國立臺灣大學生命科學院生命科學系

博士論文

Department of Life Science

College of Life Science

National Taiwan University

Doctoral Dissertation

海獸胃線蟲熱休克蛋白之研究

Study on heat shock proteins of *Anisakis* spp.

陳慧瑜

Hui-Yu Chen

指導教授：施秀惠 博士

Advisor: Hsiu-Hui Shih, Ph.D.

中華民國 104 年 1 月

January, 2015

國立臺灣大學博士學位論文
口試委員會審定書

海獸胃線蟲熱休克蛋白之研究
Study of heat shock protein of *Anisakis* spp.

本論文係陳慧瑜君（D96b41005）在國立臺灣大學生命科學系、所完成之博士學位論文，於民國 104 年 01 月 29 日承下列考試委員審查通過及口試及格，特此證明

口試委員：施秀惠（簽名）

（指導教授）鄭貽生

毛遠明

陳孝光

陳哲俊

生命科學系 系主任 潘建源代（簽名）

致謝

首先真心感謝我的指導教授施秀惠博士給予我的教導和關懷，從她身上我學到的不只是知識，更多的是待人處世和將所學的回饋社會。從我大學時期在施老師實驗室當暑期生，接著攻讀碩士和博士，轉眼間將近十年。學習研究的過程中，施老師沒有給我任何壓力，讓我擁有了很大的發揮空間，訓練我獨立自主的研究態度和解決問題的能力。感謝我的恩師陳哲俊博士，引導我走進學術殿堂，在我大學時期培養、教育和關心我。讓我了解人生就是要對自己所選擇的負責任，讓我堅持走完博士這條孤獨漫長的道路。感謝總是給學生力量和溫柔的王蓮成博士，王老師在我處於研究低潮中，點亮了一盞燈，給予我方向和幫助，真心感謝王老師。感謝鄭貽生博士讓我找回科學研究的熱情，和鄭老師對話過程中，總是有豁然開朗的感覺，了解到博士生涯只是人生中的一部分，這歷練能使小芥菜種變成大樹。感謝陳秀男博士具有宏偉的見識及胸襟，教導孕育出許多優秀的博士和教授，能成為陳老師的學生深感榮幸和感謝。一日為師，終生為父母代表這五位老師在我學習生涯中的寫照。感謝龜山里里長簡英俊，在我博士研究期間，提供我實驗魚隻，簡里長提攜學術後輩的精神，值得敬佩並真心感謝。點點滴滴的回憶，充滿了眼淚、掙扎、挫折和努力奮鬥，卻也結交一群實驗室的學長姊和學弟妹當夥伴，真的倍感幸福溫暖。感謝欣蓁、育萱、小康學姐們，曾經一同嬉笑、拚搏過、和挑燈夜戰到半夜凌晨，感謝明欣、宛菁學姊們給我鼓勵和研究上的教導。感謝阿 Len 哥、景盛、育騏、綜遠、俊諺、國洋、弘旭學長在學術和研究技術上給予許多的指導和幫助。感謝范姜、阿倖和張耀文助教，給我很多學習和學校公務上很多的幫忙和鼓勵。我感謝惠冠、韻嘉、怡婷、雪芳、Alice、易廷、茂麟、皓元、冠樑學弟妹們協助我實驗和精神上的支持。感謝我的同窗姊妹阿珊、小涵、琇婷、小君、子惠、小綾、小婷、玉珍、曉君、琤宜、士雯、幸妤、佩玲、小骷和朋友贊吉、廷彥，謝謝你們給我歡笑、鼓勵和行動上的支持。感謝德國 Uni Essen，Prof. Bernd, Daniel, Millen, Chris, Katrin, Henrike 和所有同事們在學術研究上的幫助和鼓勵。感謝台大幫英如、純慧、欣明、福明、海綾、雯芳、慧英、怡

