are efficiently electro-competent.

6.2.6.3 Cloning the cysteine-protease inhibitor (Ani p 4) gene into pCR2.1 vector

In order to ligate the Ani p 4 gene into pCR2.1 vector, the PCR reaction to amplify the required gene was repeated to obtain a fresh product using the optimal annealing temperature (55°C). Then, the ligation constituents shown in Table 1 were mixed and incubated overnight at 14°C. Afterwards, 2µl of the ligation product was transformed into electro-competent DH5α *E. coli* using the method applied for the positive control plasmid (Section 5.2.6.2), and then the transformed cells were plated on LB agar containing 100µg/ml of ampicillin, 40 µg/ml X-gal, and 0.1Mm IPTG for blue/white colonies selection.

Table 6.3 Cloning condition of the Ani p 4 gene into pCR2.1 vector

Components	Volume
Fresh PCR product	2µl
10x Ligation buffer	1µl
pCR2.1 vector	2µl
MG water	4µl
T4 DNA Ligase	1µl
Total volume	10µl

6.2.6.4 Restriction analysis

To confirm the success of ligation the PCR product into pCR2.1 vector, each purified plasmid was digested with both *Bam*HI and *Pst*1 restriction enzymes. The components shown in Table 2 were mixed in a microfuge and then incubated in a 37°C water bath for 4 hours. Afterwards the restriction products were viewed by running a 1% gel electrophoresis, which was previously described (5.2.5.5).

Table 6.4 Restriction analysis of the Ani p 3 gene into pCR2.1 vector

Components	Volume
MG water	10µl
10x Buffer H	1µl
*Bam*HI enzyme	2µl
*Pst*1 enzyme	2µl
Plasmid DNA	5µl
Total volume	10µl

6.2.7 Second cloning step

6.2.7.1 Isolation of pRSET-A vector

The pRSET-A vector was purified from DH5α *E. coli* using the Plasmid Mini Kit (BIOLINE), and by applying the isolation of high copy plasmid DNA protocol described in the kit which were previously described (Section 5.2.7.1).

6.2.7.2 Restriction analysis

In order to clone the Ani p 4 gene into the pRSET-A vector, both the insert and the dedicated cloning site of the pRSET-A vector were digested with both *Bam*-HI and *Pst*1 restriction enzymes before running on a gel. The components shown in Table 2 were mixed in a microfuge and then incubated in a 37°C water bath for 4 hours. Afterwards the restriction products were viewed by running a 1% gel electrophoresis.

Table 6.5 Conditions for double digestion of Ani p 4 gene and pRSET-A vector

Components	Volume
MG water	10µl
10x Buffer H	1µl
Bam-HI enzyme	2µl
*Pst*1 enzyme	2µl
Plasmid DNA	5µl
Total volume	10µl

6.2.7.3 Gel extraction of DNA

In order to clone the Ani p 4 gene into pRSET-A vector, both the insert and the dedicated cloning site of the pRSET-A vector should be isolated from the gel using the same method previously described (Section 5.2.7.3).

6.2.7.4 Ligation of Ani p 4 gene into pRSET-A vector

The ligation reaction was performed using 9ng of pRSET-A vector and 3.5ng of Ani p 4 insert by applying 1:1 and 1:3 ratios of insert to vector, and the calculations were done by the formula below. The ligation reactions were prepared according to the components listed in Table 3, and then the tubes were incubated overnight at 14°C.

$$\text{Ng of gene} = \frac{\text{Gene size X ng of vector X molar ratio}}{\text{Size of the vector}}$$

Subsequently, 2µl of the ligation product was transformed into electro-competent DH5α *E. coli* using the method previously described (Section 5.2.6.3), and then the transformed cells were plated on a LB agar containing 100µg/ml of ampicillin and grown overnight at 37°C.

Table 6.6 pRSET-A vector and Ani p 4 gene Ligation Conditions.

Components	Volume
Gel extracted pRSET-A backbone	1µl
Gel extracted *Ani p 3* insert	4µl
20 mM ATP	1µl
T4 DNA Ligase	1µl
10x T4 DNA Ligase Reaction Buffer containing 10mM ATP	2µl
MG water	11µl
Total volume	20µl

6.2.7.5 Sequencing of the Ani p 4 gene

One successfully cloned Ani p 4 gene into pRSET-A vector was purified from DH5α *E. coli* cells using the Plasmid Mini Kit (BIOLINE) protocol described before (Section 5.2.7.1). The purified plasmid was prepared to be sequenced in the Australian Genome Research Facility LTD (AGRF), which recommends sending a 12µl mix containing 600 to 1500ng double stranded plasmid and 0.8pmol/1µl primer. A 1% agarose gel electrophoresis was performed to determine the DNA concentration in the purified sample. The calculation below was done to determine how much of the 10µm T7 primers should be added to the sample. Hence, 1µl of the 10µm T7 forward primer was added to 11µl of the purified plasmid, and the same step was done with the T7 reverse primer. Then, the two 12µl samples were sent to AGRF for sequencing.

$$C1 \times V1 = C2 \times V2$$

10pmol/µl × V1= 0.8pmol/µl ×12µl

V1=1µl

6.2.8 Protein Expression and Purification

6.2.8.1 Preparation of electro-competent DH5α E. coli strain BL21 (DE3) pLysS

Electro-competent DH5α *E. coli* strain BL21 (DE3) pLysS cells were prepared by the same method described previously (Section 5.2.6.1) using LB broth containing 35µg/ml chloramphenicol. To assess the efficiency of the prepared electro-competent cells, 0.5µl of PUC18 plasmid (0.1ng/µl) was transformed using the method previously described (Section 5.2.6.2) and inoculated onto LB agars containing 35µg/ml chloramphenicol and 50µg/ml ampicillin. After overnight incubation at 37°C, the same calculation described was done as follows:

1) Total DNA transformed (ng) = plasmid concentration x the added volume

= 0.1ng/ µl x 1µl= 0.1ng.

2) Transformation efficiency (CFU/µg) = $\frac{\text{Number of CFU}}{\text{Total DNA transformed (ng)}} \times 10^3 \times \frac{\text{Total volume transformed}}{\text{The plated volume}}$

$= \frac{60 \text{ CFU}}{0.05\text{ng}} \times 10^3 \times \frac{(1000+52)\ \mu\text{l}}{100\mu\text{l}}$

$= 6.3 \times 10^6$ CFU/µg

Hence, it can be concluded that the prepared *E. coli* strain BL21 (DE3) pLysS is efficiently electro-competent.

6.2.8.2 Pilot expression

The expression of recombinant cysteine protease inhibitor protein from electro-competent DH5α *E. coli* strain BL21 (DE3) pLysS cells containing pRSET-A with the cloned Ani p 4 gene was performed using the same methods previously described (Section 5.2.8.2).

6.2.8.3 First protein expression approach (freezer and 37°C water bath)

The first approach was performed to express recombinant proteins using freezer and 37°C water bath methods which were previously described (Sections 5.2.8.3).

6.2.8.4 Second protein expression approach (Sonication)

The second approach was performed to express recombinant proteins using freezer and 37°C water bath methods which were previously described (Sections 5.2.8.4).

6.2.8.5 Preparation of immobilized metal affinity chromatography (IMAC) column

These methods were previously described (Sections 5.2.8.5).

6.2.8.6 Optimization of IMAC protein purification

These methods were previously described (Sections 5.2.8.6).

6.2.9 Protein electrophoresis and analysis

6.2.9.1 Tris-glycine sodium dodecyl sulphate- polyAcrylamide gel electrophoresis (SDS-PAGE)

The preparing of SDS-PAGE was previously described (Sections 5.2.9.1) with the same components of materials.

6.2.9.2 Samples preparation for SDS-PAGE (protocol in previous section 5.2.9.2)

Depending on the result of the Bradford assay, different protein amounts of each extract were prepared by diluting them in PBS to obtain a final volume of 20µl, then 5µl of 5x sample buffer was added and heated in a water bath or heater at 100°C for 5 minutes. After that, a volume of 25µl of the sample was loaded into wells using special loading tips. 5µl of protein molecular marker, Precision Plus Protein Dual Color Standards (Bio-Rad, USA) was loaded in the first well of the gel.

6.2.9.3 SDS-PAGE: Running conditions and staining

Electrophoresis apparatus was connected to the power supply and the voltage set in two stages, first stage at 80 volts for 10 minutes until the protein reached the top of resolving gel and the second stage at 160 volts for 60 minutes until the protein reached the bottom of the gel glasses.

6.2.9.4 AcquaStain Blue dye

When the electrophoresis was complete, the stacking gel region was cut away by scalpel blade and the gel was removed from the glass plates. The gel was stained with around 50ml AcquaStain Blue dye for 2 hours on a rotating shaker at room temperature. Afterwards, the stain was removed and the gel was washed with distilled water. To visualize the proteins

bands, the gel was placed on a transparency paper and scanned in Gel Doc EQ system (Bio-Rad, USA).

6.2.9.5 Western blotting

The SDS-PAGE electrophoresis was performed again for the protein samples using the Kaleidoscope pre-stained marker. After that, the proteins were transferred from SDS-PAGE gel to a nitrocellulose membrane using the iBlot TM Dry Blotting System (Invitrogen). First, the anode stack was placed on the tray, and the SDS-PAGE gel was overlaid on the top. Second, a pre-soaked iBlot TM filter paper in deionized water was placed on the top, and air bubbles were removed using the blotting roller. Third, the cathode was placed with the electrode side facing up, and the disposable sponge was positioned on the top with the metal contact on the upper side. Finally, the lid was closed on the assembled stacks and the blotting process started for 7 minutes. After that, the nitrocellulose membrane was ready for further western immunoblotting analysis.

6.2.10 Immunoblotting analysis

6.2.10.1 Immunoblotting with anti- Histidine polyclonal antibody

Briefly, after removing the nitrocellulose membrane from the device, it was treated with primary and secondary antibodies using methods, which were previously described (Sections 5.2.10.1).

6.2.12 Collection of E/S products

Briefly, *A. pegreffii* L3 were isolated and washed from Tiger flathead as described in chapter three (section 3.2.1). L3 were incubated in individual wells of a 12-well tissue culture plate (Flat bottom) containing 0.5 ml of RPMI 1640 tissue culture medium at 37 °C, 5% CO_2 for three days. Each 24 hours, the media was collected from the whole well and centrifuged at 8000 x g for 10 min and the supernatant was used as E/S products. Supernatants were pooled and stored at -20 °C. Prior to electrophoresis, supernatants were concentrated 4 –10 times by lyophilization or ultrafiltration using Centricon 10 cartridges (Amicon, Beverley, MA).

6.2.13 Phylogenetic analyses

These were performed as described in chapter five (Section 5.2.12). The recombinant cysteine protease inhibitor protein of *Anisakis pegreffii* was compared with thirteen cysteine

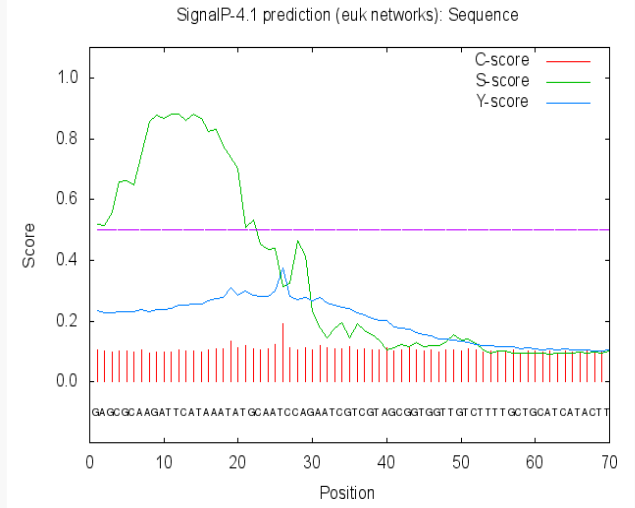
protease inhibitor proteins of some parasitic nematodes and living organism as follows: *Anisakis simplex* CPI (CAK50389), *A. lumbricoides* CPI (HQ404231), *O, volvulus* CPI *(*AF177194*), H. polygyrus* CPI (KC181863), Filarial CPI (AJ310669), Disk abalone *(H. discus discus)* CPI (JQ653304), Pacific Oyster *(C. gigas)* CPI (ADI33157), Copepod (*T. japonicas*) CPI (JN633979), Chinese mitten crab *(E. sinensis)* CPI (GU002535), Zebrafish *(D. rerio)* CPI (JQ287496), Rock bream (*O. fasciatus*) CPI (NP001096599), Human CPI (*HUMINCP1*) and Chicken CPI (NM_205500). The aim of this comparison is to examine and analyse polymorphisms and the sequence pattern through the cysteine-protease inhibitor of parasitic species and other living organisms with the cysteine-protease inhibitor of *A. pegreffii*.

For the analysis, NCBI was used to obtain and align the cysteine protease inhibitors of *Anisakis pegreffii* and amino acid sequence of the species. The Clone manager suite of software was used for alignment of the sequences. Molecular evolutionary genetics analysis (MEGA) software was used for the cysteine protease inhibitor gene sequence alignment and generation of the phylogenetic tree by the neighbour-joining dendrogram (Felsenstein 1989). Expasy was used to translate nucleotide sequence to amino acids (Artimo et al. 2012).

6.3 Results

6.3.1 Signal peptide prediction for cysteine-protease inhibitor gene.

The complete amino acid sequence of the cysteine-protease inhibitor protein of *A. simplex* was submitted to the SignalP 4.1 server, revealing a signal peptide composed of amino acids which were found with a cleavage site between position 25 and 26 of the sequence (Figure 6.1). Therefore, it is a secretory protein.




```
# Measure  Position  Value    Cutoff   signal peptide?
  max. C    26       0.193
  max. Y    26       0.374
  max. S    12       0.882
  mean S     1-25    0.701
       D     1-25    0.550   0.450   YES
Name=Sequence   SP='YES' Cleavage site between pos. 25 and 26: CAA-TC D=0.550 D-cutoff=0.450 Networks=SignalP-noTM
```

Figure 6.1 Cysteine-protease inhibitor gene of A. simplex signal peptide prediction using the SignalP 4.1 server according to signalP of several artificial neural networks (signal NN).

6.3.2 Isolation of RNA from *A. pegraffii* using the kit.

Total RNA was isolated from whole tissue of *A. pegreffii* (35 larvae) and then eluted using 30 µl of RNase-free water. Using 1% electrophoresis gel, 10 µl of the sample was loaded, and by ethidium bromide staining, there were indeed two major rRNA subunit bands (small 18S and the large 28S) that were detected in the profile of total RNAs of *A. pegreffii* (Figure 6.2). Refer to figure 6.2.

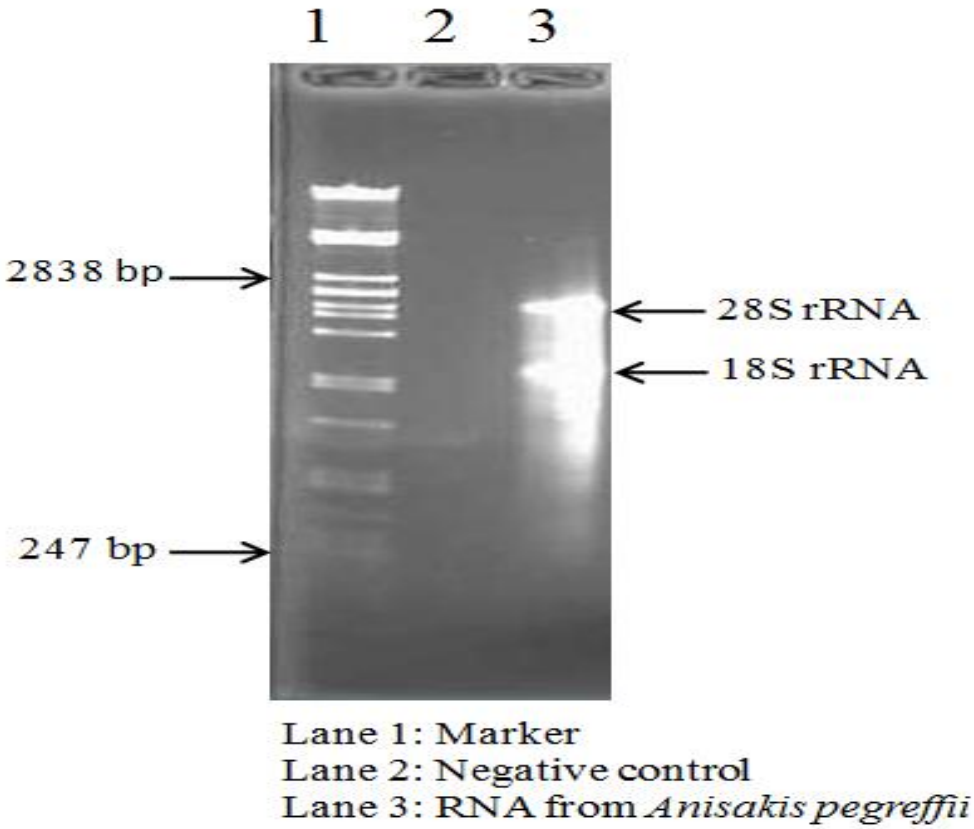


Figure 6.2 Isolation of RNA from *A. pegreffii* using ISOLATE RNA Mini Kit.

6.3.4 Double stranded cDNA synthesized from a messenger RNA (mRNA) from *A. pegreffii.*

10 µl of cDNA products were electrophoresized in a 1 % agarose gel and by ethidium bromide staining (Figure 6.3). The quality and full-length of cDNA from *A. pegreffii* were estimated and confirmed between 247 and 1159 bp.

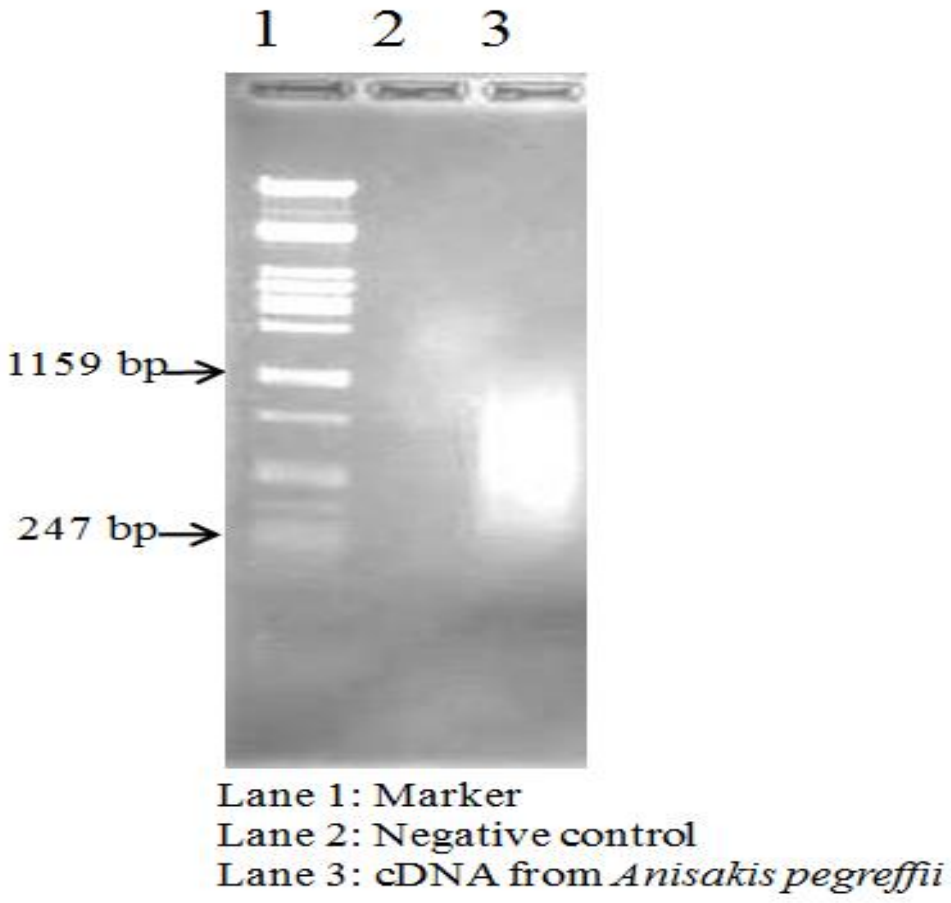
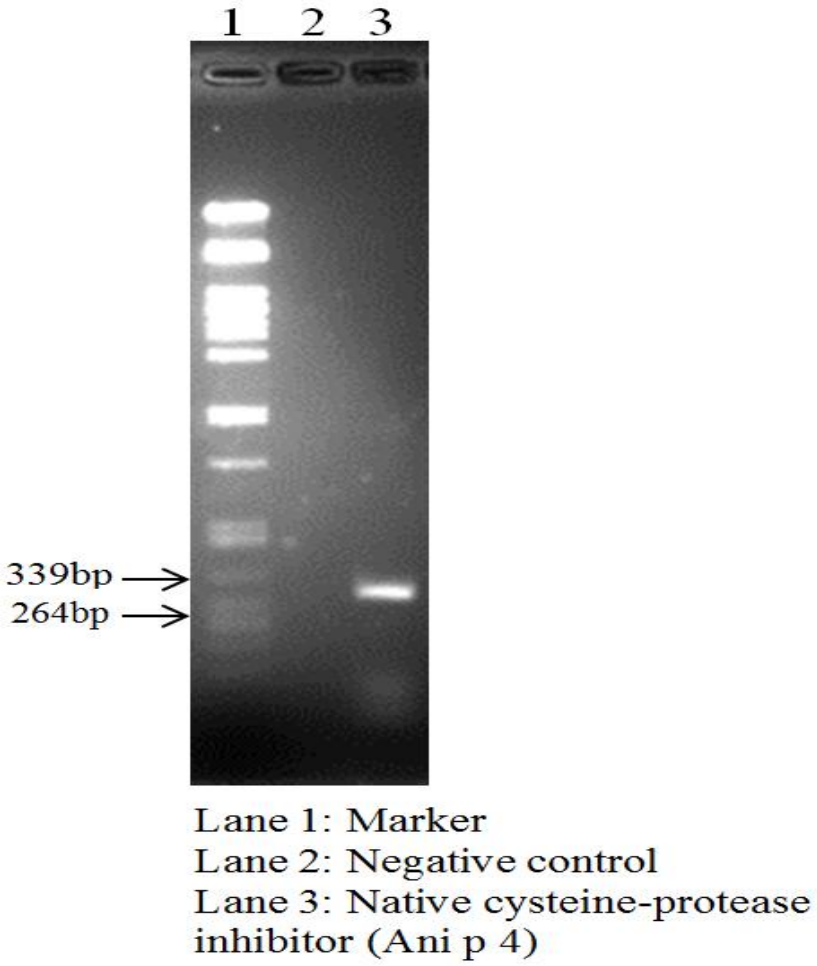


Figure 6.3 Double stranded cDNA synthesized from a messenger RNA (mRNA) using cDNA Synthesis Kit.

6.3.5 Amplification of cysteine-protease inhibitor gene from *A. pegreffii* DNA by specific designed primers.

Here the designed primers were tested with DNA to amplify the cysteine-protease inhibitor gene to determine the length of the cysteine-protease inhibitor gene and demonstrate the absence of introns (Figure 6.4).

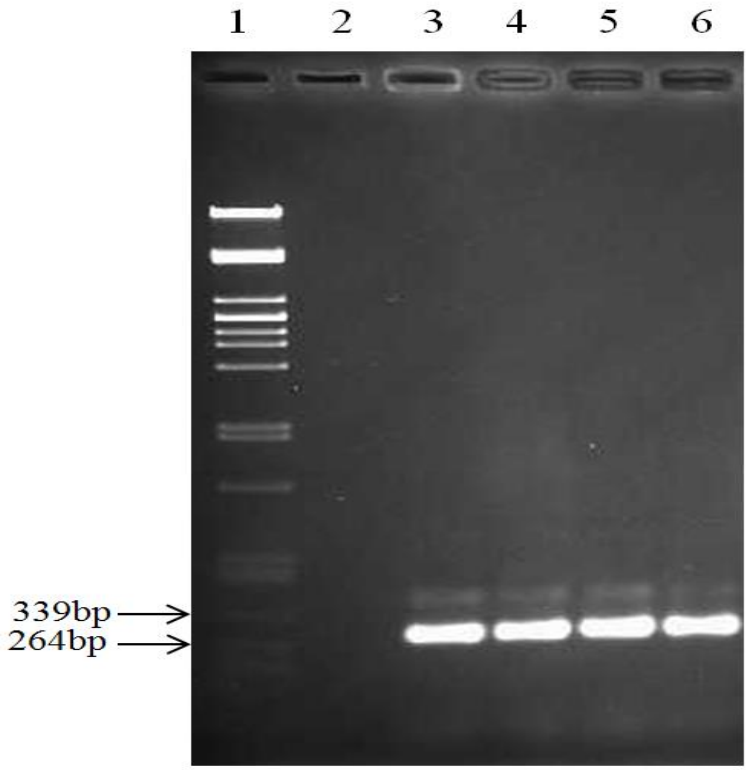


Keys:
L=Lane
L1=Marker, L2=Negative control and L3=Tropomyosin gene (Ani p 4).

Figure 6.4 Amplification of cysteine-protease inhibitor gene of *A. pegreffii* by specific designed primers.

6.3.6 Amplification of cysteine-protease inhibitor gene of *Anisakis pegreffii* from cDNA by specific designed primers.

The designed primers were tested with cDNA to amplify the cysteine-protease inhibitor gene to determine the length size of cysteine-protease inhibitor gene (Figure 6.5).



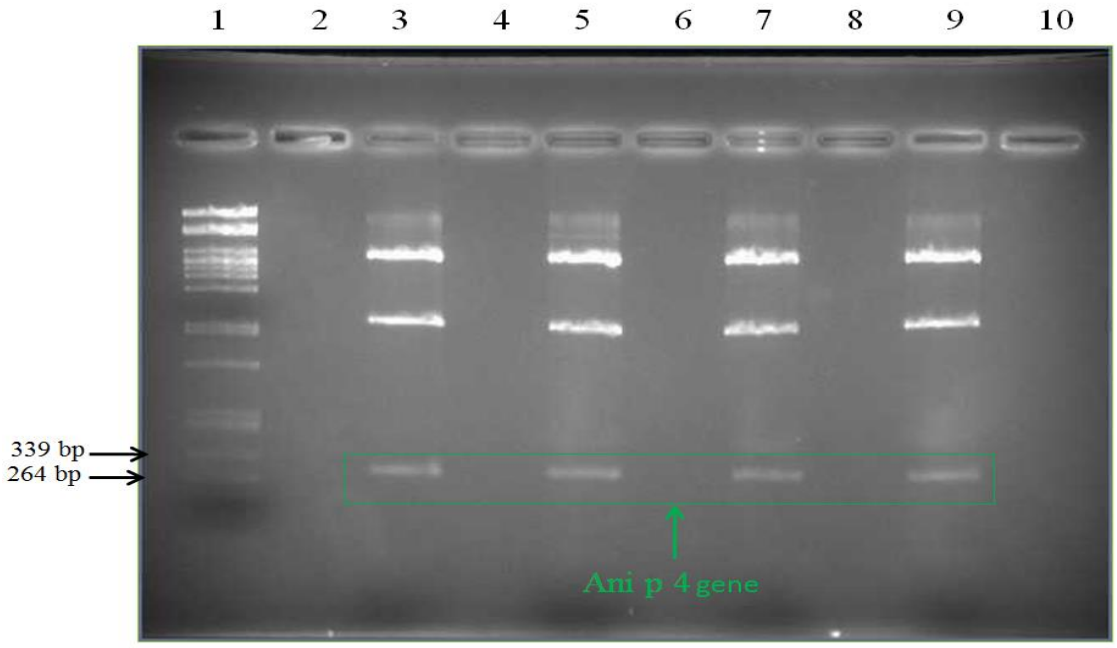
Lane 1: Marker
Lane 2: Negative control
Lane 2, 3, 4 and 5: Cysteine protease inhibitor (Ani p 4 gene)

Keys:
L=Lane
L1=Marker, L2=Negative control and L3, 4, 5 and 6=Cysteine protease inhibitor gene (Ani p 4).

Figure 6.5 Amplification of Ani p 4 genes by designed primers.

6.3.6 Digestion of pCR2.1 including Ani p 4 gene by both *Bam*HI and *Pst*I restriction enzyme, respectively.

DH5α competent cells transformed with pCR2.1 vector ligated with Ani p 4 (Cysteine protease inhibitor) inserts which were randomly selected and the vector pCR2.1 was isolated, and then digested with selected restriction enzyme to confirm the presence of the Ani p 4 gene. Agarose gel electrophoresis (1%) revealed the positive recombinant PCR2.1/Ani p 3 clones. Both the positive recombinant PCR2.1/Ani p 4 were double digested with the restriction enzymes *Bam*HI and *Pst*I which released the same three fragments bands at different sizes in each lane (3, 5, 7 and 9). Ani p 3 gene fragments from the pCR 2.1 vector were confirmed in lane 2, 3 and 4 at size 339 bp while the other two fragments from each lane belong to the rest of the PCR2.1 vector. Lane 1 represents DNA ladder marker (Figure 6.6).



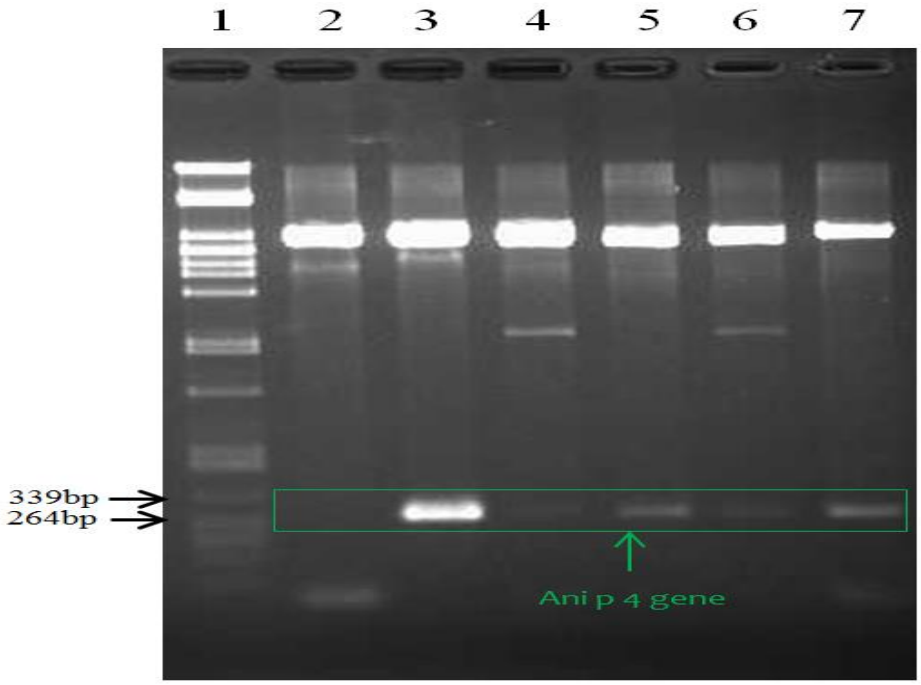
Lane 1: Marker
Lane 2, 4, 6, 8 and 10: Negative control
Lane 3, 5, 7 and 9: Cysteine protease inhibitor genes (Ani p 4)

Keys:
L=Lane
L1=Marker, L2,4,6,8 and 10=Negative control, and L3, 5, 7 and 9:= Cysteine protease inhibitor gene (Ani p 4) is labelled inside the green box.

Figure 6.6 Digestion of pCR2.1 including Ani p 3 gene by restriction enzymes.

6.3.7 Digestion of pRSET-A including Ani p 4 gene by both Bam HI and PstI restriction enzyme, respectively.

The presence of Ani p 4 excised from pCR2.1 vector and which was subcloned into pRSET- A plasmid was also confirmed in pRSET-A as described for pRC2.1. In the case of pRSET- A, two bands were obtained from this digestion. The presence of the Ani p 4 gene fragments from the pRSET-A vector was observed at size 339 bp while the other band was the rest of the pRSET-A vector (Figure 6.7).



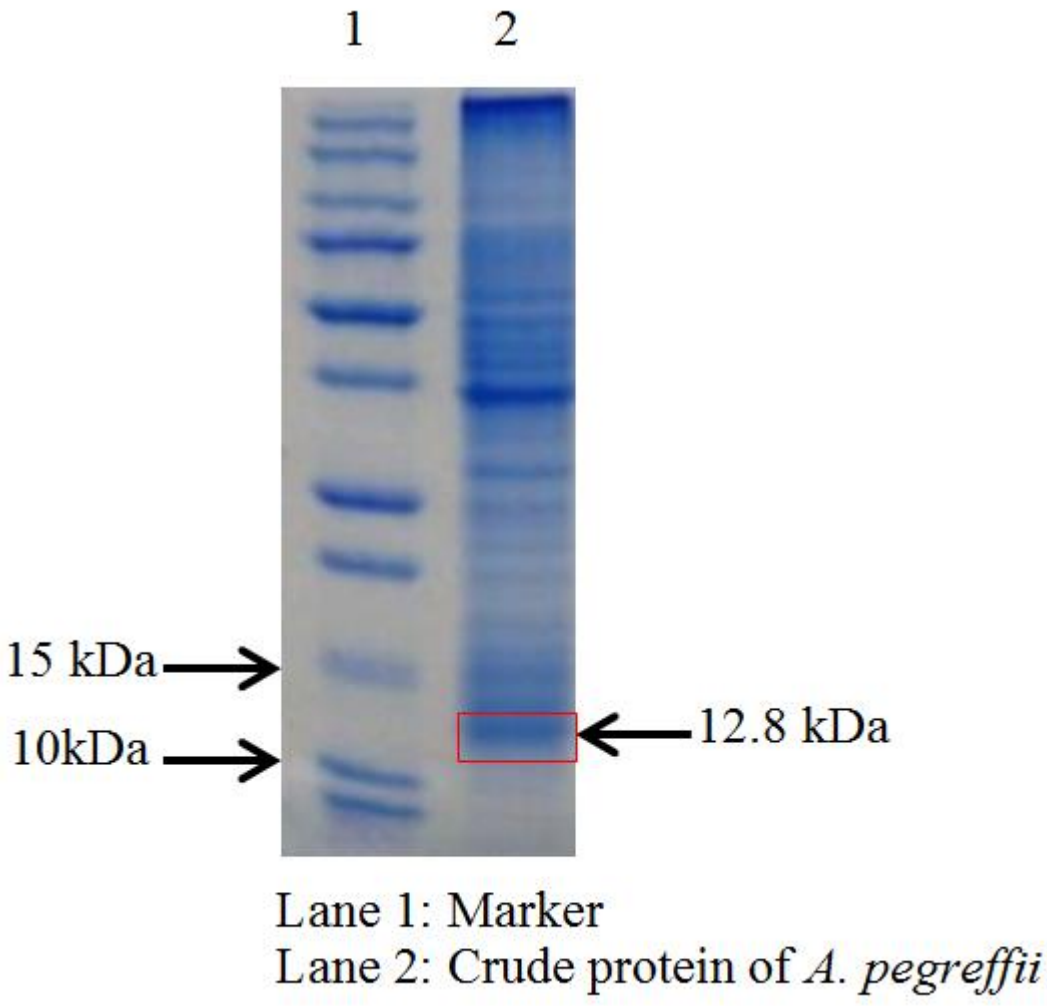
Lane 1: Marker
Lane 2, 3, 4, 5, 6 and 7: Cysteine protease inhibitor gene (Ani p 4)

Keys:
L=Lane
L1=Marker, L2, 3, 4, 5, 6 and 7 Cysteine protease inhibitor gene (Ani p 4) is labelled inside the green box.

Figure 6.7 Digestion of pRSET-A including Ani p 4 gene by restriction enzymes.

6.3.8 Identification of the presence of native cysteine-protease inhibitor protein from *A. pegreffii* using SDS PAGE gel.

SDS-PAGE electrophoresis of the raw extract protein from *A. pegreffii* (lane2) showing the expected size of native cysteine-protease inhibitor protein extract (lane 2) labelled in the red box as showed in figure 6.8.

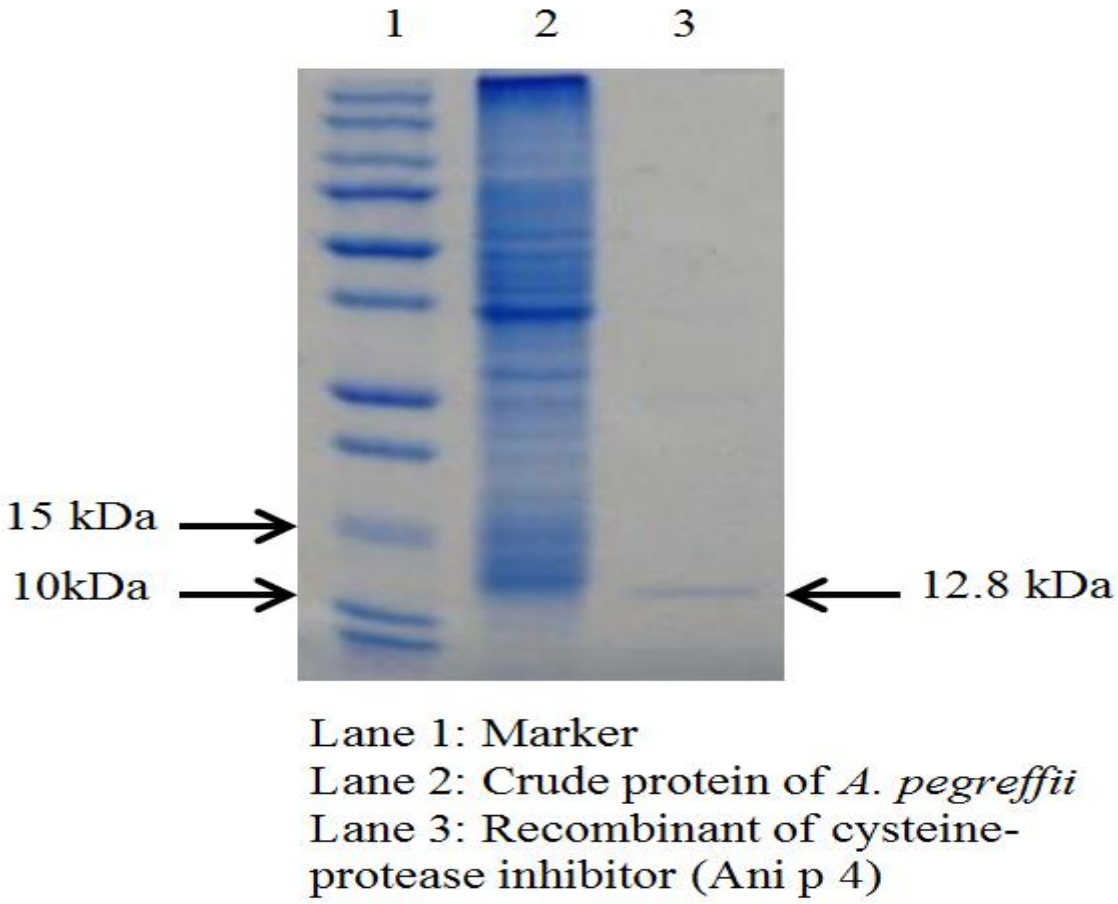


Keys:
L=Lane
From left to right: (L1) a protein ladder of markers, (L2) the expected size of the native tropomyosin protein in raw extract labelled in red box.

Figure 6.8 Identification of the presence of cysteine-protease inhibitor protein from *A. pegreffii*, using SDS PAGE gel stained with Acqua Stain Blue dye.

6.3.9 Comparison of the size of recombinant cysteine-protease inhibitor protein from *A. pegreffii* with cysteine-protease inhibitor of whole protein from *A. pegreffii*.

SDS-PAGE electrophoresis compared the expected size of raw and recombinant cysteine-protease inhibitor protein from *A. pegreffii*. The expected size of the native cysteine-protease inhibitor protein in raw extract (lane 2) and the purified recombinant cysteine-protease inhibitor protein from *A. pegreffii* (lane 3) was 12.8kDa (Figure 6.10).

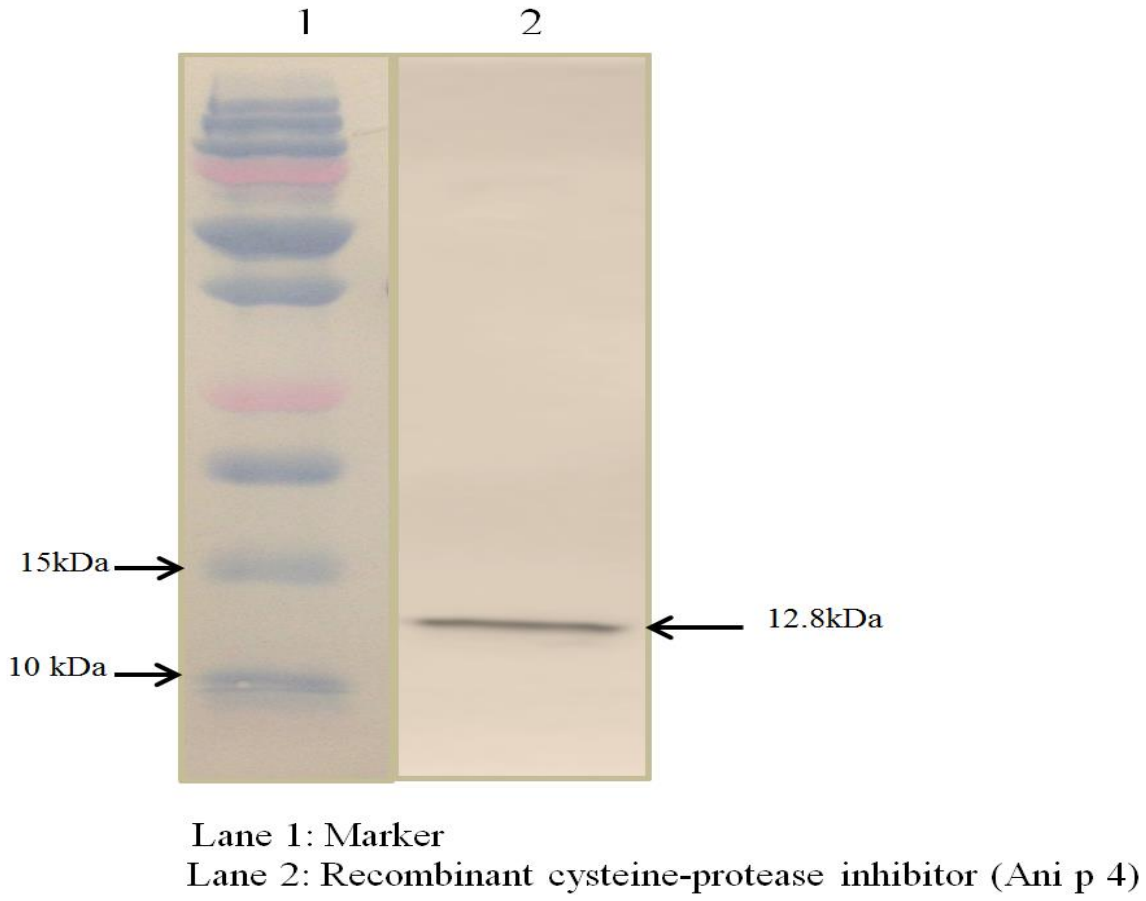


Keys:
L=Lane
From left to right: (L1) a protein ladder of markers, (L2) the expected size of native cysteine-protease inhibitor protein in raw extract, (L3) the purified recombinant cysteine-protease inhibitor protein from *A. pegreffii* at 12.8 kDa.

Figure 6.9 SDS-PAGE electrophoresis of the raw protein and recombinant cysteine-protease inhibitor protein from *A. pegreffii*.

6.3.10 Immunoblotting profile using anti-polyHistidine monoclonal antibody showing the recombinant cysteine-protease inhibitor.

Immunoblotting profile using polyHistidine polyclonal antibody showing that the blots developed a cysteine-protease inhibitor band (12.8kDa) within 5 min of exposure (Figure 6.11). This confirms the presence of the recombinant His-tagged protein.

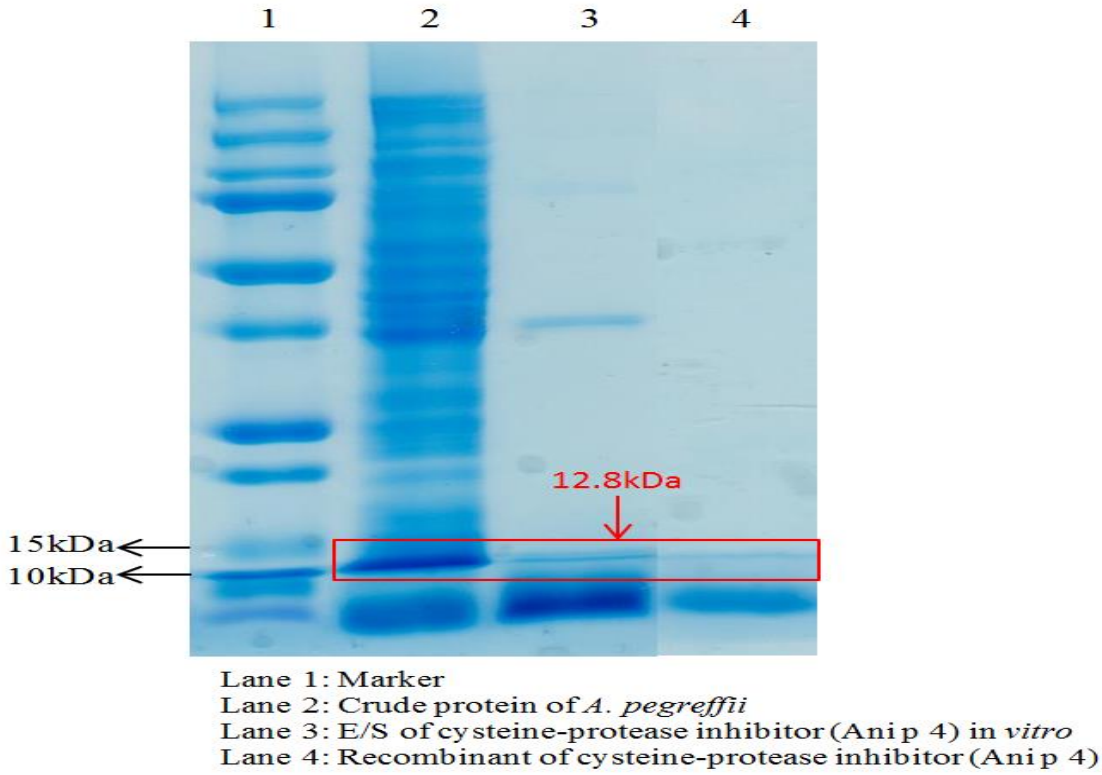


Keys:
L=Lane
From left to right: (L1) a protein ladder of markers, (L2) showing the developed cysteine-protease inhibitor band (12.8 kDa) within 5 min of exposure.

Figure 6.10 Immunoblotting profile using anti-polyHistidine polyclonal antibody.

6.3.11 Comparison of the size of recombinant cysteine-protease inhibitor protein (CPI) band (12.8 kDa) from *A. pegreffii* with CPI of crude protein from *A. pegreffii* and E/S of cysteine-protease inhibitor (Ani p 4) *in vitro* using SDS-PAGE gel.

SDS-PAGE electrophoresis of crude protein from *A. pegreffii* (lane 1), E/S of cysteine-protease inhibitor (Ani p 4) (lane 2), and recombinant cysteine-protease inhibitor protein (lane 3) showed same size band at (12.8 kDa) and labelled inside red box (Figure 6.12). This shows that there is some apparent low molecular mass contamination of the recombinant protein.



Keys:
L=Lane
From left to right: (L1) a protein ladder of markers, (L2) crude protein from *A. pegreffii*, (lane3) E/S of cysteine-protease inhibitor, (lane 4) recombinant cysteine-protease inhibitor protein (Ani p 4). All putative bands are labelled inside red box at 12.8 kDa.

Figure 6.11 SDS-PAGE electrophoresis crude protein from *A. pegreffii*, E/S of cysteine-protease inhibitor and recombinant cysteine-protease inhibitor protein (Ani p 4).

6.3.12 Nucleic acid sequence and protein sequence of recombinant cysteine-protease inhibitor of *A. pegreffii* (Anip3).

Nucleic acid sequence

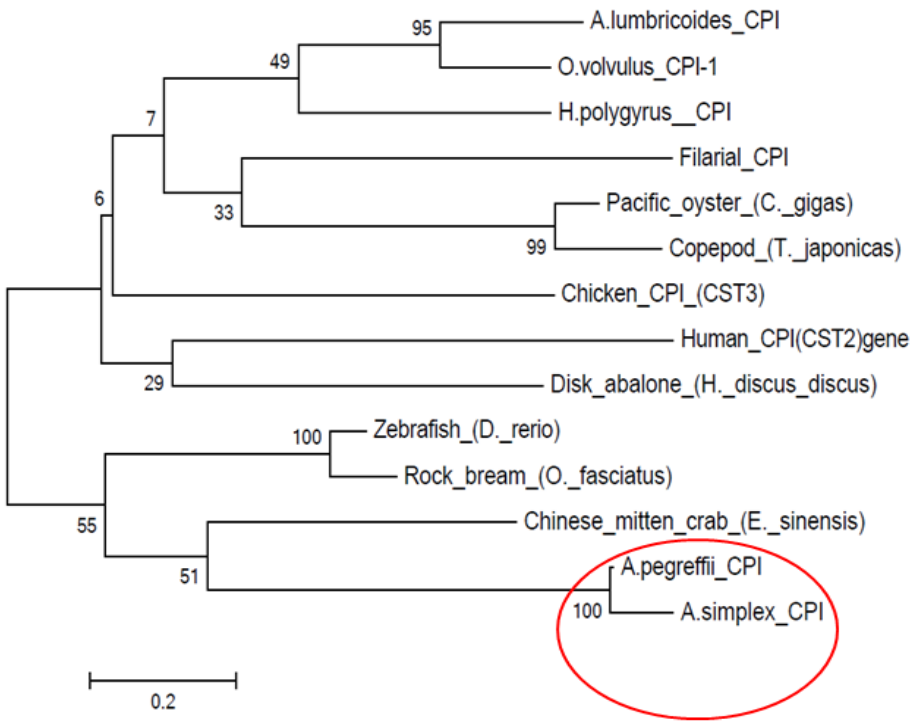
CGGGATCCATGTCCAGAATCGTCGTAGCAGTAGGTGTGGATGATCCGGAGATC
AAGGAGCTCGCTGGTAAGTCTATCGCAAAAATCAGTGCGATGATAAATGATGG
TAAGCCGCATGAACTCGTCAAAGTGGTGTCTGCAAAAAAACAAGTTGTGGCTG
GAGAGAAGTACACTCTTGAAATTCTCGTGAAAGATGGAGATCATCAAGACCTG
TGTACCGTTACCATTTGGCAAAAGAAGTGGGAGAATTTTGAAGAGGTAAAGCC
TCTTACGCTAGTAGTCATTGAGCTCTGA

Predicted Protein sequence

M S R I V V A V G V D D P E I K E L A G K S I A K I S A M I N D G K P H E L V K V
S A K K Q V V A G E K Y T L E I L V K D G D H Q D L C T V T I W Q K K W E N F E
E V K P L T L V V I E L Stop

6.3.13 Phylogenetic analysis using Clustal W programme showing the relationship of *A. pegreffii* cysteine-protease inhibitor with tropomyosin of some parasitic nematodes and living organisms.

Phylogenetic analysis using Clustal W programme showing the relationship of *A. pegreffii* cysteine-protease inhibitor with cysteine-protease inhibitors of some parasitic nematodes and living organisms, *A. simplex, A. lumbricoides, O. volvulus, H. polygyrus*, Filarial CPI, Disk abalone *(H. discus discus),* Pacific Oyster *(C. gigas),* Copepod (*T. japonicas*), Chinese mitten crab *(E. sinensis),* Zebrafish *(D. rerio),* Rock bream (*O. fasciatus*), Human, Chicken. Uniprot Accession IDs, CAK50389, HQ404231, AF177194, KC181863, AJ310669, JQ653304, ADI33157, JN633979, GU002535, JQ287496, NP001096599, HUMINCP1, NM_205500, respectively. Phylogenetic analysis using the Clustal W program revealed that the cysteine-protease inhibitor of *A. pegreffii* has the highest similarity to the cysteine-protease inhibitor from *A. simplex L3* indicating their close relatedness and common ancenstral descent. This is shown in figure 6.18.



This shows the relationship of A. pegreffii cysteine-protease inhibitor with tropomyosin of some parasitic nematodes and living organisms. `

Figure 6.12 Phylogenetic analysis using Clustal W programme.

6.4 Discussion

Proteinase inhibitors secreted by the Helminths are major active proteins involved in various physiological and pathological processes such as avoiding the immune attack of the host and inhibiting the activity of proteinases of both the host and their own (Dzik, 2006). For example; *O. volvulus* cystatin induced the release of TNF-α (an event that usually leads to proinflammatory responses), followed by a downregulation of IL-12 production and massive increase of IL-10 production and a resultant Th2 response (anti-inflammatory) (Schonemeyer *et al.,* 2001). Furthermore, the cysteine-protease inhibitor of *A. simplex* is a major allergic protein and considered a heat-resistant allergen (Rodriguez-Mahillo *et al*., 2007). It is acquired after eating of raw or undercooked fish contaminated with *L3* (Fumarola *et al*., 2009). Therefore, the aim of this present work was to clone, sequence, and obtain a recombinant cysteine-protease inhibitor derived from excretory-secretory (ES) products of *A. pegreffii* L3 that were harbored inside the body of tiger flathead.

During this study, total RNA was initially extracted from the entire bodies of the *L3 A. pegreffii* specimens using RNA Extraction mini kit (chapter five). Electrophoresis of RNA under denaturating condition showed two major rRNA subunit bands (small 18S and the large 28S) which means good quality of purified total RNA from *A. pegreffii* L3 and cDNA synthesis of the selected poly $(A)^+$ mRNA using cDNA Synthesis Kit (chapter five). The cDNA encoding Ani p 4 was amplified by normal PCR with specific primers derived from conserved amino acid sequences and nucleotide sequences of the cysteine-protease inhibitor from *A. simplex* L3 (Ani s 4) in database (Rodriguez-Mahillo *et al*., 2007).

The insert has a length of 339 bp, encoding 113 amino acids with a predicted mass of 12.8 kDa. Sequence similarity searches using the BLAST program revealed that cysteine-protease inhibitor of *A. pegreffii L3* has the highest identity to cysteine-protease inhibitor isoform from *A. simplex L3* (98%) and *A. lumbricoides* (84%), respectively (Asturias *et al*., 2000). The deduced amino acid sequences of cysteine-protease inhibitor of *A. pegreffii* L3 have revealed the cysteine-protease inhibitor has highest similarity with cysteine-protease inhibitor of *A. simplex* L3. Therefore, it agrees with previous data (Rodriguez-Mahillo *et al*., 2007).

Using SDS-PAGE electrophoresis and immunoblotting methods, the size of recombinant protein of cysteine-protease inhibitor from *A. pegreffii* L3 was detected and compared with crude protein from *A. pegreffii*. Therefore, the results showed a recombinant cysteine-protease inhibitor band at the expected size of 12.8 kDa (Figure 6.11 and 6.12). This is in agreement with the result obtained by Rodriguez-Mahillo *et al.* (2007). Furthermore, during this study, the size of E/S product of the cysteine-protease inhibitor from *A. pegreffii* was compared with the expected band of crude protein from *A. pegreffii* and recombinant cysteine-protease inhibitor protein (Ani p 4) using SDS-PAGE methods. There was a protein in the E/S product at the expected band size of 12.8 kDa, however it cannot be concluded that this is the inhibitor. Further study is being conducted to confirm if this protein is a cysteine inhibitor in the crude extract. This supposed protein in ES extract is shown in figure 6.12 in the labelled red box at 12.8 kDa.

Phylogenetic analysis using the Clustal W program revealed that the cysteine-protease inhibitor of *A. pegreffii* has the highest similarity to cysteine-protease inhibitor from *A. simplex L3* 6.18, while it showed less similarity to the cysteine-protease inhibitor from other nematodes and living organisms (humans and chickens). These results support the idea of the cysteine-protease inhibitor as a major allergen as to the cysteine-protease inhibitor from *A. simplex*. However, the clinical and allergological implications of the high similarity found between them remains to be investigated.

Allergenic cross-reactivity is probably due to the presence of highly conserved IgE-binding epitopes on invertebrate allergic proteins. Therefore, the investigation of segments of sequences conserved in the cysteine-protease inhibitor may have significant clinical implications and detection of specific IgE against helminth cysteine-protease inhibitors is a more precise diagnostic method for anisakiasis and anisakis allergy.

During this study, the highly conserved central QxVxG motif as QVVAG sequence was found in the amino acid sequence of the cysteine-protease inhibitor of *Anisakis pegreffii* (Table 6.9). The similarity of these sequences was observed between helminths, crustacean and mollusca and compared to other living organisms (Table 6.9). These conserved sequences are responsible for the binding to the catalytic site of papain-like cysteine proteases and accordingly the inhibition of proteolytic activity (Ni *et al*., 1997, Abrahamson *et al*., 2003b). Furthermore, this epitope sequence of cysteine-protease inhibitors suggests that there may be a possibility of high cross-reactivity among helminths, crustacean and

mollusca of different species (Table 6.9), and therefore may elicit hypersensitivity reactions in atopic individuals.

Table 6.7 Segments of sequences conserved in the cysteine-protease inhibitors of Helminths and living organisms

cysteine-proteinase inhibitor (CPI) species	Segments of sequences conserved in cysteine-protease inhibitors	References
A. pegreffii CPI	Q V V A G	This study
A. simplex CPI	Q V V A G	Rodriguez-Mahillo *et al*., 2007
A. lumbricoides CPI	Q V V A G	Mei *et al*., 2014
H. polygyrus CPI	Q V V A G	Sun *et al*., 2012
Filarial CPI	Q V V A G	Manoury *et al*., 2001
O. volvulus CPI	Q V V A G	Cho-Ngwa *et al*., 2010
Disk abalone (*Haliotis discus discus*)	Q V V A G	Premachandra *et al*., 2012
Pacific Oyster (*Crassostrea gigas*)	Q V V A G	Goetz and Roberts, 2008
Copepod (*Tigriopus japonicas*)	Q V V A G	Kim *et al*., 2011
Chinese mitten crab (*Eriocheir sinensis*)	Q V V S G	Fengmei *et al*., 2010
Zebrafish (*Danio rerio*)	Q L V A G	Premachandra *et al*., 2012
Rock bream (*Oplegnathus fasciatus*)	Q I V S G	Premachandra *et al*., 2012
Chicken CPI	Q V E I G	Turk *et al.*, 1983
Human CPI	Q A V A L	Saitoh *et al.*, 1985

Chapter seven: General Conclusion

Anisakid worms have a world-wide distribution (Huss and Embarek, 2004), and are found in 200 fish species and 25 cephalopod species (Abollo *et al*., 2001, Klimpel *et al*., 2004). The majority of human anisakiasis cases for which molecular species identification has been carried out are caused by infection by *A. simplex* and *A. pegreffii*, after eating raw or undercooked fish or cephalopods contaminated with larvae three (L3) of *A. simplex* and *A. pegreffii* (D'Amelio *et al*., 1999; Mattiucci *et al*., 2007, Mattiucci *et al*., 2011; Umehara *et al*., 2007; Fumarola *et al*., 2009; Arizono *et al*., 2012). In Australia, there have been several studies on the distribution (prevalence and/or number) of anisakids in fish sourced from the Northern Territory, Queensland, Western Australia, South Australia, Victoria and Tasmania (Shamsi, 2014). As indicated in Table 2, various species of anisakid nematodes *Anisakis, Terranova, Thynnascaris, Contracaecum, Hysterothylacium* and *Raphidascaris*) have been detected in fish species caught in Australian waters (Shamsi *et al*., 2014).

Although Anisakis ssp. appear to be differences in host fish preference, diverse Anisakid species may be found in the same intermediate host species, and sometimes in the same individual intermediate host (McClelland, 2002, Stobo *et al*., 2002, Mattiucci *et al*., 2005). For example, tiger flathead (*Neoplatycephalus richardsoni*) has been infected with larval *A. pegreffii, Contracaecum* larval type II and *Hysterothylacium* larval type IV (Shamsi *et al*., 2010). Moreover, silver whiting (*Sillago flindersi*) has been also infected with *A. pegreffii, Hysterothylacium* larval type IV and *Hysterothylacium* larval type VIII (Jabbar *et al*., 2012). Intensity of infection may be generally positively related to host species, age and size (McClelland, 2002). Therefore, this study was undertaken to classify the species or genotype of single Anisakidae larvae, which have life cycles involving fish and marine mammals, and to identify the major allergens in *A. pegreffii* and compare these allergens to the known allergens in fish parasites and shellfish that may be candidates for *Anisakis* allergy after consumption of fish contaminated with L3.

As it mention earlier, the most infested fish have been frequently found to harbour one or more types of Anisakid L3. Molecular biological techniques allow rapid and accurate diagnosis and identification of collected samples. In this study, fish (tiger flathead) collected from Victoria lakes were shown to be highly infected with *Anisakis* type I larva using morphological examinations and genetic analysis, and *A. pegreffii* was confirmed using SSCP analysis of the ITS-1 and ITS-2 genes. Indeed, it is the main species found in tiger flathead, in agreement with Shamsi *et al*. (2011b), who collected them from southern Australian waters. ITS-1 and ITS-2 sequences of *A. pegreffii* from tiger flathead showed

98% similarity with ITS-1 and ITS-2 sequences of *A. pegreffii* from two allergic fish species (marinated anchovies and Chub mackerel). In conclusion, this result showed the genetic identification of *A. pegreffii* in different fish species. It may also open the door to a new means of studying the probability of the host (tiger flathead) causing human Anisakiasis. In addition, ITS-1 and ITS-2 sequences of *A. pegreffii* were shown to have 95% similarity with *A. simplex s.s.*, which is also the major agent responsible for the highest number of recorded human anisakiasis and allergic reactions. In agreement with another study (Mattiucci *et al*., 2008a) the identity of sequences among the three species is that they belong to *Anisakis* type I. This finding confirms that presence of larvae of *A. pegreffii* in tiger flathead that gentetically are very close to *A. simplex s.s.*

To identify *A. pegreffii* antigens responsible for allergic reactions in tiger flathead or that can induce a strong humoral immune response, immunological tools were used to analyze the immune response to tropomyosin. Tropomyosin protein from *A. pegreffii* was confirmed by immunoblotting with the anti-tropomyosin crustacean polyclonal antibodies (PAbs) and showed a strong band at 44kDa mark, higher than crustacean tropomyosins bands at 37 kDa. Similarly, tropomyosin from *A. pegreffii* was recognized using anti-crustacean polyclonal antibodies (PAbs). In contrast, tropomyosin of *A. pegreffii* was not recognized by immunoblotting with anti- crustacean tropomyosin monoclonal antibodies (MAbs). The results of immunoblotting suggested that both tested nematode tropomyosins share one or more epitopes with crustacean tropomyosin, as the PAbs recognise multiple epitope on any one antigen while they both lack an antigenic determinant, defined by the anti-tropomyosin monoclonal antibody, which is present in crustacean tropomyosin. The size of *A. pegreffii* tropomyosin is in agreeance with previous studies on *A. simplex* and *A. lumbricoides* tropomyosin, which are known to be 41kDa and 40kDa, respectively (Acevedo *et al*., 2009, Asturias *et al*., 2000) and higher than tropomyosin from crustaceans, Cockroach (*Periplaneta americana*) and Fruitfly (*Drosophila melanogaster*) which are known to be 38kDa (Leung *et al*., 1996). The result demonstrated that size of *A. pegreffii* tropomyosin is similar with previous *A. simplex* and *A. lumbricoides* tropomyosin, as may be expected by the close relationship between these species.

Previous studies have classified the adult species using morphological characters. During this study has employed both morphological characters and molecular techniques, since larval stages of anisakid nematodes cannot be identified specifically using only morphological characters. The data obtained from this study indicated that three species of

larvae of anisakid nematodes have been identified from more than 107 species of teleost fishes in Australian waters, Lizard Island; Anisakis, Terranova and Hysterothylacium. Some of these commercial species have a high prevalence and concentration of Anisakis and Terranova such as scombrids and sphyraenids. Therefore, our results agreed with previously studying (Cannon, 1977). This finding reveals that presence of larvae of Anisakis nematodes species which can potentially cause human Anisakisis.

In addition, during this study, bioinformatics tools were used to identify genes encoding proteins that are fully or partially intercellular or extracellular antigens (somatic and excretory/secretory (ES)) in *A. pegreffii*, the latter which may induce a humoral immune response in the host and/or be responsible for the remarkable avoidance of the host immune system.. Throughout this study, recombinant tropomyosin and recombinant cysteine-protease inhibitor proteins have been investigated as major allergic proteins of *A. pegreffii*. Primers were designed to amplify, using cDNA, the tropomyosin gene present in the third stage larvae of *A. pegreffii.* The primary sequence of tropomyosin from *A. pegreffii* was used, with cDNA, to produce a recombinant tropomyosin called Ani p 3. The tropomyosin protein from *A. pegreffii* showed similar size with the tropomyosin protein from *A. simplex* (Acevedo *et al*., 2009). This finding confirms the presence of the tropomyosin gene in *A. pegreffii*.

Recombinant tropomyosin of *A. pegreffii* (Ani p 3) showed a strong band at 44 kDa by immunoblotting with anti-tropomyosin and anti-crustacean polyclonal antibodies. On the other hand, it was not detected by blotting with an anti-tropomyosin monoclonal antibody (MAbs). The data obtained from this study also indicated that the rAni p 3 binds to IgE from different serum samples of crustacean allergic patients. This is agreement with several studies that reported the reaction of serum from allergic patients with tropomyosin of *A. simplex* (Acevedo and Caraballo, 2011). This finding confirms cross reactivity between nematodes and different classes of shellfish.

Previous studies had demonstrated the function of tropomyosin protein to be associated with motility. In the current study, it is evident that the role of the tropomyosin proteins can be further inferred by the localisation of tropomyosin in the body of *A. pegreffii*. Immunohistochemistry showed Ani p 3 is located in both the muscles and under the basal layer of the cuticle in the L3 anatomy. This is supported by previous studies showing that

tropomyosin localization was established using immunocytochemistry in the muscle of larval parasite (MacGregor and Shore, 1990; Jenkins *et al*., 1998).

Results obtained from this study demonstrated that rAni p 3 retained the allergenic characteristics of nAni p 3 using IgE binding capacity assessed by immunoblot, and can therefore be used in immunodiagnosis of human anisakiasis. Bioinformatics analyses and information obtained from the phylogenetic tree validated a cross-reactivity pattern between Ani p 3 allergen with tropomyosin of some parasitic nematodes, shellfish and living organisms' allergens. In conclusion, recombinant and native tropomyosin protein present in an extract of the third stage larvae of *A. pegreffii* have been identified as a major allergen in Anisakidae.

In addition, a cysteine-protease inhibitor of *A. pegreffii* was also identified during this study. Functionally, it's known as an excretory/secretory (ES) allergic protein secreted into the extracellular environment or into the host tissues to inhibit proteases (Rodriguez-Mahillo *et al*., 2007). Bioinformatics tools were also used to analyze the complete sequence of cysteine-protease inhibitor of *A. pegreffii* as an allergic protein. Primers were designed to amplify, using cDNA, the cysteine-protease inhibitor gene present in the third stage larvae of *A. pegreffii*. The primary sequence of cysteine-protease inhibitor from *A. pegreffii* was amplified from cDNA, to produce a recombinant cysteine-protease inhibitor called Ani p 4. This finding confirms that the presence of a cysteine protease inhibitor gene of *A. pegreffii* using suitable design primers.

The results of expression and purification of the recombinant cysteine-protease inhibitor (Ani p 4) showed a band at 12.8 kDa by SDS- PAGE and immunoblotting with the anti-polyHistidine antibodies (PAbs). Therefore, these results indicate that recombinant cysteine-protease inhibitor from *A. pegreffii* has the same size as recombinant cysteine-protease inhibitor (Ani s 4) from *A. simplex* (Rodriguez-Mahillo *et al*., 2007).

The gel electrophoresis results indicated that a protein of approximately the same size was present in E/S product from *A. pegreffii*. There was a protein in the E/S product at the expected band size of 12.8 kDa as demonstrated in the crude extract and recombinant protein and most likely is the Ani p 4 protein; thus further study is being conducted to confirm if this protein is a cysteine inhibitor (Ani p 4) in the E/S material.

A phylogenetic study between the Ani p 4 allergen with cysteine-protease inhibitors of some parasitic nematodes, shellfish and living organisms was conducted. Based on the phylogenetic tree rAni p 4 is close to nAni s 4, which is considered as a major stable allergic protein from *A. simplex s.s.* and causes Anisakiasis and allergic reactions (Rodriguez-Mahillo *et al*., 2007). In conclusion, cysteine-protease inhibitor protein present in an extract of the third stage larvae of *A. pegreffii* has same similar characteristics of a cysteine-protease inhibitor protein of *A. simplex s.s.*

Due to the time constraints it was not possible to undertake bioinformatics studies on all potential proteins from *A. pegreffii*. However, the results presented in this study have provided a foundation for the future work related to bioinformatics studies, recombinant protein production and diagnostic testing.

Future perspectives

The studies that have been reported in this thesis have added new knowledge by employing bioinformatics to classify the species or genotype of single Anisakidae larvae, and identify major two allergens in *A. pegreffii*; tropomyosin and cysteine-protease inhibitor. Further studies are required to measure the functional effects of the inhibition of the cysteine protease activity on cathepsin L and cathepsin B. These studies might contribute to the identification of new drug targets, the development of antiparasite and antidisease vaccines. Moreover, investigate the cysteine protease inhibitor function may allow a study of immunomodulatory effects on dendritic cell (DC) function and immune responses. These results will be helpful to develop future immunotherapy and desensitization of individuals allergic to Anisakidae.

One future study would be to determine any reactivity of allergic patient's serum specific IgE to r Ani p 4 using the immunoblotting test. These results will facilitate the design of synthetic peptides corresponding to the B cell and T cell epitopes which would aid in developing accurate diagnosis, future immunotherapy and desensitization of individuals allergic to anisakidae. In addition, it would support our understanding of the relationship of protein allergenicity and cross reactivity while also improving diagnostic and immunotherapeutic use.

Another study should be to determine the localisation of Ani p 4 by immunohistochemical methods, using a polyclonal anti-Ani s 4 antiserum. It may be that the allergen is found in both the secretory gland and the basal layer of the cuticle of *A. simplex* L3.

In addition, each class of protease has several physicochemical properties (e.g. autolytic activity, optimum pH, sensitivity to specific inhibitors). Therefore, evaluations of effect of pH and temperature on purified r Ani p 4 activity and stability should be further examined at the enzymatic properties level by protease and proteolytic activity methods.

Appendix

General materials and methods

The materials and methods explained here relate to general laboratory procedures. The methods for Bioinformatics analyses and specific procedures can be found in the relevant chapter, and are not detailed here.

1.1 General Procedures used prior to Experiments

All glassware were placed in a warm concentrated aqueous solution of Pyroneg detergent (Diverey Pty Ltd, Melbourne, Australia), and soaked for several minutes. Glassware was washed thoroughly with tap water and given a final rinse with deionized water before use. The chemicals and reagents used in the experiments were of analytical laboratory grade. All solutions were prepared using deionised water and were obtained by filtration through a Millipore Milli-Q-water System (Liquipure, Melbourne, Australia). Sterilisation of media, reagents, glasswares, and pipette tips was performed by autoclaving at standard conditions (121^0C for 15 mins) unless specified. Micro pipettes were used to accurately measure and dispense small volumes of liquid. All solutions were dispensed using Finnpipette (Pathtech, Australia) for all volumes ranging from less than 0.5 µ l to 1000µ l and these were calibrated regularly as per manufactures recommendations.

2.1 Materials

Reagent and equipments used are detailed here.

2.1.1 Reagent:

Reagent	Supplier
Acetic acid, glacial	Sigma-Aldrich Pty. Ltd., MO, USA
Acetone	Sigma-Aldrich Pty. Ltd., MO, USA
Acrylamide, 40% (W/V) solution	Bio-Rad Laboratories, USA
Agarose, Bacteriological	Neogen, USA
Agarose (DNA grade)	Bioline, Australia
Albumin, bovine serum	Sigma-Aldrich Pty. Ltd., MO, USA
Alkaline phosphatase (calf intestinal)	Sigma-Aldrich Pty. Ltd., MO, USA
Ammonium acetate	Ajax Chemicals Ltd, Australia
Ammonium chloride	BDH Chemicals, Australia

Ammonium hydroxide	Sigma-Aldrich Pty. Ltd., MO, USA
Ammonium persulfate	Bio-Rad Laboratories, USA
Ammonium sulphate	Sigma-Aldrich Pty. Ltd., MO, USA
Ampicillin	Bioline, Australia
ATP (Adenosine Triphosphate)	Sigma
Bacteriological tryptone	Neogen, USA
Bis-acrylamide (N,N'-methylene-bis-acrylamide)	BDH Chemicals, Australia
Brilliant Blue G	Sigma-Aldrich Pty. Ltd., MO, USA
Bromo-phenol blue	Sigma-Aldrich Pty. Ltd., MO, USA
Chelating Sepharose (resin)	GE Healthcare
Chemiluminescent Peroxidise Substrate-3	Sigma-Aldrich Pty. Ltd., MO, USA
Chloroform	Ajax Chemicals Ltd, Australia
Cholesterol	ICN Pharmaceuticals, Australia
Citric acid (hydrate)	BDH Chemicals, Australia
Columbia agar base	Neogen, USA
Columbia Broth	Neogen, USA
Coomassie brilliant blue R-250	Bio-Rad Laboratories, USA
Copper sulphate	Merck, Australia
Cover slips	Mediglass, Australia
Cryovials (1.8 ml)	Nalgene Company, USA
Deoxynucleoside triphosphates (dNTPs)	Sigma-Aldrich Pty. Ltd., MO, USA
Dimethylformamide	BDH Chemicals, Australia
Dimethylsulphoxide (DMSO)	Merck, Australia
DNA Ligase (T4)	New England Biolab, Australia
DNase I (bovine pancreas, grade I)	Boehringer Mannheim, Germany
DNA Polymerase	Promega, Australia
AmpliTaq	Bioline, Australia
Dulbecco's Modified Eagle's Medium (DMEM)	Thermofisher, Australia
Ethanol	Merck, Australia
Ethidium bromide	Sigma-Aldrich Pty. Ltd., MO, USA
EDDA (Ethylene diamine-N,N'-di acetic acid)	ICN Pharmaceuticals, Australia
EDTA (Ethylene diamine tetra acetic acid)	Merck, Australia
Ficoll-400 Reagent	Merck, Australia

Folin-Ciocalteau reagent	Sigma-Aldrich Pty. Ltd., MO, USA
Gene Clean Kit	Bio101, Australia
Glycerol	Merck, Australia
Glycine	BDH Chemicals, Australia
Glucose	Merck, Australia
HEPES buffer (1 M) (N-2-hydroxyethyl piperazine-N-2-ethane sulfonic acid)	Thermofisher, Australia
Hydrochloric acid (32 %)	BDH Chemicals, Australia
Hydrogen peroxide (30 %)	BDH Chemicals, Australia
Immobilised Metal Affinity Chromatography Column (IMAC)	QIAGEN
IPTG (Isopropylthio-β-D-galactosidase)	Bioline, Australia
Isoamyl alcohol	BDH Chemicals, Australia
ISOLATE II Plasmid Mini Kit	Bioline, Australia
ISOLATE II PCR and Gel Kit	Bioline, Australia
ISOLATE II RNA Mini Kit	Bioline, Australia
Isopropanol	Merck, Australia
Kodak GBX developer replenisher	Sigma-Aldrich Pty., Ltd., USA
Kodak GBX fixer replenisher	Sigma-Aldrich Pty., Ltd., USA
Lambda DNA	New England Biolab, Australia
Low-molecular-weight Calibration Kit (for SDS electrophoresis)	Amersham Pharmacia Biotech.
Low-molecular-weight pUC19/Hpa II marker (for DNA electrophoresis)	Progen Industries Ltd., Australia
Lysozyme	Boehringer Mannheim, Germany
Magnesium chloride (Hexahydrate)	BDH Chemicals, Australia
β-Mercaptoethanol	Bio-Rad Laboratories, USA
Methanol	BDH Chemicals, Australia
Microaerobic gas mixture	Linde Gas, Australia
Microscope slides	LOMB Scientific Co., Australia
Microtitre plate (96 wells, flat bottom; v-bottom)	Nunc, Denmark
Needle (sterile 18G, 19G, 21G)	Terumo Pty, Ltd., Australia
Newborn Calf Serum (NCS)	Thermofisher, Australia
Nickal Sulfate	BDH Chemicals, Australia

Nitrocellulose membrane (Hybond-C)	Amersham, USA
Nitrophenyl phosphoryl choline (NPPC)	Sigma-Aldrich Pty., Ltd., USA
Nutrient broth No. 2	Oxoid Australia Pty. Limited
Nylon membrane (Hybond-N)	Amersham, USA
Paraffin	BDH Chemicals, Australia
Petri dish	Nunc, Denmark
Phenol/chloroform	BDH Chemicals, Australia
Phenylmethylsulfonyl fluoride (PMSF)	Sigma chemicals, Co., Australia
Phosphate buffer saline (PBS) tablets	Oxoid limited, England
Potassium chloride	BDH Chemicals, Australia
Pre-stained Protein Molecular Weight Marker	Bio-Rad Laboratories, USA
Protein standards (SDS gel marker)	Bio-Rad Laboratories, USA
Proteinase K	Promega, Australia
Restriction enzymes	Promega, Australia
RNase	Boehringer Mannheim, Germany
Skim milk	Coles, Australia
Skirrow Supplements (SR069E)	Oxoid Australia Pty. Limited
Sodium acetate	BDH Chemicals, Australia
Sodium bicarbonate (7.5 %)	Cytosystems Pty Ltd, Australia
Sodium chloride	BDH Chemicals, Australia
Sodium citrate (dihydrate)	BDH Chemicals, Australia
Sodium dodecyl sulfate (SDS)	Merck, Australia
di-Sodium hydrogen orthophosphate (anhydrous)	BDH Chemicals, Australia
Sodium hydroxide (pellets, AR)	BDH Chemicals, Australia
TEMED (N,N,N',N'-tetramethylethylenediamine)	Bio-Rad Laboratories, USA
Tetro cDNA Synthesis Kit	Bioline, Australia
Tissue culture flask (25 cm^2, 75 cm^2)	Nunc, Denmark
TOPO TA cloning Kit	Invitrogen
Tris-base (Tris (hydroxymethyl) amino methane)	Boehringer Mannheim, Germany
Tris-HCl	Boehringer Mannheim, Germany
Triton-X-100	Sigma-Aldrich Pty., Ltd, USA
trypan blue	Sigma-Aldrich Pty., Ltd, USA
Trypsin – EDTA	Trace Biosciences, Australia
Tryptone	Neogen, USA
Tween-20	BDH Chemicals, Australia

Unstained Protein Molecular Weight Marker	Bio-Rad Laboratories, USA
Whatman blotting paper	Whatman, England
Wizard® Genomic DNA Purification Kit	Promega, WI, USA
X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside)	Bioline, Australia
X-ray film, Amersham Hyperfilm ECL	GE Healthcare, UK
Yeast extract	Neogen, USA

Figure 3.1: 2.2.2 General Equipment

Equipment	**Supplier**
Anaerobic jars	Oxoid Australia Pty. Limited
Anaerobic Jars (9 & 48 plates)	Don Whitely Scientific, UK
Balance:	
(i) Analytical balance	Sartorius GMBH, Germany
(ii) Balance (0.1-500 g)	U-Lab, Australia
Camera (135 mm Polaroid MP4 Land Camera)	Polaroid, USA
Cell counting chamber (Neubauer, double ruled)	Propper MFG Co., USA
Cellulose Acetate Filter (0.65 µm)	Sartorius GMBH, Germany
Centrifuge:	
(i) Microcentrifuge (EBA12)	Zentrifugen, Germany
(ii) Bench top centrifuge (Centaur 2)	Graykon Scientific
(iii) High-speed centrifuge (L2-21 M/E)	Beckman, USA
(iv) Ultra-speed centrifuge (L8-80M)	Beckman, USA
Centrifuge tubes:	
(i) 1.5 ml Eppendorf centrifuge tubes	Sarstedt, Germany
(ii) 10 ml centrifuge tubes	Greiner Labortechnik, Germany
(iii) 50 ml centrifuge tubes	Greiner Labortechnik, German
DNA Thermocycler (for PCR)	G-Storm, England
Electrophoresis Power Supply:	
(i) PowerPac Basic	Bio-Rad Laboratories, USA
(ii) EPS 3000xi	Bio-Rad Laboratories, USA
Electrophoresis Units:	
(i) DNA	
(a) Mini gel	Bio-rad Laboratories, USA
(b) Midi gel (wide mini-sub cell GT)	Bio-rad Laboratories, USA

(ii) Protein

(a) Mini Protean II gel system Bio-Rad Laboratories, USA

(b) Maxi Protean gel system Bio-Rad Laboratories, USA

Eswab COPAN, Italy

Film:

(i) Polaroid 665 Polaroid, USA

(Black and white, positive-negative instant film)

(ii) Polaroid 667 Polaroid, USA

(Black and white, positive-negative instant film)

Filters:

Syringe Filters (0.22µm, 0.45µm) Gelman Sciences, USA

Ultrafiltration-unit filters (XM, YM) Amicon, USA

GELDOC system Bio-Rad Laboratories, USA

Incubators

(i) Bellsouth 100 still air incubator Bellsouth, USA

(ii) Tissue culture (5 % CO2) Forma Scientific, USA

Microscopes:

(i) Light microscope Olympus Optical Co., Japan

(ii) Phase contrast microscope Nikon Kogaku KK, Japan

pH meter Metrohm, Swiss

Sonicator Branson Sonic Power Co., USA

Syringe (1 ml, 5 ml, 10 ml, 20 ml, 50 ml) Terumo Pty, Ltd., Australia

Trans-blot electrophoretic transfer cell Bio-Rad Laboratories, USA

Transilluminator (Novaline UV) Novex Australia Pty Ltd

Ultrafiltration unit Amicon, USA

Vortex mixer (V ml) Ratek Instruments, Australia

3.1 Methods

Chemicals and stock solutions used are detailed here

3.3.1DNA Methods (Purification and Stock)

3.3.1.1 DNA extraction from tissue

Genomic DNA was extracted from Larvae three using the kits listed here have consistently provided high-quality results.

3.3.1.1.1 Agrose Gel Electrophoresis

(i) 0.5 M EDTA pH 8.0 (1 L)

186.1 G Na2 edta.2H2O

800 ml Milli-Q water

Adjust pH of to 8 with NaOH.

Bring volume to 1 L with Milli-Q water and autoclave

(ii) 50X TAE Running Buffer

242 gm Tris base

100 ml 0.5M EDTA pH 8

57.1 ml Glacial acetic acid

900 ml Milli-Q water

Adjust pH to 8.5 with glacial acetic acid

Bring to final volume to 1 L with Milli-Q water

To prepare 1L of 1X TAE, measure 100 ml of 10X TAE and make it up to 1L with 900 ml Milli-Q water

(iii) 1% agarose gel electrophoresis

1 gm agrose

100 ml 1X TAE buffer

Melt in microwave for 2 min

(iv) 5X loading dye for agarose gels

50 ml 50% glycerol

10 ml 0.5 M EDTA pH 8

5 ml 1M Tris pH 7.5

Add 35 ml Milli-Q water to total 100 ml

3.3.2 Protein Methods

3.3.2.1 SDS-PAGE Stock Solutions

(i) 2M Tris-HCI (pH=8.8), 100ml

24.2gm Tris (hydroxymethy1) aminomethane

50ml Milli-Q water

Adjust pH of to 8.8 with HCL, and bring up the volume to 100 ml with Milli-Q water

(ii) 1M Tris-HCI (pH=6.8), 100ml

12.1gm Tris (hydroxymethy1) aminomethane

50ml Milli-Q water

Adjust pH of to 6.8 with HCL, and bring up the volume to 100 ml with Milli-Q water

(iii) 10% SDS (W/V), 100 ml

1gm Sodium Dodeyl Sulfate (SDS)

Milli-Q water to total volume of 100 ml

(iv) 50% Glycerol (v/v), 100 ml

50ml 100% Glycerol

50ml Milli-Q water

(v) 10X Gel Electrophoresis Running Buffer (2 Litter)

60 gm Tris base

288 gm Glycine

20 gm SDS

1.6 L Milli-Q water

Adjust pH of to 8.3 with HCL, and bring up the volume to 2 L with Milli-Q water

(vi) 1% Bromophenol Blue (w/v), 10ml

100 mg Bromophenol Blue

Milli-Q water to total volume of 10 ml

3.3.2.2 SDS-PAGE work Solutions

(i) **10 % Ammonium Presulphate, (10% APS)**

50µg Ammonium Presulphate

500µl Milli-Q water

(ii) **1X Gel Electrophoresis Running Buffer**

100 ml 10X Gel Electrophoresis Running Buffer

900 ml Milli-Q water

(iii) **5X Sample Buffer**

0.6 ml 1M Tris (pH 6.8)

5 ml 50% Glycerol

2 ml 10% SDS

0.5 ml 2-mercaptoethanol

0.5 ml 1% Bromophenol Blue

0.9 ml Milli-Q water

3.3.2.3 SDS-PAGE (1 gel)

(i) 12% Resolving gel

3.13ml of 30% Acrylamide stock

1.88ml of 1.5M Tris-HCL pH 8.8

0.08ml of 10%SDS

2.38ml of MG water

37.5µl of 10% w/v APS

2.5µl of TEMED

(ii) 5% Stacking gel

0.33 ml of 30% Acrylamide stock

0.63 ml of 0.5 M Tris-HCL pH 6.8

0.03 ml of 10%SDS

1.50 ml of MG water

12.5µl of 10% w/v APS

1.25µl of TEMED

3.3.2.4 Processing with gel after running

(i) Coomassie Staining Solution (1 gel)

50 mg Coomassie Staining blue R220

10 ml 100% Methanol

5 ml 100% Acetic acid

35 ml Milli-Q water

(ii) Distaining Solution (1 L)

500 ml Methanol

300 ml Milli-Q water

100 ml of Acetic acid

Adjust the total volume to 1000 ml with Milli-Q water

(iii) Gel Storage Solution (5% Acetic acid)

5 ml of Acetic acid

95 ml Milli-Q water

3.3.2.5 Processing with gel for immunoblotting

(i) Membrane blocking Buffer (5% skim milk), 100 m

5g skim milk

100 ml TBS 0.5 Tween

(ii) Antibodies dilution buffer, 100 ml

1% skim milk TBS 0.5 Tween (1g skim milk + 100ml TBS 0.5 Tween) or

1% skim milk PBS 0.5 Tween (1g skim milk + 100ml TBS 0.5 Tween)

3.3.3 Cloning Methods (expression and purification)

(i) TA Cloning kit (Invitrogen)

The kit contains: 10X Ligation buffer, pCR®2.1 vector (20 ng/µl), and T4 DNA Ligase (4.0 Weiss units), stored at -20°C.

(ii) pRSET-A vector

pRSET-A vector (Invitrogen) in DH5α *E. coli*; stored at -80°C in 40% glycerol.

(iii) Plasmid Mini Kit (Bioline)

The kit contains: Resuspension Buffer, Lysis Buffer P, Neutralization Buffer, Spin Column P, 2 ml Collection Tube, Wash Buffer AP, Wash Buffer BP, 1.5 ml Elution Tube, and Elution Buffer; stored at RT.

(iv) Gel Extraction Kit

QIAquick gel extraction kit (Qiagen)

The kit contains: Buffer QG, Buffer EB, QIAquick spin column; stored at RT.

(v) Lysis Buffer

25mM Tris-HCL (pH8.0), 300mM NaCL, 1mg/ml or 2mg/ml lysozyme (AMERSCO) (freshly prepared), 1mM or 2mM imidazole; stored at RT.

(vi) Chelating Sepharose (resin)

Chelating sepharose fast flow containing 70% ethanol (GE Healthcare); stored at RT.

(vii) Immobilised Metal Affinity Chromatography Column (IMAC)

1ml polypropylene IMAC column (QIAGEN); stored at RT.

(viii) IMAC Equilibration Wash Buffer

25mM Tris-HCL (pH8.0), 300mM NaCL, 1mM imidazole; stored at RT.

(ix) Post sample Wash Buffer

25mM Tris-HCL (pH8.0), 300mM NaCL, 20mM imidazole; stored at RT.

(x) Elution Buffer

25mM Tris-HCL (pH8.0), 300mM NaCL, imidazole with gradual increasing concentration (30mM, 40mM, 50mM, 60mM, 70mM, 80mM, 100mM, 120mM, 140mM, 200mM, 250mM, 300mM, 500mM); stored at RT.

(xi) Nickal Sulfate ($NiSO_4$)

0.2M $NiSO_4$ (BDH); stored at RT.

3.3.3.1 Enzyme stocks

(i) DNase 1

10 mg /ml stock solution prepared in molecular grade water and stored at -20°C.

(ii) T4 DNA ligase

3 U/µl T4 DNA ligase (Promega); stored at -20°C.

(iii) Proteinase K

20 mg/ml Proteinase K (Promega), reconstituted in 50mM Tris-HCL (pH8.0) and 10mM $CaC1_2$, stored at -20°C.

(iv) Ribonuclease A (RNAse)

10 mg/ml RNAse (Sigma); stored at -20°C.

(v) 10x Ligation buffer

10x Ligation Buffer (Invitrogen); stored at -20°C.

(vi) ATP

10 mM ATP (Adenosine Triphosphate) (Sigma); stored at -20°C.

(vii) DNase (RNase-free):

RQ1 RNase –free DNase with 10x buffer (Promega) ; stored at -20°C.

(viii) Pfu DNA polymerase:

3 U/µl (Promega); stored at -20°C.

(ix) Taq DNA polymerase:

5 U/µl Ampli Taq (Perkin Elmer) ; stored at -20°C.

(x) Restriction enzymes:

All restriction enzymes used were purchased from Promega (Sydney, Australia) and were used as per manufacturer's instructions; stored at -20°C.

3.4 Bacteriological Methods

3.4.1 Media, Antibiotics and solutions related with media

3.4.1.1 Media

All media were prepared according to manufacturer's recommendations unless specified in the text. Sterilization was performed at standard conditions, 121°C for 15 minutes. Media was poured into sterile Petri plates under aseptic conditions and left to set for 20 minutes in a laminar flow cabinet. Liquid media was ready to use after autoclaving and cooling to room temperature.

(i) Luria Bertoni Agar (LBA)

Tryptone (1.0% w/v), yeast extract (0.5% w/v), NaCl (0.5% w/v) and bacteriological agar (1.0% w/v) was dissolved in dH2O and autoclaved under standard conditions. After cooling to 50°C, the agar was poured into Petri dishes and allowed to set. LBA plates were stored at 4°C.

(ii) Luria Bertoni Broth (LBB)

Tryptone (1.0% w/v), yeast extract (0.5% w/v) and NaCl (0.5% w/v) was dissolved in dH2O and autoclaved under standard conditions.

3.4.1.2 Antibiotic stock solutions

Antibiotic solutions were prepared by dissolving the antibiotics in appropriate solvents to the desired concentration. These stock solutions were stored at -20°C after being filter sterilised. Antibiotic stock concentrations were as follows:

(i) Ampicillin (CSL)

Ampicillin (500 mg/vial) was dissolved in 5 mL and 10 mL of sterile water dH2O, giving a stock solution of 100 mg/mL and 50 mg/mL, respectively. Antibiotic stock solutions were sterilised by filtration through a 0.2 μm membrane filter (Whatmann syringe filter) and one mL aliquots were stored frozen at –20°C. The ampicillin was added to media or other solution at the final concentration of 100 μg/mL and 50 μg/mL.

(ii) LBA Containing Ampicillin

LBA was prepared as stated above. After cooling agar to 50°C, ampicillin was added to a final concentration of 100 μg/mL and 50 μg/mL before pouring into Petri dishes (1μl of 100 or 50 mg/mL of ampicillin per 1ml of LBA). Plates were stored at 4°C.

(iii) LBB Containing Ampicillin

LB broth was prepared as stated above. Before use, ampicillin was added at a final concentration of 100μg/mL and 50μg/mL (1μl of 100 or 50 mg/mL of ampicillin per 1ml of LBB broth).

3.4.1.3 Solutions

(i) 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal)

A 81.72 mg of X-gal was dissolved in 5 mL dissolved in DMSO (dimethyl sulfoxide) or dimethyl formamide, giving a stock solution of 40 mg/mL. X-gal aliquots to one mL and wrapped in foil to protect it from the light, and stored frozen at –20°C.

(ii) LBA Containing X-gal

A 40 ul of Xgal was spread by a hockey stick spreader on top of the plate with LBA containing Ampicillin to get final concentration of 40 μg/mL. Then, the plates was let dry 30 minutes at least inside lamina floor before use them.

(iii) isopropyl thiogalactoside (ITPG)

A 0.238 g of ITPG was dissolved in 10 mL of sterile water dH2O, giving a stock solution of 0.1 M. ITPG solutions were sterilised by filtration on through a 0.2 μm membrane filter and one mL aliquots were stored frozen at –20°C.

(iv) LBB Containing ITPG

LBB broth was prepared as stated in (6.3.1.2.3). When the autoclaved bottle containing agar can be held with bare hands, 0.1 mM ITPG was added to LBB media at the final concentration of 1 mM (1μl of 0.1 M. ITPG per 1ml of LBB broth), with Ampicillin added as stated in (6.3.1.2.3).

(v) Chloramphenicol

A 226.19 mg of Chloramphenicol was dissolved in 20 mL dissolved in 95% EtOH, than mix/vortex vigorously so all the chloramphenicol goes into solution and giving a stock solution of 35 mg/mL. Chloramphenicol aliquots to one mL and stored frozen at –20°C.

(vi) LBB Containing Chloramphenicol

Chloramphenicol was added to LBB media prepared as stated in (6.3.1.2.3), than chloramphenicol was added LBB broth to obtain at a final concentration of 35 µg/mL (1µl of 35 mg/mL of chloramphenicol per 1ml of LBB broth), with Ampicillin added as stated in (6.3.1.2.3).

3.4.2 Anisakis, Bacterial Strains and Plasmids

Table A1. The **Anisakis,** bacterial strains and plasmids used in this study

Anisakis, Bacterial Strains and Plasmids	**Genotype or description**	**Storage**	**Reference/Source**
Anisakis	*Anisakis pegreffii*	Stored in 4°C and -20°C	
Bacterial Strains *Escherichia coli*			
DH5α Competent Cells	supE ΔlacU169 (ϕ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1	Stored in 40% glycerol at -80°C	[Hanahan, 1983]
BL21 Competent Cells	BL21(DE3) pLysS competent cells	Stored in 40% glycerol at -80°C	
Plasmids pCR2.1 with ampicillin and kanamycin resistant cassettes	3.9 kb PCR cloning plasmid, AmpR, KanR 3' T-overhang	Stored in -20°C	Invitrogen, Australia
pRSET-A with ampicillin and kanamycin resistant cassettes	2.9 kb cloning plasmid, P_{T7}	Stored in -20°C	

3.4.3 Storage of Bacterial Strains and Phage

(i) Long term.

All bacterial strains were stored at -70°C using the Protect bead storage system (Technical Service Consultants Ltd., United Kingdom).

(ii) Short term.

All bacterial strains were stored on LB or MH agar plates at 4°C. The bacteria were passaged every 4 weeks from single colonies.

3.4.4 Bacterial Culture Conditions

All *E. coli* strains were grown on solid microbiological media under aerobic conditions at 37°C for 16 h. In instances where broth cultures were used, the strains were grown aerobically at 37° C for 16 h on a Ratek orbital shaker at 200-220x g.

3.5 Primers

Table A2. The primers used in this study (5'-3')

Amplification of rDNA regions (ITS-1; ITS-2) and cDNA genes (*Ani p* 3; *Ani p 4*)

Gene	Sequencing
ITS-1	SS1 f GTTTCCGTAGGTGAACCTGCG NC13R r GCTGCGTTCTTCATCGAT
ITS-2	SS2 f TTGCAGACACATTGAGCACT NC2 r TTAGTTTCTTTTCCTCCGCT
Ani p 3	*Ani p* 3 f CGGGATCCATAGACGCAATCAAGAAGAAGATG *Ani p* 3 r CGGAATTCATATTAATATCCAGACAGCTCTTG
Ani p 4	*Ani s* 4 f CGGGATCCATATCCAGAATCGTCGTAGCG *Ani s* 4 r CGGAATTCATATTACTGATGATCGCATT

3.6 Antibodies

Types	Supplier
Mouse anti-poly Histidine antibody	Abcam, USA
Rabbit anti mouse IgG antibody	Abcam, USA
Rat monoclonal anti-tropomyosin antibody, MAC 141	Abcam, USA
Rabbit anti rat antibody conjugated to biotin	Abcam, USA
Mouse anti rabbit IgG, HRP conjugate polyclonal antibody,	Dako, USA
Goat anti rabbit IgG, AP conjugate polyclonal antibody,	Dako, USA
Rabbit anti human IgE antibody	Abcam, USA

3.7 Preparing and storage of solutions

(3.7.1) Deoxynucleotide triphosphates (dNTPs)

25 mM of each datp, dttp, dgtp, and dctp; stored at -20°C

(3.7.2) EDTA

0.5 M EDTA, pH 8.0; stored at RT.

(3.7.3) Ethanol

70 % (v/v) ethanol prepared from absolute ethanol (96%) commercial grade; stored at RT.

(3.7.4) Ethidium Bromide

0.5 µg /ml ethidium Bromide (Fluka) = 1 mg per 2 L MQ water bath; stored at RT.

(3.7.5) 10x Gel loading dye

10% (w/v) Ficoll 400, 50% (v/v) glycerol (Ajax), 0.5% (w/v) Orange G (BDH), 1% SDS, 10 mM EDTA, 50 mM Tris-base, pH to 8.0 with HCl; stored at 4°C.

(3.7.6) Glycerol

10-80% (v/v) glycerol, autoclaved; stored at RT.

(3.7.7) Lambda (λ) *Pst*I DNA ladder

100 µg λ DNA (500 µg/ml, Promega), 100 U *Pst*I (10 U/µl,), 90 µl 10x restriction enzyme Buffer H, MQ water to 900 µl; incubated overnight at 37°C, then added 100 µl 10x gel loading dye; stored at -20°C.

(3.7.8) MQ water

Sterile MQ water (Millipore) or deionised water was used to prepare reagents; stored at 4°C.

(3.7.9) Molecular grade water

Distilled water, DNAse-free and RNAse-free for PCR reactions and genomic DNA, stored at -20°C.

(3.7.10) NaCl

5 M NaCl; stored at RT.

(3.7.11) Propan-2-ol (Isopropanol)

100% (v/v) Isopropanol; stored at -20°C.

(3.7.12) Sodium acetate (NaOAc)

3 M NaOAc (BDH), pH 4.6 with HCl, autoclaved; stored at RT

(3.7.13) Sodium dodecyl sulphate (SDS)

10% (w/v) SDS; stored at RT.

(3.7.14) TAE buffer

40 mM Tris-base, 20 mM glacial acetic acid, 2 mM EDTA; stored at RT.

(3.7.15) TE buffer, pH 8.0

10 mM Tris-base, 1 mM EDTA, pH to 8.0 with HCl; for alkaline lysis method, add 20 µg/mL RNAse - fresh on day; stored at 4°C.

(3.7.16) Glycerol Cryogenic medium

50% (v/v) glycerol, 50% v/v LB broth and autoclaved under standard conditions.

(3.7.17) Ribonuclease A (RNAse)

10U/µl DNASe; stored at -20°C.

(3.7.18) Adenosine triphosphate (**ATP**)

20mM ATP (Adenosine Triphosphate); stored at -20°C.

(3.7.1) DNASe

10U/µl DNASe; stored at -20°C.

(3.7.19) Extraction solution

20 mM Tris-HCL, pH 8.0, 100 mM EDTA, 1%SDS; stored at RT.

(3.7.20) 11x Orange G loading dye

10% (w/v) Ficoll 400 (BDH), 50% (V/V) glycerol, 0.5% (w/v) Orange G (BDH), 1% SDS, 10mM EDTA, 50 mM Tris-HCL (Ph8.0); stored at 4°C.

(3.7.21) 9% Saline Solution

A 9 gm Sodium chloride (NaCl) was dissolved in 1 L Milli-Q waterd H2O, giving a stock solution of 9% Saline Solution; stored at RT.

(3.7.22) 10X PBS (Tris-Buffered Saline) Buffer (pH=7.4)

A 80 gm Sodium chloride (NaCl), 14.4gm Sodium Phosphate, dibasic (Na_2HPO_4), 2.4gm Potassium dihydrogen phosphate (KH_2PO_4), 2gm Potassium chloride (KCL) togethers were dissolved in 800 mL Milli-Q water, than adust pH of to 7.4 with HCL, and bring volume to 1L with Milli-Q water; stored at RT.

Prepare 1L of 1X PBS

Measure 100ml of 10X PBS and make it up to 1L with Milli-Q water (900ml).

(3.7.23) 10X TBS (Tris-Buffered Saline) Buffer (pH=7.4)

A 30gm Tris base ($C_4H_{11}NO_3$), 2gm Potassium chloride (KCL), 80 gm Sodium chloride (NaCl) togethers were dissolved in 800 mL Milli-Q water, than adust volume to 1L with Milli-Q water; stored at RT.

Prepare 1L of 1X TBS

Measure 100ml of 10X TBS and make it up to 1L with Milli-Q water (900ml).

(3.7.24) PBS 0.5 Tween

A 500µl Tween-20 was dissolved in 1LX PBS; stored at RT.

(3.7.25) TBS 0.5 Tween

A 500µl Tween-20 was dissolved in 1LX TBS; stored at RT.

(3.7.26) Block Buffer

5% (w/v) skim milk in TBS; freshly prepared.

(3.7.27) 0.1M Tris 0.5M Glycine Buffer (pH=8.7), 100ml

A1.2114gm Tris (hydrooxymethy1) aminomethane and 3.75gm Glycine togethers were dissolved in 80 mL Milli-Q water, than adust pH of to 7.4 with HCL, and bring volume to 100ml with Milli-Q water; stored at RT.

(3.7.28) 1% Bromophenol Blue (w/v), 10ml

A100mg Bromophenol blue was dissolved in 10ml Milli-Q water; stored at RT.

(3.7.29) 5X Sample Buffer

A 0.6 ml 1M Tris (pH 6.8), 5 ml 50% Glycerol, 2ml 10%SDS, 0.5 ml 2-mercaptoethanol and 0.5ml 1% Bromophenol Blue togethers were dissolved in 0.9 ml Milli-Q water; stored at 20°C.

(3.7.30) Lysis Buffer

25mM Tris-HCL (pH8.0), 300mM NaCL, 1mg/ml or 2mg/ml lysozyme (freshly prepared), 1mM or 2mM imidazole; stored at 4°C.

(3.7.31) Chelating Sepharose (resin)

Chelating sepharose fast flow containing 70% ethanol; stored at RT.

(3.7.32) Immobilised Metal Affinity Chromatography Column (IMAC)

1ml polypropylene IMAC column; stored at RT.

(3.7.33) IMAC Equilibration Wash Buffer

25mM Tris-HCL (pH8.0), 300mM NaCL, 1mM imidazole; stored at RT.

(3.7.34) Post sample Wash Buffer

25mM Tris-HCL (pH8.0), 300mM NaCL, 20mM imidazole; stored at RT.

(3.7.35) Elution Buffer

25mM Tris-HCL (pH8.0), 300mM NaCL, imidazole with gradual increasing concentration (30mM, 40mM, 50mM, 60mM, 70mM, 80mM, 100mM, 120mM, 140mM, 200mM, 250mM, 300mM, 500mM); stored at RT.

References

AALBERSE, R. C. 2007. Assessment of allergen cross-reactivity. *Clin Mol Allergy,* 5**,** 2.

ABATTOUY, N., VALERO, A., BENAJIBA, M. H., LOZANO, J. & MARTIN-SANCHEZ, J. 2011. Anisakis simplex s.l. parasitization in mackerel (Scomber japonicus) caught in the North of Morocco--prevalence and analysis of risk factors. *Int J Food Microbiol,* 150**,** 136-139.

ABBENANTE, G., FAIRLIE, D. P. 2005. Protease inhibitors in the clinic. *Med. Chem,* 1**,** 71–104.

ABDEL RAHMAN, A. M., KAMATH, S. D., GAGNE, S., LOPATA, A. L., HELLEUR, R. 2013. Comprehensive proteomics approach in characterizing and quantifying allergenic proteins from northern shrimp: toward better occupational asthma prevention. *J Proteome Res,* 12**,** 647-656

ABE, N., OHYA, N., YANAGIGUCHI, R. 2005. Molecular characterization of Anisakis pegreffii larvae in Pacific cod in Japan. *J Helminthol,* 79**,** 303-306.

ABE, N., TOMINAGA, K., KIMATA, I. 2006. Usefulness of PCR-restriction fragment length polymorphism analysis of the internal transcribed spacer region of rDNA for identification of anisakis simplex complex. *Japanese Journal of Infectious Diseases,* 59**,** 60–62.

ABOLLO, E., GESTAL, C. & PASCUAL, S. 2001. Anisakis infestation in marine fish and cephalopods from Galician waters: an updated perspective. *Parasitol Res,* 87**,** 492-499.

ABOLLO, E., PAGGI, L., PASCUAL, S., D'AMELIO, S. 2003. Occurrence of recombinant genotypes of Anisakis simplex s.s. and Anisakis pegreffii (Nematoda: Anisakidae) in an area of sympatry. *Infect Genet Evol,* 3**,** 175-181.

ABRAHAMSON, M. 1994. Cystatins. *Methods Enzymol,* 244**,** 685-700.

ABRAHAMSON, M., ALVAREZ-FERNANDEZ, M., NATHANSON, C. M. 2003. Cystatins. *Biochem Soc Symp,* 70**,** 179–199.

ABRAHAMSON, M., ALVAREZ-FERNANDEZ, M. & NATHANSON, C. M. 2003. Cystatins. *Biochem Soc Symp,*70**,** 179–199

ACEVEDO, N., CARABALLO, L. 2011. IgE cross-reactivity between Ascaris lumbricoides and mite allergens: possible influences on allergic sensitization and asthma. *Parasite Immunology,* 33**,** 309-321.

ADAMS A.M., K.D. MURRELL AND J.H. CROSS. 1997. Parasites of fish and risks to public health. *Office International des Epizooties, Scientific and Technical Review,* 16, 652-660.

AKAO, N., OHYAMA, T., KONDO, K., TAKAKURA, Y. 1989a. Immunoblot analysis of human gnathostomiasis. *Ann Trop Med Parasitol,* 83**,** 635-637.

AKAO, N., YOSHIMURA, H. 1989b. Latex agglutination test for immunodiagnosis of gastric anisakiasis, In: Ishikura H, Namiki M, editors. *Gastric anisakiasis in Japan. Springer-Verlag, Tokyo.* pp:97-102.

ALLEN, J. E., MAIZELS, R. M. 2011. Diversity and dialogue in immunity to helminths. *Nat Rev Immunol,* 11**,** 375-388.

ALONSO, A., DASCHNER, A., MORENO-ANCILLO, A. 1997. Anaphylaxis with Anisakis simplex in the gastric mucosa. *N Engl J Med,* 337**,** 350-351.

ALSHURAFA, H. N., STENTON, G. R., WALLACE, J. L., HOLLENBERG, M. D., BEFUS, A. D., VLIAGOFTIS, H. 2004. A protease activated receptor-2 (PAR-2) activating peptide, tc-LIGRLO-NH2, induces protease release from mast cells: role in TNF degradation. *BMC. Phamacol,* 20**,** 4-12.

ANADON, A. M., ROMARIS, F., ESCALANTE, M., RODRIGUEZ, E., GARATE, T., CUELLAR, C., UBEIRA, F. M. 2009. The Anisakis simplex Ani s 7 major allergen as an indicator of true Anisakis infections. *Clin Exp Immunol,* 156**,** 471-478.

ANDERSON, R. C. 1984. The origins of zooparasitic nematodes. *Canadian Journal of Zoology* 62**,** 317–328.

ANDERSON, R. C. E. 2000. Nematode parasites of vertebrates. Their development and transmission. 2nd edn. 650 pp. London, CAB International.

ANDREWS, R. H., CHILTON, N. B. 1999. Multilocus enzyme electrophoresis: a valuable technique for providing answers to problems in parasite systematics. *Int J Parasitol,* 29**,** 213–253.

ANIBARRO, B., SEOANE, F. J. 1998. Occupational conjunctivitis caused by sensitization to Anisakis simplex. *J. Allergy Clin. Immunol,* 102**,** 331–332.

ARAI, T., AKAO, N., SEKI, T., KUMAGAI, T., ISHIKAWA, H., OHTA, N., HIRATA, N., NAKAJI, S., YAMAUCHI, K., HIRAI, M., SHIRATORI, T., KOBAYASHI, M., FUJII, H., ISHII, E., NAITO, M., SAITOH, S., YAMAGUCHI, T., SHIBATA, N., SHIMO, M. & TOKIWA, T. 2014. Molecular Genotyping of Anisakis Larvae in Middle Eastern Japan and Endoscopic Evidence for Preferential Penetration of Normal over Atrophic Mucosa. *Plos One,* 9.

ARIZONO, N., YAMADA, M., TEGOSHI, T., YOSHIKAWA, M. 2012. Anisakis simplex sensu stricto and Anisakis pegreffii: biological characteristics and pathogenetic potential in human anisakiasis. *Foodborne Pathog Dis,* 9**,** 517-521.

ARMENTIA, A., LOMBARDERO, M., CALLEJO, A., MARTIN SANTOS, J. M., GIL, F. J., VEGA, J., ARRANZ, M. L., MARTINEZ, C. 1998. Occupational asthma by Anisakis simplex . *J Allergy Clin Immunol,* 102**,** 831–834.

ARRUDA, L. K. & SANTOS, A. B. 2005. Immunologic responses to common antigens in helminthic infections and allergic disease. *Curr Opin Allergy Clin Immunol,* 5**,** 399-402.

ASAMI, K., WATANUKI, T., SAKAI, H., IMANO, H., OKAMOTO, R. 1965. Two Cases of Stomach Granuloma Caused by Anisakis-Like Larval Nematodes in Japan. *Am J Trop Med Hyg,* 14**,** 119-123.

ASTURIAS, J. A., ERASO, E., ARILLA, M. C., GOMEZ-BAYON, N., INACIO, F., MARTINEZ, A. 2002. Cloning, isolation, and IgE-binding properties of Helix aspersa (brown garden snail) tropomyosin. *Int Arch Allergy Immunol,* 128**,** 90-96.

ASTURIAS, J. A., ERASO, E., MARTINEZ, A. 2000. Cloning and high level expression in *Escherichia coli* of an Anisakis simplex tropomyosin isoform. *Mol Biochem Parasitol,* 108**,** 263-266.

AUDÍCANA-BERASATEGUI, M. T., DEL POZO-GIL, M. D., DASCHNER, A. 2007. Alergia a Anisakis simplex. In: Tratado de Alergología Clínica. Tomo II. Ed. Sociedad Española de alergología a Inmunologia Clínica (SEAIC). Ed. Peláez A y Dávila I. Ergon, Chapter 11. pp. 1681-1705.

AUDICANA, M. T., ANSOTEGUI, I. J., DE CORRES, L. F., KENNEDY, M. W. 2002a. Anisakis simplex: dangerous--dead and alive? *Trends Parasitol,* 18**,** 20-25.

AUDICANA, M. T., FERNANDEZ DE CORRES, L., MUNOZ, D., FERNANDEZ, E., NAVARRO, J. A., DEL POZO, M. D. 1995. Recurrent anaphylaxis caused by Anisakis simplex parasitizing fish. *J Allergy Clin Immunol,* 96**,** 558-560.

AUDICANA, M. T., KENNEDY, M. W. 2008. Anisakis simplex: from obscure infectious worm to inducer of immune hypersensitivity. *Clin Microbiol Rev,* 21**,** 360-79.

AUDICANA, M. T., MUNOZ, D., DEL POZO, M. D., FERNANDEZ, E., GASTAMINZA, G., FERNANDEZ DE CORRES, L. 2002c. Allergic contact dermatitis from mercury antiseptics and derivatives: study protocol of tolerance to intramuscular injections of thimerosal. *Am J Contact Dermat,* 13**,** 3-9.

AUERSWALD, L., LOPATA, A. 2005. Insects –Diversity and Allergy. *Current Allergy* 18**,** 58-60.

AYUSO, R., GRISHINA, G., BARDINA, L., CARRILLO, T., BLANCO, C., IBANEZ, M. D., SAMPSON, H. A., BEYER, K. 2008. Myosin light chain is a novel shrimp allergen, Lit v 3. *J Allergy Clin Immunol,* 122**,** 795-802.

BARRETT A.J., RAWLINGS N.D., WOESSNER, J. F. 2003. The Handbook of Proteolytic Enzymes, 2nd ed. Academic Press.

BARRY, R. A., KENT, S. B., MCKINLEY, M. P., MEYER, R. K., DEARMOND, S. J., HOOD, L. E., PRUSINER, S. B. 1986. Scrapie and cellular prion proteins share polypeptide epitopes. *J Infect Dis,* 153**,** 848–854.

BEALE, J. E., JEEBHAY, M. F., LOPATA, A. L. 2009. Characterisation of purified parvalbumin from five fish species and nucleotide sequencing of this major allergen from Pacific pilchard, Sardinops sagax. *Mol Immunol,* 46**,** 2985-2993.

BEN-SHOSHAN, M., KAGAN, R.S., ALIZADEHFAR, R., JOSEPH, L., TURNBULL, E., ST PIERRE, Y, CLARKE, A.E. 2009. Is the prevalence of peanut allergy increasing? . *J Allergy Clin Immunol,* 123**,** 783-788.

BINDER, M., MAHLER, V., HAYEK, B., SPERR, W. R., SCHOLLER, M., PROZELL, S., WIEDERMANN, G., VALENT, P., VALENTA, R. & DUCHENE, M. 2001. Molecular and immunological characterization of arginine kinase from the indianmeal moth, Plodia interpunctella, a novel cross-reactive invertebrate pan-allergen. *J of Immunol,* 167**,** 5470-5477.

BINDROO, B., B., & MOORTHY, S., M. 2014. Genetic Divergence, Implication of Diversity, and Conservation of Silkworm, Bombyx mori. *Int J Biodiversity.* **,** 15 pages.

BLACK, W. C. & PIESMAN, J. 1994. Phylogeny of hard- and soft-tick taxa (Acari: Ixodida) based on mitochondrial 16S rDNA sequences. *Proc Natl Acad Sci. U.S.A.* 91**,** 10034–10038.

BLAXTER, M. L., DE LEY, P., GAREY, J. R., LIU, L. X., SCHELDEMAN, P., VIERSTRAETE, A., VANFLETEREN, J. R., MACKEY, L. Y., DORRIS, M., FRISSE, L. M., VIDA, J. T. & THOMAS, W. K. 1998. A molecular evolutionary framework for the phylum Nematoda. *Nature*, 392**,** 71-75.

BLOUIN, M., DAME, J.B., TARRANT, C.A., COURTNEY, C.H. 1992. Unusual population genetics of a parasitic nematode: mtDNA variation within and among populations. *Evolution,* 46**,** 470–476.

BLOUIN, M., YOWELL, C.A., COURTNEY, C.H., DAME, J.B. 1995. Host movement and the genetic structure of populations of parasitic nematodes. *Genetics,* 141**,** 1007–1014.

BLOUIN, M., YOWELL, C.A., COURTNEY, C.H., DAME, J.B. 1998. Substitution bias, rapid saturation, and the use of mtDNA for nematode systematics. *Mol Biol Evol* 15**,** 1719–1727.

BOCK, S. A. 1987. Prospective appraisal of complaints of adverse reactions to foods in children during the first 3 years of life. *Pediatrics,* 79**,** 683-688.

BØGH, H., ZHU XQ., QIAN, B.Z ., GASSER, R.B. 1999. Scanning for nucleotide variations in mitochondrial DNA fragments of Schistosoma japonicum by single-strand conformation polymorphism. *Parasitology*. 118, 73–82.

BOGITSH, B. J., CARTER, C. E. & OELTMANN, T. N. 2005. Human parasitology. *3 rd ed. New York: Elsevier Academic Press.* **,** 299-317.

BOHM, S. K., KONG, W., BROMME, D., SMEEKENS, S. P., ANDERSON, D. C., CONNOLLY, A., KAHN, M., NELKEN, N. A., COUGHLIN, S. R., PAYAN, D. G. & BUNNETT, N. W. 1996. Molecular cloning, expression and potential functions of the human proteinase-activated receptor-2. *Biochem J,* 314**,** 1009-1016.

BOTT, N. J., CAMPBELL, B. E., BEVERIDGE, I., CHILTON, N. B., REES, D., HUNT, P. W. & GASSER, R. B. 2009 A combined microscopic-molecular method for the diagnosis of strongylid infections in sheep. *Inter J Parasitol,* 39**,** 1277–1287.

BOUREE, P., JUETTE, P. & G., K. 1987. Anisakiase: diagnostic et extraction par endoscopie gastrique. *Presse Med,* 1484-1485

BOUREE, P., PAUGAM, A. & PETITHORY, J. C. 1995. Anisakidosis: report of 25 cases and review of the literature. *Comp Immunol Microbiol Infect Dis,* 18**,** 75-84.

BOWLES, J., BLAIR, D., MCMANUS, D. P. 1992. Genetic variants within the genus *Echinococcus* identified by mitochondrial sequencing. *Mol Biochem Parasitol,* 54**,** 165-174.

BOWLES, J., BLAIR, D., MCMANUS, D. P. 1995b Amolecular phylogeny of the genus Echinococcus. *Parasitol Res,* 110:**,** 317-328.

BOWLES, J., BLAIR, D. & MCMANUS, D. P. 1995a. Amolecular phylogeny of the human schistosomes. *Mol Phylogenetic Evol.* 4**,** 103-109.

BOYCE, J., ASSA'AD, A., BURKS, A.W., JONES, S.M., SAMPSON, H.A., WOOD, R.A., PLAUT, M., COOPER, S.F., FENTON, M.J., ARSHAD, S.H., BAHNA, S.L, BECK, L.A., BYRD-BREDBENNE, R. C., CAMARGO CA, J.R., EICHENFIELD, L., FURUTA, G.T., HANIFIN, J.M., JONES, C., KRAFT, M., LEVY, B.D., LIEBERMAN, P., LUCCIOLI, S., MCCALL, K.M., SCHNEIDER, L.C., SIMON, R.A., SIMONS, F.E., TEACH, S.J., YAWN, B.P., JM., S. 2010. Guidelines for the diagnosis and management of food allergy in the United States: report of the NIAID-sponsored expert panel. *J Allergy Clin Immunol.1,* 26**,** 1-58.

BRADFORD, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem,* 72**,** 248-254.

BRANUM, A., SL, L. 2008. Food allergy among U.S. children: trends in prevalence and hospitalizations. *NCHS Data Brief***,** 1-8.

BRANUM, A., SL, L. 2009. Food allergy among children in the United States. *Pediatrics.* 124**,** 1549-1555.

BROOKER, S., CLEMENTS, A. C., BUNDY, D. A. 2006. Global epidemiology, ecology and control of soil-transmitted helminth infections. *Adv Parasitol,* 62**,** 221-61.

BROWN, W. M. 1985. The mitochondrial genome of animals. In: MacIntyre RJ (ed) Molecular Evolutionary Genetics. Plenum Press, New York, pp 95-130.

BROWNRIDGE, P., DE MELLO, L. V., PETERS, M., MCLEAN, L., CLAYDON, A., COSSINS, A. R., WHITFIELD, P. D., YOUNG, I. S. 2009. Regional variation in parvalbumin isoform expression correlates with muscle performance in common carp (Cyprinus carpio). *J Exp Biol,* 212**,** 184-93.

BRUCE, N. L. 1990a. Hysterothylacium Ward and Magath, 1917, and Ichthyascaris Wu, 1949, ascaridoid nematodes from Australian demersal fishes. *Memoirs of the Queensland Museum,* 28**,** 389–426. .

BRUCE, N. L. 1990b. Redescription of the ascaridoid nematode Hysterothylacium scomberomori (Yamaguti) from Australian Spanish mackerel Scomberomorus commerson (Lacépède). *Memoirs of the Queensland Museum* 28**,** 427–434. .

BRUCE, N. L. & CANNON, L. R. G. 1989. Hysterothylacium, Iheringascaris and Maricostula new genus, nematodes (Ascaridoidea) from Australian pelagic marine fishes. *J Natur Hist,* 23**,** 1397–1441.

BRUCE, N. L. & CANNON, L. R. G. 1990. Ascaridoid nematodes from sharks from Australia and the Solomon Islands, southwestern Pacific Ocean. *Invertebrate Taxonomy,* 4**,** 763–783.

BUGAJSKA-SCHRETTER, A., PASTORE, A., VANGELISTA, L., RUMPOLD, H., VALENTA, R. & SPITZAUER, S. 1999. Molecular and immunological characterization of carp parvalbumin, a major fish allergen. *Int Arch Allergy Immunol,* 118**,** 306-8.

BUSH, A. O., LAFFERTY, K. D., LOTZ, J. M. & SHOSTAK, A. W. 1977. Parasitology meets ecology on its own terms: Margolis et al. revisited. . *The Journal of Parasitology* 83:**,** 575–583.

CABALLERO, M. L., MONEO, I. 2004. Several allergens from Anisakis simplex are highly resistant to heat and pepsin treatments. *Parasitol Res ,* 93**,** 248-251.

CABALLERO, M. L., MONEO, I., GOMEZ-AGUADO, F., CORCUERA, M. T., CASADO, I. & RODRIGUEZ-PEREZ, R. 2008. Isolation of Ani s 5, an excretory-secretory and highly heat-resistant allergen useful for the diagnosis of Anisakis larvae sensitization. *Parasitol Res,* 103**,** 1231-1233.

CANCRINI, G., MAGRO, G. & GIANNONE, G. 1997. Primo caso di anisakiosi extragastrointestinale nell'uomo diagnosticato in Italia. *Parassitologia,* 39:**,** 13-17. .

CANNON, L. R. 1977b. Some larval ascaridoids from south-eastern Queensland marine fishes. *Int J Parasitol,* 7**,** 233–243.

CANNON, L. R. G. 1977a. Some ecological relationships of larval ascaridoids from south-eastern Queensland marine fishes. *Int J Parasitol,* 7**,** 227–232.

CARABALLO, L. & ACEVEDO, N. 2011. Allergy in the tropics: the impact of cross-reactivity between mites and ascaris. *Front Biosci (Elite Ed),* 3**,** 51-64.

CARRETERO ANIBARRO, P., BLANCO CARMONA, J., GARCIA GONZALEZ, F., MARCOS DURANTEZ, M., ALONSO GIL, L., GARCES SOTILLOS, M., PEREZ GIMENEZ, R., JUSTE PICON, S., GUTIERREZ & ORTEGA, M. C. 1997. Protein contact dermatitis caused by Anisakis simplex. *Contact Dermatitis***,** 37, 247.

CAVALLERO, S., LIGAS, A., BRUSCHI, F. & D'AMELIO, S. 2012. Molecular identification of Anisakis spp. from fishes collected in the Tyrrhenian Sea (NW Mediterranean). *Vet Parasitol,* 187**,** 563-566.

CHAI, J. Y., DARWIN MURRELL, K. & LYMBERY, A. J. 2005. Fish-borne parasitic zoonoses: status and issues. *Int J Parasitol,* 35**,** 1233-1254.

CHAPMAN, M. D. 2003. Allergen nomenclature. In: Lockey RF, Bukantz SC, Bousquet J, editors. Allergens and allergen immunotherapy. New York: Marcel Decker. p. 51-64.

CHAPMAN, M. D., POMES, A., BREITENEDER, H. & FERREIRA, F. 2007. Nomenclature and structural biology of allergens. *J Allergy Clin Immunol,* 119**,** 414-420.

CHEN, H. J., TSAI, J. C., CHANG, T. C., HUNG, W. C., TSENG, S. P., HSUEH, P. R. & TENG, L. J. 2008. PCR-RFLP assay for species and subspecies differentiation of the Streptococcus bovis group based on groESL sequences. *J Med Microbiol,* 57**,** 432-438.

CHILTON, N. B., GASSER, R. B. & BEVERIDGE, I. 1997. Phylogenetic relationships of Australian strongyloid nematodes inferred from ribosomal DNA sequence data. *Int J Parasitol,* 27**,** 1481–1494.

CHO, T. H., . PARK, H. Y., CHO, S., SOHN, .J., YOON, Y. W., CHO, J. E., CHO, S. W. 2006. The time-course of biological and immunochemical allergy states induced by Anisakis simplex larvae in rats. *ClinExp Immunol,* 143**,** 203-208.

CHOI, S. J., LEE, J. C., KIM, M. J., HUR, G. Y., SHIN, S. Y., PARK, H. S. 2009. The clinical characteristics of Anisakis allergy in Korea. *Korean J Intern Med,* 24**,** 160–163.

CHOU, Y. Y., WANG, C. S., CHEN, H. G., CHEN, H. Y., CHEN, S. N. & SHIH, H. H. 2011. Parasitism between Anisakis simplex (Nematoda: Anisakidae) third-stage larvae and the spotted mackerel Scomber australasicus with regard to the application of stock identification. *Veterinary Parasitology,* 177**,** 324-331.

CHRISTENSEN, N. D., HOPfl, R., DIANGELO, S. L., CLADEL, N. M., PATRICK, S. D., WELSH, P. A., BUDGEON, L. R., REED, C. A., KREIDER, J. W. 1994a. Assembled baculovirus-expressed human papillomavirus type 11 L1 capsid protein virus-like particles are recognized by neutralizing monoclonal antibodies and induce high titres of neutralizing antibodies. *J Gen Virol ,* 75**,** 2271–2276.

CHRISTENSEN, N. D., KIRNBAUER, R., SCHILLER, J. T., GHIM, S. J., SCHLEGEL, R., JENSON, A. B. & KREIDER, J. W. 1994b Human papillomavirus type 6 and 11 have antigenically distinct strongly immunogenic conformationally dependant neutralizing epitopes. *Virol,* 205**,** 329–335.

CHU, K. H., WONG, S. H., LEUNG, P. S. C. 2000. Tropomyosin is the major mollusk allergen: Reverse transcriptase polymerase chain reaction, expression and IgE reactivity. *Mar Biotechnol,* 2**,** 499-509.

CHUNG, Y. B. & YANG, H. J. 2008. Partial purification and characterization of a cysteine protease inhibitor from the plerocercoid of Spirometra erinacei. *Korean J Parasitol* 46**,** 183-186.

COCA, A. F. & COOKE, R. A. 1923. On the classification of the phenomena of hypersensitiveness. *Journal of Immunology,* 8**,** 163-182.

COCKS, T. M., FONG, B., CHOW, J. M., ANDERSON, G. P., FRAUMAN, A. G., GOLDIE, R. G., HENRY, P. J., CARR, M. J., HAMILTON, J. R. & MOFFATT, J. D. 1999. A protective role for protease-activated receptors in the airways. *Nature,* 398**,** 156-60.

COOMBS R.R.A., GELL, P. G. H. 1963. The classification of allergic reactions underlying diseases. In: Gell PGH, Coombs RRA, eds. *Clinical Aspects of Immunology, chapter 13. Oxford: Blackwell Scientific Publications.,* 217–37.

COLE, A. J., PRATCHETT, M. S. & JONES, G. P. 2008. Diversity and functional importance of coral-feeding fishes on tropical coral reefs. *Fish and Fisheries,* 9**,** 286-307.

COUTURE, C., MEASURES, L. & GAGNON, J., ET AL. 2003. Human intestinal anisakiosis due to consumption of raw salmon. *Am J Surg Pathol,* 27**,** 1167–1172.

COX, F. E. 1997. Designer vaccines for parasitic diseases. *Int J Parasitol,* 27**,** 1147-1157.

D'AMELIO, S., MATHIOPOULOS, K. D., BRANDONISIO, O., LUCARELLI, G., DORONZO, F. & PAGGI, L. 1999. Diagnosis of a case of gastric anisakidosis by PCR-based restriction fragment length polymorphism analysis. *Parassitologia,* 41**,** 591-593.

D'AMELIO, S., MATHIOPOULOS, K. D., SANTOS, C. P., PUGACHEV, O. N., WEBB, S. C., PICANCO, M., PAGGI, L. 2000. Genetic markers in ribosomal DNA for the identification of members of the genus Anisakis (Nematoda: ascaridoidea) defined by polymerase-chain-reaction-based restriction fragment length polymorphism. *Int J Parasitol,* 30**,** 223-226.

D'ANDREA, M. R., DERIAN, C. K., LETURCQ, D., BAKER, S. M., BRUNMARK, A., LING, P., DARROW, A. L., SANTULLI, R. J., BRASS, L. F., ANDRADE-GORDON, P. 1998. Characterization of protease-activated receptor-2 immunoreactivity in normal human tissues. *J Histochem Cytochem,* 46**,** 157-164.

DAINICHI, T., MAEKAWA, Y., ISHII, K., ZHANG, T., NASHED, B. F., SAKAI, T., TAKASHIMA, M., HIMENO, K. 2001. Nippocystatin, a cysteine protease inhibitor from Nippostrongylus brasiliensis, inhibits antigen processing and modulates antigen-specific immune response. *Infect Immun,* 69**,** 7380–7386.

DAMIN, L. & HEQING, H. 2001. Heliconema minnanensis n. sp. (Physalopteroidae: Physalopteridae) and Raphidascaris trichiuri (Yin and Zhang) n. comb. (Ascaridoidea: Anisakidae) in marine fishes. . *The Journal of Parasitology,* 87, **,** 1090–1094.

DAS DORES, S., CHOPIN, C., ROMANO, A., GALLAND-IRMOULI, A. V., QUARATINO, D., PASCUAL, C., FLEURENCE, J. & GUEANT, J. L. 2002. IgE-binding and cross-reactivity of a new 41 kDa allergen of codfish. *Allergy,* 57**,** 84-87.

DASCHNER, A., ALONSO-GOMEZ, A., CABANAS, R., SUAREZ-DE-PARGA, J. M., LOPEZ-SERRANO, M. C. 2000. Gastroallergic anisakiasis: borderline between food allergy and parasitic disease-clinical and allergologic evaluation of 20 patients with confirmed acute parasitism by Anisakis simplex. *J Allergy Clin Immunol,* 105**,** 176-181.

DASCHNER, A., CUELLAR, C., SANCHEZ-PASTOR, S., PASCUAL, C. Y., MARTIN-ESTEBAN, M. 2002. Gastro-allergic anisakiasis as a consequence of simultaneous primary and secondary immune response. *Parasite Immunol,* 24**,** 243-251.

DASCHNER, A., CULLÉAR, C. A. & RODERO, M. 2012. "The Anisakis allergy debate: does an evolutionary approach help?". *Trends Parasitol.,* 28**,** 9–15

DASCHNER, A., VEGA DE LA OSADA, F. & PASCUAL, C. Y.2005. Allergy and parasites reevaluated: wide-scale induction of chronic urticaria by the ubiquitous fishnematode Anisakis simplex in an endemic region. *Allergol. Immunopathol. (Madr.)* 33**,** 31–37.

DAUL, C. B., SLATTERY, M., REESE, G. & LEHRER, S. B. 1994. Identification of the Major Brown Shrimp (Penaeus-Aztecus) Allergen as the Muscle Protein Tropomyosin. *Int Arch Allergy Immunol,* 105**,** 49-55.

DAVEY, J. T. 1971. A case of Anisakis sp. infection in a brown bear from Alaska. *Trans R Soc Trop Med Hyg,* 65**,** 433-434.

DE SILVA, N. R., BROOKER, S., HOTEZ, P. J., MONTRESOR, A., ENGELS, D., SAVIOLI, L. 2003. Soil-transmitted helminth infections: updating the global picture. *Trends in Parasitology,* 19**,** 547–551.

DEARDORFF, T. L., ALTMAN, J., NOLAN, C. M. 1987. Human Anisakiasis - 2 Case-Reports from the State of Washington. *Proceedings of the Helminthological Society of Washington,* 54**,** 274-275.

DEARDORFF, T. L., FUKUMURA, T., RAYBOURNE, R. B. 1986. Invasive Anisakiasis - a Case-Report from Hawaii. *Gastroenterol,* 90**,** 1047-1050.

DERY, O., CORVERA, C. U., STEINHOFF, M. & BUNNETT, N. W. 1998. Proteinase-activated receptors: novel mechanisms of signaling by serine proteases. *Am J Physiol,* 274**,** C1429-52.

DESOWITZ, R. S., RAYBOURNE, R. B., ISHIKURA, H., KLIKS, M. M. 1985. The radio allergo sorbent test (RAST) for the serological diagnosis of human anisakiasis. *Trans Roy Soc trop Med Hyg ,* 75.

DEWITT, A. M., MATTSSON, L., LAUER, I., REESE, G., LIDHOLM, J. 2004. Recombinant tropomyosin from Penaeus aztecus (rPen a 1) for measurement of

specific immunoglobulin E antibodies relevant in food allergy to crustaceans and other invertebrates. *Mol Nutr Food Res,* 48**,** 370-379.

DIETRICH, W. F., MILLER, J., STEEN, R., MERCHANT, M. A., DAMRON-BOLES, D., HUSAIN, Z. E. A. 1996. A comprehensive genetic map of the mouse genome. *Nature,* 380**,** 149–152.

DOMINGUEZ-ORTEGA, J., ALONSO-LLAMAZARES, A., RODRIGUEZ, L., CHAMORRO, M., ROBLEDO, T., BARTOLOME, J. M., MARTINEZ-COCERA, C. 2001. Anaphylaxis due to hypersensitivity to Anisakis simplex. *Int Arch Allergy Immunol,* 125**,** 86-88.

DONNELLY, S., DALTON, J. P., LOUKAS, A. 2006. Proteases in helminth- and allergen-induced inflammatory responses. *Chem Immunol Allergy,* 90**,** 45-64.

DOOLEY, J. R. & NEAFIE, R. C. 1976. Anisakiasis. In Pathology of Tropical and Extraordinary Diseases, (Edited by C. Binford and D. Conner), pp. 475-481 Armed Forces Inst. Pathol., Washington, U.S.A. .

DORRIS, M., DE LEY, P., BLAXTER, M. L. 1999. Molecular analysis of nematode diversity and the evolution of parasitism. *Parasitol. Today,* 15**,** 188–193.

DOUPÉ, R. G., LYMBERY, A. J., WONG, S. & HOBBS, R. P. 2003. Larval anisakid infections of some tropical fish species from north-west Australia. *J Helminthol.* 77**,** 363–365.

DRAKE, L. J., JUKES, M. C. H., STERNBERG, R. J., BUNDY, D. A. P. 2000. Geohelminth infections (Ascariasis, Trichuriasis and Hookworm): cognitive and developmental impacts. . *Seminars in Pediatric Infectious Diseases ,* 11**,** 245–251

DZIK, J. M. 2006. Molecules released by helminth parasites involved in host colonization. *Acta Biochim Pol,* 53**,** 33–64.

ELSAYED, S., AAS, K. 1971. Isolation of purified allergens (cod) by isoelectric focusing. *Int Arch Allergy Appl Immunol,* 40**,** 428-38.

EISENBARTH, A. 2009. Occurrence of Intestinal Fish Parasites in Australia: Identification of Anisakid Nematodes in Commercially Available Fish Species from South Australian Waters. *Hamburg Diplomica Verlag GmbH, P-92*

ELSAYED, S., BENNICH, H. 1975. The primary structure of allergen M from cod. *Scand J Immunol,* 4**,** 203-208.

ELSAYED, S. M., AAS, K. 1970. Characterization of a major allergen (cod.) chemical composition and immunological properties. *Int Arch Allergy Appl Immunol,* 38**,** 536-48.

FAGERHOLM, H. P. 1991. Systematic Implications of Male Caudal Morphology in Ascaridoid Nematode Parasites. *Systematic Parasitology,* 19**,** 215-228.

FARJALLAH, S., SLIMANE, B. B., BUSI, M., PAGGI, L., AMOR, N., BLEL, H., SAID, K. & D'AMELIO, S. 2008. Occurrence and molecular identification of Anisakis

spp. from the North African coasts of Mediterranean Sea. *Parasitol Res,* 102**,** 371-379.

FEAGIN, J. E. 2000 Mitochondrial genome diversity in parasites . *Int J Parasitol,* 30**,** 371-390.

FINKELMAN, F. D., SHEA-DONOHUE, T., GOLDHILL, J., SULLIVAN, C. A., MORRIS, S. C., MADDEN, K. B., GAUSE, W. C., URBAN, J. F., JR. 1997. Cytokine regulation of host defense against parasitic gastrointestinal nematodes: lessons from studies with rodent models. *Annu Rev Immunol,* 15**,** 505-533.

FINKELMAN, F. D., SHEA-DONOHUE, T., MORRIS, S. C., GILDEA, L., STRAIT, R., MADDEN, K. B., SCHOPF, L., URBAN, J. F., J.R. 2004. Interleukin-4- and interleukin-13-mediated host protection against intestinal nematode parasites. *Immunol Rev,* 201**,** 139-155.

FISCHER, S. G. & LERMAN, L. S. 1983. DNA fragments differing by single base-pair substitutions are separated in denaturing gradient gels: correspondence with melting theory. . *Proc. Natl. Acad. Sci. U. S. A.,* 80**,** 1579–1583.

FOTI, C., NETTIS, E., CASSANO, N., DI MUNDO, I., VENA, G. A. 2002. Acute allergic reactions to Anisakis simplex after ingestion of anchovies. *Acta Derm Venereol,* 82**,** 121-123.

FRENKEL, M. J., SAVIN, K. W., BAKKER, R. E., WARD, C. W. 1989. Characterization of Cdna Clones Coding for Muscle Tropomyosin of the Nematode Trichostrongylus-Colubriformis. *Molecular and Biochemical Parasitology,* 37**,** 191-199.

FROESE, R., D, P. 2012. Fishbase. www.fishbase.org Accessed 20 January 2012

FUJINO, T., OOIWA, T. & ISHII, Y. 1984. Clinical, epidemiological and morphological studies on 150 cases of acute gastric anisakiasis in Fukuoka Prefecture. *Japanese J Parasitol*. 33**,** 73-92.

FUJITA, K. & SILVER, J. 1994. Single-strand conformational polymorphism. peR Method and Applications 4**,** S 137-S 139.

FUMAROLA, L., MONNO, R., IERARDI, E., RIZZO, G., GIANNELLI, G., LALLE, M., POZIO, E. 2009. Anisakis pegreffi etiological agent of gastric infections in two Italian women. *Foodborne Pathog Dis,* 6**,** 1157-1159.

FURMONAVICIENE, R., GHAEMMAGHAMI, A. M., BOYD, S. E., JONES, N. S., BAILEY, K., WILLIS, A. C., SEWELL, H. F., MITCHELL, D. A., SHAKIB, F. 2007. The protease allergen Der p 1 cleaves cell surface DC-SIGN and DC-SIGNR: experimental analysis of in silico substrate identification and implications in allergic responses. *Clin Exp Allergy.* 37**,** 231-242.

GARCIA, B. E., LIZASO, M. T. 2011. Cross-reactivity syndromes in food allergy. *J Investig Allergol Clin Immunol,* 21**,** 162-70; quiz 2 p following 170.

GARCIA, F., BLANCO, J. G., GARCES, M., JUSTE, S., FUENTES, M., HERRERO, D., 2001. 2001. Freezing protects against allergy to Anisakis simplex. *J. Investig. Allergol. Clin. Immunol,* 11**,** 49–52.

GARCIA, M., MONEO, I., AUDICANA, M.T., DEL POZO, M. D., MUNOZ, D., FERNANDEZ, E., DÍEZ, J., ETXENAGUSIA, M.A, ANSOTEGUI, I .J., FERNÁNDEZ, D.E., CORRES, L. 1997. The use of IgE immunoblotting as a diagnostic tool in Anisakis simplex allergy. *J Allergy Clin Immunol.* 99**,** 497–501.

GARDINER, D. L., MCCARTHY, J. S., TRENHOLME, K. R. 2005. Malaria in the post-genomics era: light at the end of the tunnel or just another train? *Postgrad Med J,* 81**,** 505-509.

GASSER, R., NB, C. 2001a. Applications of single-strand conformation polymorphism (SSCP) to taxonomy, diagnosis, population genetics and molecular evolution of parasitic nematodes. . *Vet Parasitol* 101:**,** 201–213.

GASSER, R. B. 1999 PCR-based technology in veterinary parasitology. *Veterinary Parasitology.,* 84: **,** 229-258.

GASSER, R. B. 2001. Identification of parasitic nematodes and study of genetic variability using PCR approaches. pp. 53–82 in Kennedy, M.W. & Harnett, W. (Eds) Parasitic nematodes: Molecular biology, biochemistry and immunology. London, CAB International.

GASSER, R. B. 2006a. Molecular tools—Advances, opportunities and prospects. *Vet Parasitol,* 136 **,** 69–89.

GASSER, R. B. & CHILTON, N. B. 2001b. Applications of single-strand conformation polymorphism (SSCP) to taxonomy, diagnosis, population genetics and molecular evolution of parasitic nematodes. *Vet Parasitol,* 101**,** 201-213.

GASSER, R. B., CHILTON, N. B., HOSTE, H., BEVERIDGE, I. 1993 Rapid sequencing of rDNA from single worms and eggs of parasitic helminths. *Nucleic Acids Research* 21**,** 2525-2526.

GASSER, R. B., LEGOFF, L., PETIT, G., Bain O. 1996a. Rapid delineation of closely related filarial parasites using genetic markers in spacer rDNA . *Acta Tropica,* 62**,** 143–150.

GASSER, R. B., MONTI, J. R. 1997. Identification of parasitic nematodes by PCR-SSCP of ITS-2 rDNA. . *Mol Cell.Probes,* 11**,** 201–209.

GASSER, R. B., NEWTON, L. A. 2000. Genomic and genetic research on bursate nematodes: significance, implications and prospects. *Int J Parasitol,* 30**,** 509–534.

GASSER, R. B., ZHU, X. Q. & MCMANUS, D. P. 1998c. Display of sequence variation in PCR-amplified mitochondrial DNA regions of Echinococcus by single-strand conformation polymorphism. *Acta Trop,* 71**,** 107–115.

GASSER, R. B., HU, M., CHILTON, N. B., CAMPBELL, B. E., JEX, A. J., OTRANTO, D., CAFARCHIA, C., BEVERIDGE, I. & ZHU, X. 2006. Single-strand

conformation polymorphism (SSCP) for the analysis of genetic variation. *Nature Protocols,* 1**,** 3121-3128.

GLASS, W. I., POWER, P., BURT, R., FISHWICK, D., BRADSHAW, L. M., PEARCE, N. E. 1998. Work-related respiratory symptoms and lung function in New Zealand mussel openers. *Am J Ind Med,* 34**,** 163-168.

GLAZEBROOK, J., S., CAMPBELL, R. F. 1990. A survey of the diseases of marine turtles in northern Australia. I. Farmed turtles. *Dis Aquat Organ,* 9**,** 83–104.

GONZALEZ-FERNANDEZ, J., DASCHNER, A., NIEUWENHUIZEN, N. E., LOPATA, A. L., FRUTOS, C., VALLS, A. & CUELLAR, C. 2015. Haemoglobin, a new major allergen of Anisakis simplex. *Int J Parasitol*.

GOOD, M. F., STANISIC, D., XU, H., ELLIOTT, S., WYKES, M. 2004. The immunological challenge to developing a vaccine to the blood stages of malaria parasites. *Immunol Rev,* 201**,** 254-267.

GOTUA, M., LOMIDZE, N, DOLIDZE N, GOTUA, T. 2008. IgE-mediated food hypersensitivity disorders. *Georgian Med News,* 157**,** 39-44.

GRANT, W. N. 1994. Genetic variation in parasitic nematodes and its implications. *Int J Parasitol* 24**,** 821-830.

GREGORY, W., Maizels, R.M. 2007. Cystatins from filarial parasites: evolution, adaptation and function in the host-parasite relationship. *Int J Biochem Cell Biol,* 40**,** 1389-1398.

GRUNDY, J., MATTHEWS, S., BATEMAN, B., DEAN, T., SH, A. 2002. Rising prevalence of allergy to peanut in children. *J Allergy Clin Immunol. 2002,* 110**,** 784-789.

GUARNERI, F., GUARNERI, C. & BENVENGA, S. 2007. Cross-reactivity of Anisakis simplex: possible role of Ani s 2 and Ani s 3. *Int J Dermatol,* 46**,** 146-150.

GÜLEN, A. L. 2007. Gastrointestinal food hypersensitivity: Symptoms, diagnosis and provocation tests.*Turk J Gastroenterol,* 18, 5-13.

HALL, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series,* 41**,** 95–98.

HAMADA, Y., TANAKA, H., ISHIZAKI, S., ISHIDA, M., NAGASHIMA, Y., SHIOMI, K. 2003. Purification, reactivity with IgE and cDNA cloning of parvalbumin as the major allergen of mackerels. *Food Chem Toxicol,* 41**,** 1149-1156.

HARTMANN, S., LUCIUS, R. 2003. Modulation of host immune responses by nematode cystatins. International Journal for Parasitology . *Int J Parasitol,* 33**,** 1291-302.

HASHMI, S., ZHANG, J., OKSOV, Y., JI, Q., LUSTIGMAN, S. 2006. The Caenorhabditis elegans CPI-2a cystatin-like inhibitor has an essential regulatory role during oogenesis and fertilization. *J Biol Chem.* , 281**,** 28415-28429.

HASZPRUNAR, G. & WANNINGER, A. 2012. Molluscs. *Current Biology,* 22**,** R510-R514.

HAYASAKA, H., ISHIKURA, H., TAKAYAMA, T. 1971. Acute regional ileitis due to anisakis larvae. *Int Surg,* 55**,** 8-14.

HEDSTROM, L. 2002. Serine protease mechanism and specificity. *Chem Rev,* 102**,** 4501-4524.

HEWITSON, J. R., HARCUS, Y. M., CURWENB, R. S., DOWLE, A. A., ATMADJA, A. K., ASHTON, P. D., WILSON, A. & MAIZELS, R. M. 2008. The secretome of the filarial parasite, Brugia malayi: Proteomic profile of adult excretory-secretory products. *Molecular and Biochemical Parasitology,* 160**,** 8-21.

HILL, D. E., CHIRUKANDOTH, S., DUBEY, J. P. 2005. Biology and epidemiology of Toxoplasma gondii in man and animals. *Anim Health Res Rev,* 6**,** 41-61.

HILLIS, D. M. & DIXON, M. T. 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. *Q Rev Biol.* 66:**,** 411–453.

HOFFMAN, D., DAY ED JR & JS, M. 1981. The major heat stable allergen of shrimp. *Ann Allergy 1981,* 47**,** 17-22.

HOSTE, H., CHILTON, N. B., BEVERIDGE, I., GASSER, R. B. 1998. A comparison of the first internal transcribed spacer of ribosomal DNA in seven species of Trichostrongylus (Nematoda: Trichostrongylidae). *Int J Parasitol,* 28**,** 1251-1260.

HOTEZ, P. J., BUNDY, D. A. P., BEEGLE, K., BROOKER, S., DE SILVA, N., MONTRESOR, A., ENGELS, D., DRAKE, L., CHITSULO, L., MICHAUD, C., BETHONY, J. M., OLIVEIRA, R., XIAO, S. H., FENWICK, A., SAVIOLI, L. 2005. Disease Control Priorities in Developing Countries. 2nd Edition. WHO, World Bank, NIH, Oxford University Press. Helminth Infections. in press.

HU, M., CHILTON, N.B., ABS EL-OSTA, Y.G., GASSER, R.B. 2003a Comparative analysis of mitochondrial genome data for Necator americanus from two endemic regions reveals substantial genetic variation. *Int J Parasitol* 33**,** 955–963.

HU, M., CHILTON, N.B., GASSER, R.B. 2002. The mitochondrial genomes of the human hookworms, Ancylostoma duodenale and Necator americanus (Nematoda: Secernentea). *Int J Parasitol* 32**,** 145–158.

HU, M., CHILTON, N.B., GASSER, R.B. 2003b. The mitochondrial genome of Strongyloides stercoralis (Nematoda)—idiosyncratic gene order and evolutionary implications. *Int J Parasitol* 33**,** 1393–1408.

HU, M., GASSER, R. B. 2006. Mitochondrial genomes of parasitic nematodes - progress and perspectives. *Trends Parasitol,* 22**,** 78–84.

HU, Y., CHEN, J., LI, H. 2010. Comparison of food allergy prevalence among Chinese infants in Chongqing, 2009 versus 1999. *Pediatr Int.* 52**,** 820-824.

HUELSENBECK, J. P., and RONQUIST, F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. . *Bioinformatics,,* 17**,** 754–755.

HUFBAUER, R. A.,RODERICK, G. K. 2005. Microevolution in biological control: mechanisms, patterns, and processes. *Biological Control,* 35:**,** 227–239.

HUNG, G. C., CHILTON, N. B., BEVERIDGE, I., MCDONNELL, A., LICHTENFELS, J. R., GASSER, R. B. 1997. Molecular delineation of Cylicocyclus nassatus and C. ashworthi (Nematoda:Strongylidae). *Int J Parasitol,* 27**,** 601-605.

HUNG, G. C., JACOBS, D. E., KRECEK, R. C., GASSER, R. B., CHILTON, N. B. 1996. Strongylus asini (Nematoda, Strongyloidea): genetic relationships with other Strongylus species determined by ribosomal DNA. *Int J Parasitol,* 26**,** 1407-1411.

HUSS, H. H., EMBAREK, P.K.B. 2004. Assessment and management of seafood safety and quality. . *FAO Fisheries Technical Paper,* 444**,** 60-70.

IGLESIAS, R., LEIRO, J., SANTAMARINA, M. T., SANMARTIN, M. L., UBEIRA, F. M. 1997. Monoclonal antibodies against diagnostic Anisakis simplex antigens. *Parasitol Res,* 83**,** 755-761.

IGLESIAS, R., LEIRO, J., UBEIRA, F. M., SANTAMARINA, M. T., NAVARRETE, I., SANMARTIN, M. L. 1996. Antigenic cross-reactivity in mice between third-stage larvae of Anisakis simplex and other nematodes. *Parasitol Res,* 82**,** 378-381.

IKEDA, K., KUMASHIRO, R., KIFUNE, T. 1989. Nine cases of acute gastric anisakiasis. *Gastrointest Endosc,* 35**,** 304-308.

ISHIKAWA, M., ISHIDA, M., SHIMAKURA, K., NAGASHIMA, Y., SHIOMI, K. 1998a. Purification and IgE-binding epitopes of a major allergen in the gastropod Turbo cornutus. *Biosci Biotechnol Biochem,* 62**,** 1337-1343.

ISHIKAWA, M., ISHIDA, M., SHIMAKURA, K., NAGASHIMA, Y., SHIOMI, K. 1998b. Tropomyosin, the major oyster Crassostrea gigas allergen and its IgE-binding epitopes. *Journal of Food Science,* 63**,** 44-47.

ISHIKAWA, M., SHIMAKURA, K., NAGASHIMA, Y., SHIOMI, K. 1997. Isolation and properties of allergenic proteins in the oyster Crassostrea gigas. *Fish Sci,* 63**,** 610-614.

ISHIKAWA, M., SUZUKI, F., ISHIDA, M., NAGASHIMA, Y., SHIOMI, K. 2001. Identification of tropomyosin as a major allergen in the octopus Octopus vulgaris and elucidation of its IgE-binding epitopes. *Fish Sci,* 67**,** 934-942.

ISHIKURA, H., KIKUCHI, K., NAGASAWA, K., OOIWA, T., TAKAMIYA, H., SATO, N., SUGANE, K. 1993. Anisakidae and anisakidosis. *Prog Clin Parasitol,* 3**,** 43-102.

ISHIKURA, H., KIKUCHI, K., NAGASAWA, K., OOIWA, T., TAMIYA, H., SATO, N., SUGANE, K. 1990. Anisakidae and anisakidosis. In: Sun T, ed. Progress in clinical parasitology. Vol III. *New York: Springer-Verlag***,** 43–102.

SHIKURA, H., KOBAYASHI, Y., MIYAMOTO, K., YAGI, K., NAKAJIMA, O., FUJITA, O., OIKAWA, Y., MAEJIMA, J., AJI, T., AKAO, Y., et al. 1988. Transition of occurrence of anisakiasis and its paratenic host fishes in Japan, with pathogenesis of anisakiasis. *Hokkaido Igaku Zasshi,* 63**,** 376-391.

INIGUEZ, A. M., SANTOS, C. P. & VICENTE, A. C. 2009. Genetic characterization of Anisakis typica and Anisakis physeteris from marine mammals and fish from the Atlantic Ocean off Brazil. *Vet Parasitol,* 165**,** 350-356.

ISHIKURA, H., TAKAHASHI, S., YAGI, K., NAKAMURA, K., KON, S., MATSUURA, A., SATO, N., KIKUCHI, K. 1998. Epidemiology: global aspects of anisakidosis. *Icopa Ix - 9th International Congress of Parasitology***,** 379-382.

ISHIZAKA, K., ISHIZAKA, T., HORNBROOK, M. M. 1967a. Allergen-binding activity of gamma-E, gamma-G and gamma-A antibodies in sera from atopic patients. In vitro measurements of reaginic antibody. *J Immunol,* 98**,** 490-501.

ISHIZAKA, K., ISHIZAKA, T., TERRY, W. D. 1967b. Antigenic structure of gamma-E-globulin and reaginic antibody. *J Immunol,* 99**,** 849-858.

ITO, Y., IKEMATSU, Y., YUZAWA, H., NISHIWAKI, Y., KIDA, H., WAKI, S., UCHIMURA, M., OZAWA, T., IWAOKA, T., KANEMATSU, T. 2007. Chronic gastric anisakiasis presenting as pneumoperitoneum. *Asian Journal of Surgery,* 30**,** 67-71.

JABBAR, A., KHOON, A. T., HUI, T. X., SCHAEFFNER, B. C., JEX, A. R., NOLAN, M. J., LOPATA, A., GASSER, R. B., BEVERIDGE, I. 2012. Mutation scanning-based analysis of anisakid larvae from Sillago flindersi from Bass Strait, Australia. *Electrophoresis,* 33**,** 499-505.

JABBAR, A., FONG, R. W., KOK, K. X., LOPATA, A. L., GASSER, R. B. & BEVERIDGE, I. 2013. Molecular characterization of anisakid nematode larvae from 13 species of fish from Western Australia. *Int J Food Microbiol,* 161**,** 247-53.

JABBAR, A., KHOON, A. T., HUI, T. X., SCHAEFFNER, B. C., JEX, A. R., NOLAN, M. J., LOPATA, A., GASSER, R. B. & BEVERIDGE, I. 2012. Mutation scanning-based analysis of anisakid larvae from Sillago flindersi from Bass Strait, Australia. *Electrophoresis,* 33**,** 499-505.

JEEBHAY, M. F. & LOPATA, A. L. 2012. Chapter 2 – Occupational Allergies in Seafood-Processing Workers. *Adv Food Nutr Res,* 66**,** 47–73.

JEEBHAY, M. F., ROBINS, T. G., LEHRER, S. B., LOPATA, A. L. 2001. Occupational seafood allergy: a review. *Occup Environ Med,* 58**,** 553-62.

JEFFREYS, A. J., WILSON, E., THEIN, S. L. 1985. Hypervariable `minisatellite' regions in Human DNA',. *Nature, ,* 314:**,** 67-73.

JENKINS, R. E., TAYLOR, M. J., GILVARY, N. J., BIANCO, A. E. 1998. Tropomyosin implicated in host protective responses to microfilariae in onchocerciasis. *Proceedings of the National Academy of Sciences of the United States of America,* 95**,** 7550-7555.

JEONG, K. Y., HWANG, H., LEE, J. E. A. 2004a. Allergenic characterization of tropomyosin from the dusky brown cockroach, Periplaneta fuliginosa. *Clin Diagn Lab Immunol* 11**,** 680–685.

JOHANSSON, A., BOMAN, K., CEDERQUIST, K., FORSBERG, H., OLSSON, T. 2001. Increased levels of tPA antigen and tPA/PAI-1 complex in myotonic dystrophy. *Journal of Internal Medicine,* 249**,** 503-510.

JOHANSSON, S. G., BENNICH, H. 1967. Immunological studies of an atypical (myeloma) immunoglobulin. *Immunol,* 13**,** 381-394.

JOHNSON, P., MARSH, D. G. 1965. 'Isoallergens' from rye grass pollen. *Nature,* 206**,** 935-937.

JOHNSON, P., MARSH, D. G. 1966. Allergens from common rye grass pollen (Lolium perenne). II. The allergenic determinants and carbohydrate moiety. *Immunochemistry,* 3**,** 101-110.

JOHNSTON, T. H. 1910. Exhibit of a series of entozoa. *Proc Linn Soc. N.S.W. ,* 35**,** 309–310.

JOHNSTON, T. H. 1913. Notes on some entozoa. *Proc R Soc Qld,* 24**,** 63–91.

JOHNSTON, T. H. 1937. Entozoa from Australian hair seal. *Proc Linn Soc. N.S.W,* 62**,** 9–16.

JOHNSTON, T. H., MAWSON, P. M. 1939. Internal parasites of the pigmy sperm whale. *Rec S Aust Mus,* 6**,** 263–274.

JOHNSTON, T. H., MAWSON, P. M. 1940a. Some filarial parasites of Australian birds. *Trans R Soc S Aust,* 64**,** 355–361.

JOHNSTON, T. H., MAWSON, P. M. 1940b. Some nematode parasitic in Australian freshwater fish. *Trans R Soc S Aust,* 64**,** 340–352.

JOHNSTON, T. H., MAWSON, P. M. 1942b. Nematodes from Australian albatrosses and petrels. *Trans R Soc S Aust,* 66**,** 66–70.

JOHNSTON, T. H., MAWSON, P. M. 1942d. Some new and known Australian parasitic nematodes. *Proc Linn Soc. N.S.W,* 67**,** 90–94.

JOHNSTON, T. H., MAWSON, P. M. 1943a. Some ascarid nematodes from Australian marine fish. *Trans R Soc S Aust,* 67**,** 20–35.

JOHNSTON, T. H., MAWSON, P. M. 1943b. Some nematodes from Australian elasmobranchs. *Trans R Soc S Aust ,* 67**,** 187–190.

JOHNSTON, T. H., MAWSON, P. M. 1944. Remarks on some parasitic nematodes from Australia and New Zealand. *Trans R Soc Aus,* 68**,** 60–66.

JOHNSTON, T. H., MAWSON, P. M. 1947a. Some avian and fish nematodes, chiefly from Tailem Bend, South Australia. *Rec S Aust Mus,* 8**,** 547–553.

JOHNSTON, T. H., MAWSON, P. M. 1949. Some nematodes from Australian hosts together with a note on Rhabditis allgeni. *Trans R Soc S Aust,* 73**,** 63–71.

JOHNSTON, T. H., MAWSON, P. M. 1951a. Additional nematodes from Australian fish. *Trans R Soc S Aust,* 74**,** 18–24.

JOHNSTON, T. H., MAWSON, P. M. 1952. Some nematodes from Australian birds and mammals. *Trans R Soc S Aust,* 75**,** 30–37.

JOHNSTON, T. H., MAWSON, P. M. 1953. Parasitic nematodes and trematodes from Campbell and Auckland Islands (cape expedition). *Rec Dom Mus,* 2**,** 63–71.

JOHNSTON, T. H., MAWSON, P. M. 1945a. Parasitic nematodes. British, Australian and New Zealand Antarctic Research Expedition. *Reports Series B.* 5**,** 73–159.

JOHNSTON, T. H., MAWSON, P. M. 1941a. Additional nematodes from Australian birds. *Trans R Soc S Aust,* 65**,** 254–262.

JOHNSTON, T. H., MAWSON, P. M. 1945b. Some parasitic nematodes from south Australian marine fish *Trans R Soc S Aust,* 69**,** 114–117.

JOHNSTON, T. H.,MAWSON, P. M. 1942c. Remarks on some parasitic nematodes. *Rec S Aust Mus,* 7**,** 183–186.

JONES, R., BROWN, D. S., HARRIS, E., JONES, J., SYMONDSON, W. O. C., BRUFORD, M. W., CABLE, J. 2012. First record of Neoxysomatium brevicaudatum through the noninvasive sampling of Anguis fragilis: complementary morphological and molecular detection. *J Helminthol.* 86**,** 125–129.

KAGAN, R., JOSEPH, L., DUFRESNEC, E. A. 2003. Prevalence of peanut allergy in primary-school children in Montreal, Canada. *J Allergy Clin Immunol,* 112**,** 1223-1228.

KAKIZOE, S., KAKIZOE, H., KAKIZOE, K., KAKIZOE, Y., MARUTA, M., KAKIZOE, T., KAKIZOE, S. 1995. Endoscopic findings and clinical manifestation of gastric anisakiasis. *Am J Gastroenterol,* 90**,** 761-763.

KANG, D. B., OH, J. T., PARK, W. C., LEE, J. K. 2010. Small bowel obstruction caused by acute invasive enteric anisakiasis. *Korean J Gastroenterol,* 56**,** 192-195.

KANG, J., LEE, K.H., SOHN, W.M, E. A. 2011. Identification and functional characterization of CsStefin-1, a cysteine protease inhibitor of Clonorchis sinensis. *Mol Biochem Parasitol.* 177**,** 126-34.

KASSAI, T., CORDERO DEL CAMPILLO, M., EUZEBY, J., GAAFAR, S., HIEPE, T., HIMONAS, C. A. 1988. Standardized nomenclature of animal parasitic diseases (SNOAPAD). *Vet Parasitol,* 29**,** 299-326.

KASUYA, S., HAMANO, H., IZUMI, S. 1990. Mackerel-induced urticaria and Anisakis. *Lancet* ;335:665.

KENNEDY, M. W. 2000. Immune response to Anisakis simplex and other ascarid nematodes. *Allergy 55(Suppl. 59)***,** 7-13.

KENNEDY, M. W., TIERNEY, J., YE, P., MCMONAGLE, F. A., MCINTOSH, A., MCLAUGHLIN, D., SMITH, J. W. 1988. The secreted and somatic antigens of the third stage larva of Anisakis simplex, and antigenic relationship with Ascaris suum, Ascaris lumbricoides, and Toxocara canis. *Mol Biochem Parasitol,* 31**,** 35-46.

KHAZNADJI, E., COLLINS P., DALTON J. P., BIGOT Y., N, M. 2005. A new multi-domain member of the cystatin superfamily expressed by Fasciola hepatica. *Int. J. Parasitol,* 35**,** 1115–1125.

KIJEWSKA, A., ROKICKI, J., SITKO, J., WEGRZYN, G. 2002. Ascaridoidea: a simple DNA assay for identification of 11 species infecting marine and freshwater fish, mammals, and fish-eating birds. *Exp Parasitol,* 101**,** 35-39.

KIJEWSKA, A., CZARNA, A., FERNANDEZ, M., ZDZITOWIECKI, K., ROKICKI, J. & WROBEL, B. 2008. Analysis of 5.8S rDNA and internal transcribed spacer 1 (ITS1) sequences of ascaridoid nematodes: Phylogenetic signal and hypothesis testing. *Genes & Genomics,* 30**,** 291-306.

KIJEWSKA, A., DZIDO, J. & ROKICKI, J. 2009. Mitochondrial DNA of Anisakis Simplex Ss as a Potential Tool for Differentiating Populations. *Journal of Parasitology,* 95**,** 1364-1370.

KING, T. P., HOFFMAN, D., LOWENSTEIN, H., MARSH, D. G., PLATTS-MILLS, T. A. & THOMAS, W. 1995. Allergen nomenclature. *Allergy,* 50**,** 765-774.

KING, T. P. NORMAN, P. S. 1962. Isolation Studies of Allergens from Ragweed Pollen. *Biochemistry,* 4**,** 709-720.

KIRSTEIN, F., HORSNELL, W. G., NIEUWENHUIZEN, N., RYFFEL, B., LOPATA, A. L., BROMBACHER, F. 2010. Anisakis pegreffii-induced airway hyperresponsiveness is mediated by gamma interferon in the absence of interleukin-4 receptor alpha responsiveness. *Infect Immun,* 78**,** 4077-4086.

KLIKS, M. M. 1983a. Anisakiasis in the western united-states - 4 new case-reports from california. *Am J Tro Med and Hyg,* 32**,** 526-532.

KLIMPEL, S., PALM, H. W. 2001. Anisakid nematode (Ascaridoidea) life cycles and distribution. Increasing zoonotic potential in the time of climate change?. *Parasitology Research Monographs* 2**,** 201–222.

KLIMPEL, S., PALM, H. W., RUCKERT, S., PIATKOWSKI, U. 2004. The life cycle of Anisakis simplex in the Norwegian Deep (northern North Sea). *Parasitology Research,* 94**,** 1-9.

KOBAYASHI, A., TSUJI, M., WILBUR, D. L. 1985. Probable pulmonary anisakiasis accompanying pleural effusion. *Am J Trop Med Hyg,* 34**,** 310-313.

KOBAYASHI, Y., ISHIZAKI, S., SHIMAKURA, K., NAGASHIMA, Y., SHIOMI, K. 2007a. Molecular cloning and expression of two new allergens from Anisakis simplex. *Parasitology Research,* 100**,** 1233-1241.

KOBAYASHI, Y., SHIMAKURA, K., ISHIZAKI, S., NAGASHIMA, Y., SHIOMI, K. 2007b. Purification and cDNA cloning of a new heat-stable allergen from Anisakis simplex. *Molecular and Biochemical Parasitology,* 155**,** 138-145.

KOIE, M., BERLAND, B., BURT, M. D., B. 1995. Development to third-stage larvae occurs in the eggs of Anisakis simplex and Pseudoterranova decipiens (Nematoda, Ascaridoidea, Anisakiadae). National Research Council of Canada, Ottawa, ON, CANADA, 260 p.

KOYAMA, T. 1974. I. Anisakidae larvae. 1. Morphology and classification. Fish and anisakis (The Japanese Society of Fisheries Science), Koseisha koseikaku, pp.9-19. .

KUHN, T., GARCÍA-MÀRQUEZ, J., KLIMPEL, S. 2011. Adaptive radiation within marine anisakid nematodes: a zoogeographical modeling of cosmopolitan, zoonotic parasites. PLoS One 6, e28642. doi:10.1371/journal.pone.0028642.

KUITER, R. H. 2000. Coastal Fishes of South-eastern Australia. Gary Allen pp. 437.

LARSEN, J. N. & LOWENSTEIN, H. 1996. Allergen nomenclature. *J Allergy Clin Immunol,* 97**,** 577-578.

KUNZELMANN, K., SCHREIBER, R., KONIG, J., MALL, M. 2002. Ion transport induced by proteinase-activated receptors (PAR2) in colon and airways. *Cell Biochem Biophys,* 36**,** 209-214.

KWEE, H. G., SAUTTER, R. L. 1987. Anisakiasis. *American Family Physician,* 36**,** 137-140.

LAFFERTY, K. D., ALLESINA, S., ARIM, M., BRIGGS, C. J., DE LEO, G., DOBSON, A. P., et al. 2008. Parasites in food webs: the ultimate missing links. *Ecology Letters,* 11:**,** 533–546.

LEE, E. J., KIM, Y. C., JEONG, H. G., LEE, O. J. 2009. [The mucosal changes and influencing factors in upper gastrointestinal anisakiasis: analysis of 141 cases]. *Korean J Gastroenterol,* 53**,** 90-97.

LEES-MILLER, J. P., HELFMAN, D. M. 1991. The molecular basis for tropomyosin isoform diversity. *Bioessays,* 13**,** 429-437.

LEUNG, D. Y. M., SAMPSON, H. A., GEHA, R. S. & SZEFLER, S. J. 2003. Pediatric Allergy: Principles and Practice. *Missouri: Mosby.***,** p234.

LEUNG, P. S. C., CHEN, Y. C., GERSHWIN, M. E., WONG, H., KWAN, H. S., CHU, K. H. 1998a. Identification and molecular characterization of Charybdis feriatus tropomyosin, the major crab allergen. *J Allergy Clin Immunol,* 102**,** 847-852.

LEUNG, P. S. C., CHEN, Y. C., MYKLES, D. L., CHOW, W. K., LI, C. P., CHU, K. H. 1998b. Molecular identification of the lobster muscle protein tropomyosin as a seafood allergen. *Mol Mar Biol Biotechnol,* 7**,** 12-20.

LEUNG, P. S. C., CHU, K. H. 2001. cDNA cloning and molecular identification of the major oyster allergen from the Pacific oyster Crassostrea gigas. *Clin Exp Allergy,* 31**,** 1287-1294.

LEUNG, P. S. C., CHU, K. H., CHOW, W. K., ANSARI, A., BANDEA, C. I., KWAN, H. S., NAGY, S. M., GERSHWIN, M. E. 1994. Cloning, Expression, and Primary Structure of Metapenaeus-Ensis Tropomyosin, the Major Heat-Stable Shrimp Allergen. *J Allergy Clin Immunol,* 94**,** 882-890.

LEWONTIN, R. C., HUBBY, J. L. 1966. A molecular approach to the study of genic heterozygosity in natural populations. II. Amount of variation and degree of heterozygosity in natural populations of Drosophila pseudoobscura. *Genetics* 54:**,** 595–609.

LIANG, C. H., CHAN, L. P., CHOU, T. H., CHIANG, F. Y., YEN, C. M., CHEN, P. J., DING, H. Y., LIN, R. J. 2013. Brazilein from Caesalpinia sappan L. Antioxidant Inhibits Adipocyte Differentiation and Induces Apoptosis through Caspase-3 Activity and Anthelmintic Activities against Hymenolepis nana and Anisakis simplex. *Evidence-Based Complementary and Alternative Medicine*. Article ID 864892, 14 pages. http://dx.doi.org/10.1155/2013/864892.

LIEW, W., WILLIAMSON, E., MLK, T. 2009. Anaphylaxis fatalities and admissions in Australia. *J Allergy Clin Immunol* 123**,** 434-442.

LILLYWHITE, J. E., BUNDY, D. A., DIDIER, J. M., COOPER, E. S. & BIANCO, A. E. 1991. Humoral immune responses in human infection with the whipworm Trichuris trichiura. *Parasite Immunol,* 13**,** 491-507.

LINDSTROM, C. D., VAN DO, T., HORDVIK, I., ENDRESEN, C., ELSAYED, S. 1996. Cloning of two distinct cDNAs encoding parvalbumin, the major allergen of Atlantic salmon (*Salmo salar*). *Scand J Immunol,* 44**,** 335-44.

LITT, M., LUTY, J. A. 1989. A hypervariable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. *Am J Hum Genet, ,* 44**,** 397–401.

LITTLE, M. D., MACPHAIL, J. C. 1972. Large nematode larva from the abdominal cavity of a man in Massachusetts. *Am J Trop Med Hyg,* 21**,** 948-950.

LONG, E. O., DAWID, I. B. 1980 Repeated genes in eukaryotes. *Ann Rev Biochem,* 49**,** 727-764.

LOPATA, A. L., FENEMORE, B., JEEBHAY, M. F., GADE, G., POTTER, P. C. 2005a. Occupational allergy in laboratory workers caused by the African migratory grasshopper Locusta migratoria. *Allergy,* 60**,** 200-205.

LOPATA, A. L., FENEMORE, B. & POTTER, P. C. 2001a. Crustacean allergy in South Africa. . *Curr Allergy Clin Immunol* 14.

LOPATA, A. L., JEEBHAY, M. F. 2001b. Seafood allergy in South Africa ñ Studies in the domestic and occupational setting. *ACI International,* 13**,** 204-210.

LOPATA, A. L., JEEBHAY, M. F., GROENEWALD, M., POTTER, P. C., MOTALA, C., FENEMORE, B., SCHINKEL, M., LEE, S., CALVERT, J., SIBANDA, E. N., DU TOIT, G., MANJRA, A. 2005b. Sensitisation to three different cockroach species in Southern Africa. . *Current Allergy & Clinical Immunolog.,* 18**,** 62-66.

LOPATA, A. L. & LEHRER, S. B. 2009. New insights into seafood allergy. *Current Opinion in Allergy and Clinical Immunology,* 9**,** 270–277.

LOPATA, A. L., O'HEHIR, R. E., LEHRER, S. B. 2010. Shellfish allergy. *Clin Exp Allergy,* 40**,** 850-858.

LOPATA, A. L., POTTER, P. C. 2000. Allergy and other adverse reactions to seafood. *Allergy Clin Immunol Int.,* 12**.,** 271-281.

LOPATA, A. L., ZINN, C., POTTER, P. C. 1997. Characteristics of hypersensitivity reactions and identification of a unique 49 kd IgE-binding protein (Hal-m-1) in abalone (Haliotis midae). *J Allergy Clin Immunol,* 100**,** 642-648.

LOPEZ-SERRANO, M. C., GOMEZ, A. A., DASCHNER, A., MORENO-ANCILLO, A., DE PARGA, J. M. S., CABALLERO, M. T., BARRANCO, P., CABANAS, R. 2000. Gastroallergic anisakiasis: Findings in 22 patients. *J Gastroenterol Hepatol,* 15**,** 503-506.

LOWENSTEIN, H., KING, T. P., GOODFRIEND, L., HUSSAIN, R., ROEBBER, M., MARSH, D. G. 1981. Antigens of Ambrosia elatior (short ragweed) pollen. II. Immunochemical identification of known antigens by quantitative immunoelectrophoresis. *J Immunol,* 127**,** 637-42.

LUSTIGMAN, S., BROTMAN, B., HUIMA, T., AM, P. 1991. Characterization of an Onchocerca volvulus cDNA clone encoding a genus specific antigen present in infective larvae and adult worms. *Mol Biochem Parasitol,* 45**,** 65–75.

LUSTIGMAN, S., BROTMAN B, HUIMA T, P. A. & JH., M. 1992. Molecular cloning and characterisation of onchocystatin, a cysteine proteinase inhibitor of Onchocerca volvulus. *J Biol Chem,* 267**,** 17339–17346.

LUSTIGMAN, S., MCKERROW JH, SHAH K, L. J. & HUIMA T, E. A. 1996. Cloning of a cysteine protease required for the molting of Onchocerca volvulus third stage larvae. *J Biol Chem,* 271**,** 30181–30189.

LYMBERY, A. J., DOUPE, R. G., MUNSHI, M. A. & WONG, T. 2002. Larvae of Contracaecum sp. among inshore fish species of southwestern Australia. *Dis Aquat Organ,* 51**,** 157-159.

MACGREGOR, A. N. & SHORE, S. J. 1990. Immunocytochemistry of cytoskeletal proteins in adult Schistosoma mansoni. *Int J Parasitol,* 20**,** 279-84.

MAGGI, P., CAPUTI IAMBRENGHI, O., SCARDIGNO, A., SCOPETTA, L., SARACINO, A., VALENTE, M., PASTORE, G. & ANGARANO, G. 2000. Gastrointestinal infection due to Anisakis simplex in Southern Italy. *Europ J Entomol* 16**,** 75-78.

MARNANE, M. & BELLWOOD, D. R. 2002. Diet and nocturnal foraging in cardinal fishes (Apogonidae) at One Tree Reef, Great Barrier Reef, Australia. *Marine Ecology Progress Series,* 231**,** 261–268.

MARCOGLIESE, D. J. 2002. Food webs and the transmission of parasites to marine fish. *Parasitol,* 124**,** S83–99.

MARCOGLIESE, D. J. 2004. Parasites: small players with crucial roles in the ecological theater. *EcoHealth,* 1**,** 151–164.

MARSH, D. G., BIAS, W. B., SANTILLI, J., JR., SCHACTER, B., GOODFRIEND, L. 1975. Ragweed allergen Ra5: a new tool in understanding the genetics and immunochemistry of immune response in man. *Immunochemistry,* 12**,** 539-543.

MARTIN-SANCHEZ, J., ARTACHO-REINOSO, M. E., DIAZ-GAVILAN, M. & VALERO-LOPEZ, A. 2005. Structure of Anisakis simplex s.l. populations in a region sympatric for A-pegreffii and A-simplex s.s. Absence of reproductive isolation between both species. *Mol Biochemical Parasitol,* 141**,** 155-162.

MARTINEZ, A., MARTINEZ, J., PALACIOS, R., PANZANI, R. 1997. Importance of tropomyosin in the allergy to household arthropods. Cross-reactivity with other invertebrate extracts. *Allergol Immunopathol (Madr)* 25**,** 118-126.

MARQUARDT, W. C. 2000. Parasitology and vector biology. *2nd ed. New York: Academic Press.* **,** 442-443.

MASON, J. M. & ARNDT, K. M. 2004. Coiled coil domains: stability, specificity, and biological implications. *Chembiochem,* 5**,** 170-176.

MATA, L. 1982. Sociocultural factors in the control and prevention of parasitic diseases. *Rev Infect Dis,* 4**,** 871-879.

MATTIUCCI, S., NASCETTI, G., DAILEY, M., WEBB, S. C., BARROS, N. B., CIANCHI, R. & BULLINI, L. 2005. Evidence for a new species of Anisakis Dujardin, 1845: morphological description and genetic relationships between congeners (Nematoda : Anisakidae). *Systematic Parasitology,* 61**,** 157-171.

MATTIUCCI, S., ABAUNZA, P., RAMADORI, L. & NASCETTI, G. 2004. Genetic identification of Anisakis larvae in European hake from Atlantic and Mediterranean waters for stock recognition. *Journal of Fish Biology,* 65**,** 495-510.

MATTIUCCI, S. & NASCETTI, G. 2006. Molecular systematics, phylogeny and ecology of anisakid nematodes of the genus Anisakis Dujardin, 1845: an update. *Parasite,* 13**,** 99-113.

MATTIUCCI, S., FAZII, P., DE ROSA, A., PAOLETTI, M., MEGNA, A. S., GLIELMO, A., DE ANGELIS, M., COSTA, A., MEUCCI, C., CALVARUSO, V., SORRENTINI, I., PALMA, G., BRUSCHI, F., NASCETTI, G. 2013. Anisakiasis and gastroallergic reactions associated with Anisakis pegreffii infection, Italy. *Emerg Infect Dis,* 19**,** 496-499.

MATTIUCCI, S., NASCETTI, G. 2007. Genetic diversity and infection levels of anisakid nematodes parasitic in fish and marine mammals from Boreal and Austral hemispheres. *Vet Parasitol,* 148**,** 43-57.

MATTIUCCI, S., NASCETTI, G. 2008a. Advances and trends in the molecular systematics of anisakid nematodes, with implications for their evolutionary ecology and host-parasite co-evolutionary processes. *Adv Parasitol,* 66**,** 47-148.

MATTIUCCI, S., NASCETTI, G., CIANCHI, R., PAGGI, L., ARDUINO, P., MARGOLIS, L., BRATTEY, J., WEBB, S., DAMELIO, S., ORECCHIA, P., BULLINI, L. 1997. Genetic and ecological data on the Anisakis simplex complex, with evidence for a new species (Nematoda, Ascaridoidea, Anisakidae). *J Parasitol,* 83**,** 401-416.

MATTIUCCI, S., PAGGI, L., NASCETTI, G., ABOLLO, E., WEBB, S. C., PASCUAL, S., CIANCHI, R., BULLINI, L. 2001. Genetic divergence and reproductive isolation between Anisakis brevispiculata and Anisakis physeteris (Nematoda: Anisakidae)s. *Int J Parasitol,* 31**,** 9-14.

MATTIUCCI, S., PAGGI, L., NASCETTI, G., PORTES SANTOS, C., COSTA, G., DI BENEDITTO, A. P., RAMOS, R., ARGYROU, M., CIANCHI, R., BULLINI, L. 2002. Genetic markers in the study of Anisakis typica (Diesing, 1860): larval identification and genetic relationships with other species of Anisakis Dujardin, 1845 (Nematoda: Anisakidae). *Syst Parasitol,* 51**,** 159-170.

MATTIUCCI, S., PAOLETTI, M., BORRINI, F., PALUMBO, M., PALMIERI, R. M., GOMES, V., CASATI, A., NASCETTI, G. 2011. First molecular identification of the zoonotic parasite *Anisakis pegreffii* (Nematoda: Anisakidae) in a paraffin-embedded granuloma taken from a case of human intestinal anisakiasis in Italy. *BMC Infect Dis,* 11**,** 82.

MATTIUCCI, S., PAOLETTI, M., WEBB, S. C. 2009. Anisakis nascettii n. sp. (Nematoda: Anisakidae) from beaked whales of the southern hemisphere: morphological description, genetic relationships between congeners and ecological data. *Syst Parasitol,* 74**,** 199-217.

MATTIUCCI, S., PAOLETTI, M., WEBB, S. C., SARDELLA, N., TIMI, J. T., BERLAND, B., NASCETTI, G. 2008b. Genetic relationships among species of Contracaecum Railliet & Henry, 1912 and Phocascaris Host, 1932 (Nematoda : Anisakidae) from pinnipeds inferred from mitochondrial cox2 sequences, and congruence with allozyme data. *Parasite,* 15**,** 408-419.

MATTIUCCI, S., PAOLETTI, M., WEBB, S. C., SARDELLA, N., TIMI, J. T., BERLAND, B., NASCETTI, G. 2008c. Genetic relationships among species of Contracaecum Railliet & Henry, 1912 and Phocascaris Host, 1932 (Nematoda: Anisakidae) from pinnipeds inferred from mitochondrial cox2 sequences, and congruence with allozyme data. *Parasite,* 15**,** 408-419.

MAWSON, P. 1953. Parasitic nematoda collected by the Australian national Antarctic research expedition: Heard Island and Macquarie Island, 1948–1951. *Parasitol Res Monographs,* 43:**,** 291–297.

MAWSON, P. 1957. Some nematodes from fish from Heron Island, Queensland. *Trans R Soc S Aust,* 80**,** 177–179.

MAWSON, P. 1969. Some nematodes from Australian gulls and terns. *J Fish Res Res Bd Canada* 26**,** 1103–1111.

MAWSON, P. M., ANGEL, M., EDMONDS, S. J. 1986. A checklist of helminths from Australian birds *Rec S Aust Mus,* 19**,** 219–325.

MCCLELLAND, G. 2002. The trouble with sealworms (Pseudoterranova decipiens species complex, Nematoda): a review. *Parasitology,* 124**,** S183-S203.

MCMANUS, D. P., BOWLES, J. 1996. Molecular genetic approaches to parasite identification: their value in diagnostic parasitology and systemic. *Int J Parasitol,* 26**,** 687–704.

MIYAZAWA, H., FUKAMACHI, H., INAGAKI, Y., REESE, G., DAUL, C. B., LEHRER, S. B., INOUYE, S., SAKAGUCHI, M. 1996. Identification of the first major allergen of a squid (Todarodes pacificus). *J Allergy Clin Immunol,* 98**,** 948-53.

MLADINEO, I., ŠIMAT, V., MILETIĆ, J.,BECK, R., POLJAK, V 2012. Molecular identification and population dynamic of Anisakis pegreffii (Nematoda: Anisakidae Dujardin, 1845) isolated from the European anchovy (Engraulis encrasicolus L.) in the Adriatic Sea. *Int J Food Microbiol* 157**,** 224–229.

MONEO, I., CABALLERO, M. L., GONZALEZ-MUNOZ, M., RODRIGUEZ-MAHILLO, A. I., RODRIGUEZ-PEREZ, R., SILVA, A. 2005b. Isolation of a heat-resistant allergen from the fish parasite Anisakis simplex. *Parasitol Res,* 96**,** 285-289.

MONTORO, A., PERTEGUER, M. J., CHIVATO, T., LAGUNA, R., CUELLAR, C. 1997. Recidivous acute urticaria caused by Anisakis simplex. *Allergy & Clinical Immunology International,* 52**,** 985–991.

MORAND, S., CRIBB, T. H., KULBICKI, M., RIGBY, M. C., CHAUVET, C., DUFOUR, V., FALIEX, E., GALZIN, R., LO, C. M., LO-YAT, A., PICHELIN, S. & SASAL, P. 2000. Endoparasite species richness of New Caledonian butterfly fishes: host density and diet matter. *Parasitology,* 121**,** 65-73.

MORENO-ANCILLO, A., CABALLERO, M. T., CABANAS, R. 1997. Allergic reactions to Anisakis simplex parasitizing seafood. *Ann Allergy Asthma Immunol* 79**,** 246–250.

MORGAN, J. A. T., BLAIR, D. 1998. Relative merits of nuclear ribosomal internal transcribed spacers and mitochondrial CO1 and ND1 genes for distinguishing among Echinostoma species (Trematoda). *Parasitol Res,* 116**,** 289–297.

MORIKAWA, A., KATO, M., TOKUYAMA, K., KUROUME, T., MINOSHIMA, M. & IWATA, S. 1990. Anaphylaxis to grand keyhole limpet (abalone-like shellfish) and abalone. *Ann Allergy,* 65**,** 415-417.

MOSCHELLA, C. M., MATTIUCCI, S., MINGAZZINI, P., DE ANGELIS, G., ASSENZA, M., LOMBARDO, F., MONACO, S., PAGGI, L., MODINI, C. 2004a. Intestinal anisakiasis in Italy: case report. *J. Helminthol. ,* 78:**,** 271–273.

MOSCHELLA, C. M., MATTIUCCI, S., MINGAZZINI, P., MONGARDINI, M., CHEIN, A., MICCOLIS, D., MODINI, C. 2005. Intestinal anisakiasis in Italy: a case treated by emergence surgery. *G Chir,* 26**,** 201-205.

MOSCHELLA, S. L., KAY, J., MACKOOL, B. T., LIU, V. 2004b. Case records of the Massachusetts General Hospital. Weekly clinicopathological exercises. Case 35-2004. A 68-year-old man with end-stage renal disease and thickening of the skin. *N Engl J Med,* 351**,** 2219-2227.

MULLINS, R. 2007. Paediatric food allergy trends in a community-based specialist allergy practice, 1995-2006. *Med J Aust* 186**,** 618-621.

MULLINS, R., DEAR, K., ML, T. 2009. Characteristics of childhood peanut allergy in the Australian Capital Territory 1995-2007. *J Allergy Clin Immunol* 123**,** 689-993.

MUNÕZ, G., GRUTTER, A. S. & CRIBB, T. H. 2007. Structure of the parasite communities of a coral reef fish assemblage (Labridae): testing ecological and phylogenetic host factors. *J Parasitol,* 93, 17-30.

MURATA, R., SUZUKI, J., SADAMASU, K., KAI, A. 2011. Morphological and molecular characterization of Anisakis larvae (Nematoda: Anisakidae) in Beryx splendens from Japanese waters. *Parasitol Int,* 60**,** 193-198.

MYKLES, D. L., COTTON, J. L. S., TANIGUCHI, H., SANO, K. I., MAEDA, Y. 1998. Cloning of tropomyosins from lobster (Homarus americanus) striated muscles: fast and slow isoforms may be generated from the same transcript. *J Muscle Res Cell Motil,* 19**,** 105-115.

NADLER, S. A. 1995b. Genetic structure of midwestern Ascaris suum populations: a comparison of isoenzyme and RAPD markers. *J. Parasitol.,* 81**,** 385-394.

NADLER, S. A. 1995a Microevolution and the genetic structure of parasite populations. *J. Parasitol,* 81**,** 395-403.

NADLER, S. A. 1990. Molecular approaches to studying helminth population genetics and phylogeny. *Int J Parasitol.*20**,** 11 -29.

NADLER, S. A., D'AMELIO, S., DAILEY, M. D., PAGGI, L., SIU, S. & SAKANARI, J. A. 2005. Molecular phylogenetics and diagnosis of Anisakis, Pseudoterranova, and Contracaecum from Northern Pacific marine mammals. *Journal of Parasitology,* 91**,** 1413-1429.

NADLER, S. A., D'AMELIO, S., FAGERHOLM, H. P., BERLAND, B. & PAGGI, L. 2000. Phylogenetic relationships among species of Contracaecum Railliet & Henry, 1912 and Phocascaris Host, 1932 (Nematoda : Ascaridoidea) based on nuclear rDNA sequence data. *Parasitology,* 121**,** 455-463.

NAGARAJ, S. H., GASSER, R. B. & RANGANATHAN, S. 2008. Needles in the EST haystack: largescale identification and analysis of excretory–secretory (ES) proteins in parasitic nematodes using expressed sequence tags (ESTs).

NAKANO, S., YOSHINUMA, T. & YAMADA, T. 2008. Reactivity of shrimp allergy-related IgE antibodies to krill tropomyosin. *Int Arch Allergy Immunol,* 145**,** 175–181.

NAMIKI, M. 1989. A history of research into gastric anisakiasis in Japan. In Gastric Anisakiasis in Japan (Edited by H. Ishikura and N. Namiki), pp. 13-18. Springer-Verlag, New York.

NASCETTI, G., PAGGI, L., ORECCHIA, P., SMITH, J. W., MATTIUCCI, S., BULLINI, L. 1986. Electrophoretic studies on the Anisakis simplex complex (Ascaridida:Anisakidae) from the Mediterranean and North-East Atlantic. *Int J Parasitol,* 16**,** 633-40.

NASH, J. A. 1998. The ecology of endoparasites of whiting, Sillago flindersi. The University of New England, Armidale, NSW, Australia.

NDAO, M. 2009. Diagnosis of parasitic diseases: old and new approaches. *Interdiscip Perspect Infect Dis,* 2009**,** 278246.

NEWLANDS, G. F., SKUCE, P. J., KNOX, D. P., SMITH, W. D. 2001. Cloning and expression of cystatin, a potent cysteine protease inhibitor from the gut of Haemonchus contortus. *Parasitol Res,* 122**,** 371–378.

NI, J., ABRAHAMSON, M., ZHANG, M., FERNANDEZ, M. A., GRUBB, A., SU, J., YU, G. L., LI, Y. L., PARMELEE, D., XING, L., COLEMAN, T. A., GENTZ, S., THOTAKURA, R., NGUYEN, N., HESSELBERG, M. & GENTZ, R. 1997. Cystatin E is a novel human cysteine proteinase inhibitor with structural resemblance to family 2 cystatins. *J Biol Chemist,* 272**,** 10853-10858.

NICKLIN, M. J. H., BARRET, A. J. 1984. Inhibition of cysteine proteinases and dipeptidyl peptidase I by egg-white cystatin. *Biochem J,* 223**,** 245–253.

NIELSEN, C. 1998. Systematics - Sequences lead to tree of worms. *Nature,* 392**,** 25-26.

NIEUWENHUIZEN, N., HERBERT, D. R., BROMBACHER, F., LOPATA, A. L. 2009. Differential requirements for interleukin (IL)-4 and IL-13 in protein contact dermatitis induced by Anisakis. *Allergy Clin Immunol Int,* 64**,** 1309–1318.

NIEUWENHUIZEN, N., LOPATA, A. L., JEEBHAY, M. F., HERBERT, D. R., ROBINS, T. G., BROMBACHER, F. 2006. Exposure to the fish parasite Anisakis causes allergic airway hyperreactivity and dermatitis. *J Allergy Clin Immunol,* 117**,** 1098-105.

NIEUWENHUIZEN, N. E., LOPATA, A. L. 2013. Anisakis - A food-borne parasite that triggers allergic host defences. *Int J Parasitol,* 43**,** 1047-1057.

NYSTEDT, S., EMILSSON, K., WAHLESTEDT, C., SUNDELIN, J. 1994. Molecular cloning of a potential proteinase activated receptor. *Proc Natl Acad Sci U S A,* 91**,** 9208-9212.

O'NEIL, C., HELBLING, A. A., LEHRER, S. B. 1993. Allergic reactions to fish. *Clin Rev Allergy,* 11**,** 183-200.

O'DONNELL, I. J., DINEEN, J. K., WAGLAND, B. M., LETHO, S., DOPHEIDE, T. A. A., GRANT, W. N., WARD, C. W. 1989. Characterization of the Major Immunogen in the Excretory Secretory Products of Exsheathed 3rd-Stage Larvae of Trichostrongylus-Colubriformis. *Int J Parasitol,* 19**,** 793-802.

OHMAN, J. L., JR., LOWELL, F. C., BLOCH, K. J. 1974. Allergens of mammalian origin. III. Properties of a major feline allergen. *J Immunol,* 113**,** 1668-1677.

OHTAKI, H., OHTAKI., R. 1989. Clinical manifestation of gastric anisakiasis, p. 37-46. In H. Ishikura and M. Namiki (ed.), Gastric anisakiasis in Japan. Epidemiology, diagnosis, treatment. Springer-Verlag, Tokyo, Japan.

ORITA, M., SUZUKI, Y., SEKIYA, T., HAYASHI, K. 1989. Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics* 5**,** 874–879.

OSHIMA, T. 1972. Anisakis and anisakiasis in Japan and adjacent area. In: Progress of Medical Parasitology in Japan, Volume 4. Morishita K, Komiya Y, and Matsubayashi H (eds.). Tokyo: Meguro Parasitological Museum. 301–393.

PAGGI, L., MATTIUCCI, S. & D'AMELIO, S. 2001. Allozyme and PCR-RFLP markers in anisakid nematodes, aethiological agents of human anisakidosis. *Parassitologia (Rome),* 43**,** 21-27.

PAGGI, L., NASCETTI, G., WEBB, S. C., MATTIUCCI, S., CIANCHI, R. & BULLINI, L. 1998. A new species of Anisakis Dujardin, 1845 (Nematoda, Anisakidae) from beaked whales (Ziphiidae): allozyme and morphological evidence. *Systematic Parasitology,* 40**,** 161-174.

PALM, H. W., LINDA, L., DAMRIYASA, I. M., OKA, I. B. M. 2008. Molecular genotyping of Anisakis Dujardin, 1845 (Nematoda: Ascaridoidea: Anisakidae) larvae from marine fish of Balinese and Javanese waters, Indonesia. *Helminthol,* 45**,** 3–12.

PAMPIGLIONE, S., RIVASI, F., CRISCUOLO, M., DE BENEDETTIS, A., GENTILE, A., RUSSO, S., TESTINI, M., VILLANI, M. 2002. Human anisakiasis in Italy: A report of eleven new cases. *Path Res Pract,* 198**,** 429-434.

PANDEY, K. 2013. Macromolecular inhibitors of malarial cysteine proteases —An invited review. *J BiomedSci Eng,* 6**,** 885-895.

PASCUAL, C. Y., CRESPO, J. F., SANMARTIN, S., ORNIA, N., ORTEGA, N., CABALLERO, T., MUNOZPEREIRA, M. 1997a. Cross-reactivity between IgE-binding proteins from Anisakis, German cockroach, and chironomids. *Allergy,* 52**,** 514-520.

PASCUAL, D. W., WALTERS, N., HELLEMEYER, P., LOETTERLE, L., POWELL, R. J., HONE, D. M. 1997b. Placement of vaccine antigen onto surface of Salmonella typhimurium alters the Th cell phenotype from Th1 to Th2 type response following oral immunization. *J Allergy Clin Immunol,* 99**,** 141-141.

PATWARY, M. U., REITH, M., KENCHINGTON, E. L. 1999. Cloning and characterization of tropomyosin cDNAs from the sea scallop Placopecten magellanicus (Gmelin, 1791). *J Shellfish Res,* 18**,** 67-70.

PEREIRA, T., FONSECA, G., MUNDO-OCAMPO, M., GUILHERME, B.C., A, R.-O. 2010 Diversity of free-living marine nematodes (Enoplida) from Baja California assessed by integrative taxonomy. . *Mar Biol* 157**,** 1665-1678.

PEREZ-PEREZ, J., FERNANDEZ-CALDAS, E., MARANON, F., et al. 2000. Molecular cloning of ˜ paramyosin, a new allergen of Anisakis simplex. *Int Arch Allergy Immunol,* 123**,** 120–129.

PERMYAKOV, S. E., KHOKHLOVA, T. I., NAZIPOVA, A. A., ZHADAN, A. P., MOROZOVA-ROCHE, L. A., PERMYAKOV, E. A. 2006. Calcium-binding and temperature induced transitions in equine lysozyme: New insights from the pCa-temperature "phase diagrams". *Proteins,* 65**,** 984-998.

PETITHORY, J. C., CHAUMEIL, C., LIOTET, S., ROSSEAU, M, , BISOGNANI, A. C., IAFUSCO, D. 1993. Immunological studies on ocular larva migrans. In: Lewis JW, Maizels RM, editors. Toxocara and Toxocariasis, clinical, epidemiological and molecular perspectives. London: British Society for Parasitology and Institute of Biology;. 81-89.

PETITHORY, J. C., LAPIERRE, J., ROUSSEAU, M., CLIQUE, M. T. 1986. Serology of Anisakiase (Eosinophilic Digestive Granuloma) by Gel-Diffusion Precipitin Test (Ouchterlony, Immunodiagnosis). *Medecine Et Maladies Infectieuses,* 16**,** 157-162.

PIBOONPOCANUN, S., MALAINUAL, N., JIRAPONGSANANURUK, O., VICHYANOND, P., THOMAS, W. R. 2006. Genetic polymorphisms of major house dust mite allergens. *Clin Exp Allergy,* 36**,** 510-516.

PITTENGER, M. F., KAZZAZ, J. A., HELFMAN, D. M. 1994. Functional properties of non-muscle tropomyosin isoforms. *Curr Opin Cell Biol,* 6**,** 96-104.

PITTENGER, M. F., KAZZAZ, J. A. & HELFMAN, D. M. 1994. Functional properties of non-muscle tropomyosin isoforms. *Curr Opin Cell Biol,* 6**,** 96-104.

POCZAI, P., HYVÖNEN, J. 2010. Nuclear ribosomal spacer regions in plant phylogenetics: problems and prospects. *Mol Biol Rep***,** 37, 4, pp 1897-1912.

PONTES, T., D'AMELIO, S., COSTA, G., PAGGI, L. 2005. Molecular characterization of larval anisakid nematodes from marine fishes of Madeira by a PCR-based approach, with evidence for a new species. *J Parasitol,* 91**,** 1430-1434.

POSADA, G. & CRANDALL, K. A. 1998. Model test: testing the model of DNA substitution. *Bioinformatics,,* 14**,,** 817–818.

PRAUSNITZ AND KUSTNER, H. 1921. Studien uber die Uberempfindlichkeit. *Zentr. Bakteriol. Parasitenk. Abt. I Orig,* 66**,** 160-190.

PRITCHARD, D. I., WILSON, R.A. 1997. Helminth infections. *In Robert A Stockley, Pulmonary Defences. John Wiley and Sons Ltd.*

PROKOPOWICH, C., GREGORY, T.R,, CREASE, T. J. 2003. The correlation between rDNA copy number and genome size in eukaryotes. *Genome* 46**,** 48–50.

PUENTE, P., ANADON, A. M., RODERO, M., ROMARIS, F., UBEIRA, F. M., CUELLAR, C. 2008. Anisakis simplex: the high prevalence in Madrid (Spain) and its relation with fish consumption. *Exp Parasitol,* 118**,** 271-274.

QUIAZON, K. M. A., YOSHINAGA, T., OGAWA, K. 2011. Experimental challenge of *Anisakis simplex sensu stricto* and *Anisakis pegreffii* (Nematoda: Anisakidae) in rainbow trout and olive flounder. *Parasitol Int,* 60**,** 126-131.

QUIAZON, K. M. A., YOSHINAGA, T., OGAWA, K., YUKAMI, R. 2008. Morphological differences between larvae and in vitro-cultured adults of Anisakis simplex (sensu stricto) and *Anisakis pegreffii* (Nematoda: Anisakidae). *Parasitol Int,* 57**,** 483-489.

RAHMAN, A. M. A., KAMATH, S., LOPATA, A. L., HELLEUR, R. J. 2010. Analysis of the allergenic proteins in black tiger prawn (Penaeus monodon) and characterization of the major allergen tropomyosin using mass spectrometry. *Rapid Commun Mass Spectrom,* 24**,** 2462-2470.

RANDALL, R. W., GIBBS, H. C. 1981. Effects of clinical and subclinical gastrointestinal helminthiasis on digestion and energy metabolism in calves. *Am J Vet Res,* 42**,** 1730-1734.

REED, C. E. 2007a. Inflammatory effect of environmental proteases on airway mucosa. *Curr Allergy Asthma Rep,* 7**,** 368-374.

REESE, G., JEOUNG, B. J., DAUL, C. B., LEHRER, S. B. 1997. Characterization of recombinant shrimp allergen Pen a 1 (Tropomyosin). *International Archives of Allergy and Immunology,* 113**,** 240-242.

REESE, G., AYUSO, R. & LEHRER, S. B. 1999. Tropomyosin: an invertebrate pan-allergen. *Int Arch Allergy Immunol,* 119, 247-258.

REPISO ORTEGA, A., ALCANTARA TORRES, M., GONZALEZ DE FRUTOS, C., DE ARTAZA VARASA, T., RODRIGUEZ MERLO, R., VALLE MUNOZ, J. & MARTINEZ POTENCIANO, J. L. 2003. Gastrointestinal anisakiasis. Study of a series of 25 patients. *Gastroenterol Hepatol,* 26**,** 341-346.

RING, J., BROCKOW, K. & BEHRENDT, H. 2001. Adverse reactions to foods. *J Chromatography B,* 756**,** 3–10.

RODERO, M., CHIVATO, T., MURO, A., CUELLAR, C. 2005. Enzyme-linked immunosorbent assay and Western blot antibody determination in sera from patients

diagnosed with different helminthic infections with Anisakis simplex antigen purified by affinity chromatography. *Mem Inst Oswaldo Cruz,* 100**,** 293-301.

RODRIGUEZ-MAHILLO, A. I., GONZALEZ-MUN˜OZ, M., GOMEZ-AGUADO, F., RODRı´-GUEZ-PEREZ, R., CORCUERA, M. T., CABALLERO, M. L., MONEO, I. 2007. Cloning and characterization of the Anisakis simplex allergen Ani s4 as a cysteine-protease inhibitor. *Int J Parasitol,* 37**,** 907–917.

RODRIGUEZ-PEREZ, R., MONEO, I., RODRIGUEZ-MAHILLO, A., CABALLERO, M. L. 2008. Cloning and expression of Ani s 9, a new Anisakis simplex allergen. *Mol Biochem Parasitol,* 159**,** 92-97.

ROEBER, F., JEX, A., GASSER, R. 2013. Impact of gastrointestinal parasitic nematodes of sheep, and the role of advanced molecular tools for exploring epidemiology and drug resistance - an Australian perspective. *Parasit Vectors,* 6:153.

ROMERO, M. D., VALERO, A., NAVARRO-MOLL, M. C., MARTIN-SANCHEZ, J. 2013. Experimental comparison of pathogenic potential of two sibling species *Anisakis simplex s.s*. and *Anisakis pegreffii* in Wistar rat. *Trop Med Int Health,* 18**,** 979-984.

RONQUIST, F., ; AND HUELSENBECK, J. P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics,,* 19, **,** 1572–1574.

ROOS, M. H., GRANT, W. N. 1993 Species-specific PCR for the parasitic nematodes Haemonchus contortus and Trichostrongylus colubriformis. *Int J Parasitol,* 23**,** 419-421.

RUITENBERG, E. J., VAN KNAPEN, F., WEISS, J. W. 1979. Food-borne parasitic infections - old stories and new facts. *Vet Q,* 1**,** 5-13.

RUSHOVICH, A. M., RANDALL, E. L., CAPRINI, J. A., WESTENFELDER, G. O. 1983. Omental anisakiasis: a rare mimic of acute appendicitis. *Am J Clin Pathol,* 80**,** 517-20.

SAKANARI, J. A., LOINAZ, H. M., DEARDORFF, T. L., RAYBOURNE, R. B., MCKERROW, J. H., FRIERSON, J. G. 1988. Intestinal anisakiasis. A case diagnosed by morphologic and immunologic methods. *Am J Clin Pathol,* 90**,** 107-13.

SAKANARI, J. A., MCKERROW, J. H. 1989. Anisakiasis. *Clin Microbiol Rev ,* 2**,** 278–284.

SAMBROOK, J., FRITSCH, E. F., MANIATIS, T. 1989. Molecular Cloning: A Laboratory Manual, second ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

SAMPSON, H. 1999. Food allergy. Part 1: immunopathogenesis and clinical disorders. *J Allergy Clin Immunol,* 103**,** 717-728.

SANTOS, A. B., CHAPMAN, M. D., AALBERSE, R. C., VAILES, L. D., FERRIANI, V. P., OLIVER, C., RIZZO, M. C., NASPITZ, C. K., ARRUDA, L. K. 1999.

Cockroach allergens and asthma in Brazil: identification of tropomyosin as a major allergen with potential cross-reactivity with mite and shrimp allergens. *J Allergy Clin Immunol,* 104**,** 329-37.

SANTOS, A. B. R., ROCHA, G. M., OLIVER, C., FERRIANI, V. P. L., LIMA, R. C., PALMA, M. S., SALES, V. S. F., AALBERSE, R. C., CHAPMAN, M. D., ARRUDA, L. K. 2008. Cross-reactive IgE antibody responses to tropomyosins from Ascaris lumbricoides and cockroach. *J Allergy Clin Immunol,* 121**,** 1040-1046.

SANTORO, M., MATTIUCCI, S., PAOLETTI, M., LIOTTA, A., UBERTI, B. D., GALIERO, G. & NASCETTI, G. 2010. Molecular identification and pathology of Anisakis pegreffii (Nematoda: Anisakidae) infection in the Mediterranean loggerhead sea turtle (Caretta caretta). *Vet Parasitol,* 174**,** 65-71.

SCALA, E., GIANI, M., PIRROTTA, L., GUERRA, E. C., CADONI, S., GIRARDELLI, C. R., DE PITA, O., PUDDU, P., 2001 2001. Occupational generalised urticaria and allergic airborne asthma due to Anisakis simplex. Eur. *J. Dermatol.,* 11:.

SCHIERACK, P., LUCIUS, R., SONNENBURG, B., SCHILLING, K. & HARTMANN, S. 2003. Parasite-specific immunomodulatory functions of filarial cystatin. *Infection and Immunity,* 71**,** 2422-2429.

SCHEVZOV, G., VRHOVSKI, B., BRYCE, N. S., ELMIR, S., QIU, M. R., O'NEILL G, M., YANG, N., VERRILLS, N. M., KAVALLARIS, M., GUNNING, P. W. 2005. Tissue-specific tropomyosin isoform composition. *J Histochem Cytochem,* 53**,** 557-570.

SCHMIDLIN, F., AMADESI, S., VIDIL, R., TREVISANI, M., MARTINET, N., CAUGHEY, G., TOGNETTO, M., CAVALLESCO, G., MAPP, C., GEPPETTI, P., BUNNETT, N. W. 2001. Expression and function of proteinase-activated receptor 2 in human bronchial smooth muscle. *Am J Respir Crit Care Med,* 164**,** 1276-1281.

SCHÖNEMEYER, A., LUCIUS, R., SONNENBURG, B., BRATTIG, N., SABAT, R., SCHILLING, K., BRADLEY, J. HARTMANN S. 2001. Modulation of human T cell responses and macrophage functions by onchocystatin, a secreted protein of the filarial nematode Onchocerca volvulus. *J Immunol,* 167**,** 3207-3215.

SCHWARTZ, E., HATZ, C., BLUM, J. 2006. New world cutaneous leishmaniasis in travellers. *Lancet Infect Dis,* 6**,** 342-9.

SEREDA, M. J., HARTMANN, S., LUCIUS, R. 2008. Helminths and allergy: the example of tropomyosin. *Trends Parasitol,* 24**,** 272-278.

SEQUEIRA, V., GORDO, L. S., NEVES, A., PAIVA, R. B., CABRAL, H. N. & MARQUES, J. F. 2010. Macroparasites as biological tags for stock identification of the bluemouth, Helicolenus dactylopterus (Delaroche, 1809) in Portuguese waters. *Fisheries Research,* 106**,** 321-328.

SHAMSI, S. 2007. Morphologic and genetic characterisation of selected ascaridoid nematodes. PhD Thesis, The University of Melbourne.

SHAMSI, S., BUTCHER, A. R. 2011a. First report of human anisakidosis in australia. *Med J Aust,* 194**,** 199-200.

SHAMSI, S., EISENBARTH, A., SAPTARSHI, S., BEVERIDGE, I., GASSER, R. B., LOPATA, A. L. 2011b. Occurrence and abundance of anisakid nematode larvae in five species of fish from southern Australian waters. *Parasitol Res,* 108**,** 927-934.

SHAMSI, S., GASSER, R., BEVERIDGE, I. 2012. Genetic characterisation and taxonomy of species of Anisakis (Nematoda:Anisakidae) parasitic in Australian marine mammals. *Invert Sys,* 26**,** 204-212.

SHAMSI, S., GASSER, R., BEVERIDGE, I., SHABANI, A. A. 2008. Contracaecum pyripapillatum n. Sp. And a description of c. Multipapillatum (von drasche, 1882) from the australian pelican, pelecanus conspicillatus. *Parasitol Res,* 103**,** 1031-1039.

SHAMSI, S., NORMAN, R., GASSER, R., BEVERIDGE, I. 2009a. Genetic and morphological evidences for the existence of sibling species within contracaecum rudolphii (hartwich, 1964) (nematoda: Anisakidae) in australia. *Parasitol Res,* 105**,** 529-538.

SHAMSI, S., NORMAN, R., GASSER, R., BEVERIDGE, I. 2009b. Redescription and genetic characterization of selected contracaecum spp. (nematoda: Anisakidae) from various hosts in australia. . *Parasitol Res,* 104**,** 1507-1525.

SHAMSI, S. 2014. Recent advances in our knowledge of Australian anisakid nematodes. *International Journal for Parasitology: Parasites and Wildlife,* 3**,** 178–187.

SHAMSI, S., GASSER, R. B. & BEVERIDGE, I. 2011. Mutation scanning-coupled sequencing of nuclear ribosomal DNA spacers as a tool for the specific identification of different Contracaecum (Nematoda: Anisakidae) larval types. *Molecular and Cellular Probes,* 25**,** 13-18.

SHANTI, K. N., MARTIN, B. M., NAGPAL, S., METCALFE, D. D., RAO, P. V. S. 1993. Identification of Tropomyosin as the Major Shrimp Allergen and Characterization of Its Ige-Binding Epitopes. *J Immunol,* 151**,** 5354-5363.

SHARP, M. F., LOPATA, A. L. 2013. Fish Allergy: In Review. *Clin Rev Allergy Immunol*.

SHIBASAKI, M., EHARA, T., TAKITA, H. 1989. Late anaphylactic reaction to cuttlefish. *Ann Allergy,* 63**,** 421-422.

SHIH, H. H., KU, C. C. & WANG, C. S. 2010. Anisakis simplex (Nematoda: Anisakidae) third-stage larval infections of marine cage cultured cobia, Rachycentron canadum L., in Taiwan. *Vet Parasitol,* 171**,** 277-285.

SHIOMI, K., SATO, Y., HAMAMOTO, S., MITA, H., SHIMAKURA, K. 2008. Sarcoplasmic calcium-binding protein: Identification as a new allergen of the black tiger shrimp Penaeus monodon. *International Arch Allergy Immunol,* 146**,** 91-98.

SHIRAKI, T. 1974. Larval nematodes of family Anisakidae (Nematoda) in the northern sea of Japan as a causative agent of eosinophilic phlegmone or granuloma in the human gastro-intestinal tract. *Acta Med Biol,* 22**,** 57–98.

SILES, M., CUELLAR, C. & PERTEGUER, M. J. 1997. Genomic identification of Anisakis simplex isolates. *J Helminthol,* 71**,** 73-75.

SICHERER, S., MU~NOZ-FURLONG, A., GODBOLD. J.H., SAMPSON, H.A. 2010. US prevalence of self-reported peanut, tree nut, and sesame allergy: 11-year follow-up. *J Allergy Clin Immunol.*125**,** 1322-1326.

SLOANE, B. E., HONN, K. V. 1984. Cysteine proteinases and metastasis. *Cancer Metast.* 3**,** 249-263.

SMITH, A. M., BENJAMIN, D. C., HOZIC, N., DEREWENDA, U., SMITH, W. A., THOMAS, W. R., GAFVELIN, G., VAN HAGE-HAMSTEN, M., CHAPMAN, M. D. 2001. The molecular basis of antigenic cross-reactivity between the group 2 mite allergens. *J Allergy Clin Immunol,* 107**,** 977-984.

SMITH, J. L. 1999. Foodborne infections during pregnancy. *J Food Prot.,* 62**,** 818–829.

SMITH, J. W., WOOTTEN, R. 1978. Anisakis and anisakiasis. *Adv Parasitol,* 16**,** 93-163.

SMITH, S. K., NISBET, A. J., MEIKLE, L. I., INGLIS, N. F., SALES, J., BEYNON, R. J. & MATTHEWS, J. B. 2009. Proteomic analysis of excretory/secretory products released by Teladorsagia circumcincta larvae early post-infection. *Parasite Immunology,* 31**,** 10-19.

SOHN, W.-M., CHAI, J.-Y. 2005a. Infection status with helminthes in feral cats purchased from a market in Busan, Republic of Korea. *Korean J Parasitol,* 43**,** 93-100.

SOLAS, M. T., GARCÍA, M. L., DE LAS HERAS, C., RODRIGUEZ-MAHILLO, A. I., GONZALEZ-MUNOZ, M., MONEO, I., MENDIZÁBAL, A., TEJADA, M. 2009. Anisakis simplex antigens in fresh and frozen-thawed muscle of anchovies in vinegar. *Food Sci Technol Int,* 15**,** 139–148.

SPEHN, J., SCHONBECK, S., KOPERSKI, K., BUTZOW, G. H. 1988. [Atypical anisakiasis in acquired immunodeficiency syndrome]. *Dtsch Med Wochenschr,* 113**,** 983-984.

STALLONE, O., PAGGI, L., BALESTRAZZI, A., MATTIUCCI, S., MONTINARI, M. 1996. Gastric Anisakiasis in Italy: Case Report. *Med J Sur Med,* 4 **,** 13-6.

STEPHENSON, L. S., LATHAM, M. C., OTTESEN, E. A. 2000. Malnutrition and parasitic helminth infections. *Parasitol,* 121(Suppl):S23–S38.

STOBO, W. T., FANNING, L. P., BECK, B. & FOWLER, G. M. 2002. Abundance and maturity of three species of parasitic anisakine nematodes (Pseudoterranova decipiens, Contracaecum osculatum, Anisakis simplex) occurring in Sable Island harbour seals (Phoca vitulina). *Canadian J Zoology-Revue Canadienne De Zoologie,* 80**,** 442-449.

SUBBA RAO, P. V., RAJAGOPALM, D. M. & K.A., G. 1998. B- and T-cell epitopes of tropomyosin, the major shrimp allergen. *Allergy.* 53**,** 44–47.

SUDHA, V. T., ARORA, N., GAUR, S. N., PASHA, S., SINGH, B. P. 2008. Identification of a serine protease as a major allergen (Per a 10) of Periplaneta americana. *Allergy,* 63**,** 768-776.

SUNNUCKS, P., WILSON, A. C. C., BEHEREGARAY, L. B., ZENGER,K., FRENCH, J., TAYLOR, A. C. 2000 SSCP is not so diffi cult: The application and utility of single-stranded conformation polymorphism in evolutionary biology and molecular ecology. *Mol Ecol*, 9**,** 1699 –1710.

SUZUKI, J., MURATA, R., HOSAKA, M. & ARAKI, J. 2010b. Risk factors for human Anisakis infection and association between the geographic origins of Scomber japonicus and anisakid nematodes. *Int J Food Microbiol,* 137**,** 88-93.

SZOSTAKOWSKA, B., MYJAK, P., KUR, J. 2002. Identification of anisakid nematodes from the Southern Baltic Sea using PCR-based methods. *Mol Cell Probes,* 16**,** 111-118.

TALIAFERRO, W. 2009. Chapter 2, Basic Principles and Concepts I: Parasite Systematics, Ecology, and Evolution In Foundation of Parasitology, Eighth Edition, Roberts L.S and ovy J.R. (ed), Page:11-24. Boston Burr Ridge, IL Dubuque, IA New York, San Francisco.[this presents description of parasitism, ecology and epidemiology of parasitic diseases].

TAYLOR, S. L. 2008. Molluscan shellfish allergy. *Adv Food Nutr Res,* 54**,** 139-177.

THOMPSON, J. D., GIBSON, T. J., PLEWNIAK, F., JEANMOUGIN, F. & HIGGINS, D. G. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research,* 25**,** 4876-4882.

TOMASZUNAS, S., WECLAWIK, Z., LEWINSKI, M. 1988. Allergic reactions to cuttlefish in deep-sea fishermen. *Lancet,* 1**,** 1116-1117.

TORRES, J., FELIU, C., FERNANDEZ-MORAN, J., RUIZ-OLMO, J., ROSOUX, R., SANTOS-REIS, M., MIQUEL, J., FONS, R. 2004a. Helminth parasites of the Eurasian otter Lutra lutra in southwest Europe. *J Helminthol,* 78**,** 353-359.

TSUJI, M. 1989. In: Ishikura, H., Namiki, M. (Eds.), Gastric Anisakiasis in Japan. Epidemiology, Diagnosis, Treatment. Springer–Verlag, Tokyo.

UMEHARA, A., KAWAKAMI, Y., ARAKI, J., UCHIDA, A. 2007. Molecular identification of the etiological agent of the human anisakiasis in Japan. *Parasitol Int,* 56**,** 211-215.

UMEHARA, A., KAWAKAMI, Y., ARAKI, J., UCHIDA, A. 2008. Multiplex PCR for the identification of Anisakis simplex sensu stricto, *Anisakis pegreffii* and the other anisakid nematodes. *Parasitol Int,* 57**,** 49-53.

UMEHARA, A., KAWAKAMI, Y., MATSUI, T., ARAKI, J., UCHIDA, A. 2006. Molecular identification of Anisakis simplex sensu stricto and Anisakis pegreffii (Nematoda: Anisakidae) from fish and cetacean in Japanese waters. *Parasitol Int,* 55**,** 267-71.

UMEHARA, A., KAWAKAMI, Y., OOI, H. K., UCHIDA, A., OHMAE, H. & SUGIYAMA, H. 2010. Molecular identification of Anisakis type I larvae isolated from hairtail fish off the coasts of Taiwan and Japan. *International Journal of Food Microbiology,* 143**,** 161-165.

VALENTINI, A., MATTIUCCI, S., BONDANELLI, P., WEBB, S. C., MIGNUCCI-GIANNONE, A. A., COLOM-LLAVINA, M. M., NASCETTI, G. 2006b. Genetic relationships among Anisakis species (Nematoda: Anisakidae) inferred from mitochondrial cox2 sequences, and comparison with allozyme data. *J Parasitol,* 92**,** 156-166.

VALINAS, B., LORENZO, S., EIRAS, A., FIGUEIRAS, A., SANMARTIN, M. L., UBEIRA, F. M. 2001. Prevalence of and risk factors for IgE sensitization to Anisakis simplex in a Spanish population. *Allergy* . 56:**,** 667–671.

VAN THIEL, P. H. 1962. Anisakiasis. *Parasitology* 52**,** 16–17.

VAN THIEL, P. H. 1976. The present state of anisakiasis and its causative worms. *Trop Geogr Med,* 28**,** 75-85.

VIDAČEK, S., DE LAS HERAS, C., SOLAS, M., MENDIZABAL, A., RODRIGUEZ-MAHILLO, A., GONZÁLEZ-MUÑOZ, M.,TEJADA, M. 2009. Anisakis simplex allergens remain active after conventional or microwave heating and pepsin treatments of chilled and frozen L3 larvae. *J Sci Food Agric,* 89 **,** 1997-2002.

VIDAČEK, S., DE LAS HERAS, C., SOLAS M., G. M., MENDIZÁBAL, A., TEJADA, M. 2011. Viability and antigenicity of Anisakis simplex after conventional and microwave heating at fixed temperatures. *J. Food Protect,* 74**,** 2119-2126.

VIDACEK, S. E. A. 2010. Antigenicity and viability of Anisakis larvae infesting hake heated at different time-temperature conditions. *J Food Protoc,*73**,** 62–68.

VON PIRQUET, C. 1906. Allergie. *Munch Med Worchenschr***,** 53,1457. Cited in http://en.wikipedia.org/wiki/Allergy

WAN, H., WINTON, H. L., SOELLER, C., TOVEY, E. R., GRUENERT, D. C., THOMPSON, P. J., STEWART, G. A., TAYLOR, G. W., GARROD, D. R., CANNELL, M. B., ROBINSON, C. 1999. Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions. *J Clin Invest,* 104**,** 123-133.

WHITE, A. C., GARCIA, H. H. 1999. Recent Developments in the Epidemiology, Diagnosis, Treatment, and Prevention of Neurocysticercosis. *Curr Infect Dis Rep,* 1**,** 434-440.

WIDE, L., BENNICH, H. & JOHANSSON, S. G. O. 1967. Diagnosis of allergy by an in vitro test for allergen antibodies. . *Lancet ii, 1105.*

WILLIAMS, J. G. K., KUBELIK, A. R., LIVAK, K. J., RAFALSKI, J. A., TINGEY, S. V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res,* 18**,** 6531-6535.

YAGI, K., NAGASAWA, K., ISHIKURA, H., NAKAGAWA, A., SATO, N., KIKUCHI, K., ET AL. 1996. Female worm Hysterothylacium aduncum excreted from human: a case report. . *Japanese J Parasitol,* 45**,** 12–23.

YOKOGAWA, M., YOSHIMURA, H. 1967. Clinicopathologic studies on larval anisakiasis in Japan. *Am J Trop Med Hyg,* 16**,** 723-728.

YOSHINAGA, T., KINAMI, R., HALL, K. A. & OGAWA, K. 2006. A preliminary study on the infection of anisakid larvae in juvenile greater amberjack Seriola dumerili imported from China to Japan as mariculture seedlings. *Fish Pathol,* 41**,** 123-126.

YU, C. J., LIN, Y. F., CHIANG, B. L. & CHOW, L. P. 2003. Proteomics and immunological analysis of a novel shrimp allergen, Pen m 2. *J Immunol,* 170**,** 445-453.

ZEYL, C., G, B. & DM, G. 1996. Sex and the spread of retrotransposon Ty3 in experimental populations of Saccharomyces cerevisiae. *Genetics Mol Biol,* 143**,** 1567–1577.

ZHANG, L., HU, M., SHAMSI, S., BEVERIDGE, I., LI, H., XU, Z., LI, L., CANTACESSI, C., GASSER, R. B. 2007. The specific identification of anisakid larvae from fishes from the Yellow Sea, China, using mutation scanning-coupled sequence analysis of nuclear ribosomal DNA. *Mol Cell Probes,* 21**,** 386-390.

ZHANG, Z., P. BERMAN, W., MILLER. 1998. Alignments without low-scoring regions. *J Comput Biol,* 5**,** 197-210.

ZHU, H., ZHOU, W. Y., XU, M., SHEN, Y. L., WEI, D. Z. 2007. Molecular characterization of Serratia marcescens strains by RFLP and sequencing of PCR-amplified 16S rDNA and 16S-23S rDNA intergenic spacer. *Lett Appl Microbiol,* 45**,** 174-178.

ZHU, X. Q., GASSER, R. B., PODOLSKA, M. & CHILTON, N. B. 1998. Characterisation of anisakid nematodes with zoonotic potential by nuclear ribosomal DNA sequences. *International Journal for Parasitology,* 28**,** 1911-1921.

ZHU, X. Q., PODOLSKA, M., LIU, J. S., YU, H. Q., CHEN, H. H., LIN, Z. X., LUO, C. B., SONG, H. Q. & LIN, R. Q. 2007. Identification of anisakid nematodes with zoonotic potential from Europe and China by single-strand conformation polymorphism analysis of nuclear ribosomal DNA. *Parasitology Research,* 101**,** 1703-1707.

ZINN, C., LOPATA, A., VISSER, M., POTTER, P. C. 1997. The spectrum of allergy to South African bony fish (Teleosti). Evaluation by double-blind, placebo-controlled challenge. *S Afr Med J,* 87**,** 146-152.