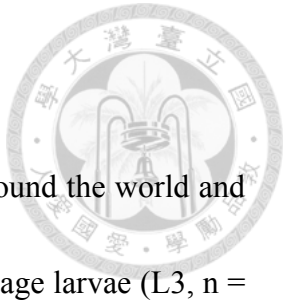
菁、若慈、雯琇、瑞緹、玳君、阿梅、思儀、韶巖、威博、政緯、俊毅，謝謝你們的關心、支持和學術研究上的幫助。博士學位的完成，最重要的幕後主角，就是我的父母親和家人。感謝爸爸和媽媽毫無保留的愛，無限支持我的每一個決定，總是以愛和笑容支持我，包容和體諒我假日都在實驗室做實驗、家庭出遊時，我無法參與但總是記得買紀念品給我、難過時給我關心和安慰、給我生活費用、為我的學業能順利而努力禱告，讓我有勇氣走在這條研究路上，真心的獻上感謝和愛。感謝我親愛的阿媽照顧我、愛我、每天為我準備愛心便當，真心的獻上感謝。感謝我的舅舅、媽咪和爹地經常早上或半夜來學校接我回家，為我準備便當，給我關心和鼓勵，謝謝你們的愛。感謝我的姐姐婷婷和弟弟們銘宇、廷宇、韋廷、家佑和品賢，你們常幫我弄資料、做雜事和帶給我歡笑，謝謝你們。感謝姑姑們和親戚們給我很多關心和鼓勵。所以這博士學位和榮耀是屬於我的每一位家人。感謝明月教會和 Essen Kirche 的每一位弟兄姊妹，總是關心幫助我和為我順利亨通禱告，謝謝你們。感謝鄭明析博士牧師，成為我人生的導師，教導我真誠的愛和堅持到底的精神，也教導我科學的真諦，在我博士生涯中不斷幫助和鼓勵我，讓我看得見希望和更遠的未來。最後，感謝 神 聖靈 聖子主，每時每刻與我同在，陪我經歷酸甜苦辣的人生旅途，總是教導和管理我，並賜下祝福與愛，您就是道路、真理、生命，將我所有一切感激感謝榮耀歸與您們。

中文摘要

海獸胃線蟲寄生於多種海水魚類和頭足類，廣泛分布於世界各大洋，屬於人畜共通傳染病。本研究自台灣東北海域宜蘭(北緯 25 度，東經 121 度)捕撈 250 尾花腹鯖(*Scomber australasicus*)。魚體內採集之線蟲經過初步的型態鑑定後，共檢獲 502 條海獸胃線蟲第三期幼蟲。利用聚合酶連鎖反應-限制酶片段長度多型性技術(Polymerase chain reaction-restriction fragment length polymorphism)可精確鑑定物種。由於 DNA 的多型性使限制酶切位點及片段數目發生改變，藉此可建立簡易的海獸胃線蟲屬內分子分類檢索表。本研究共檢測到六種海獸胃線蟲，分別為 *Anisakis pegreffii*, *A. typica*, *A. paggiae*, *A. brevispiculata*, *A. physeteris*, 和一種重組種(*A. pegreffii* × *A. simplex* sensu stricto)。其中三種海獸胃線蟲為新寄主和新地理紀錄：*A. paggiae*, *A. brevispiculata*, *A. physeteris*。*A. pegreffii* 為優勢種(盛行率=57.2%)，同時亦是造成海獸胃線蟲症的主要病原之一。最大似然估計（maximum likelihood）和近鄰相接法（neighbor-joining method）建立演化樹之結果形成為兩大演化支，一分支為單系群的 *A. paggiae*, *A. brevispiculata*, *A. physeteris*，另一分支為獨立出來的 *A. typica*。為了瞭解海獸胃線蟲在溫度壓力下細胞的反應機制，我們選擇熱休克蛋白 90 為研究目標。目前已知在其他生物體中，它在細胞過程和壓力環境下扮演重要角色。首先，我們利用 cDNA 末端快速擴增技術(Rapid amplification of cDNA ends)選殖放大五種海獸胃線蟲熱休克蛋白 90 的全長(重組種除外)。藉由定量即時聚合酶鏈鎖反應分析，可偵測熱休克蛋白 90 轉錄表現。結果顯示在熱刺激(50˚C)下，熱休克蛋白 90 mRNA 有逐漸增加的趨勢；在 37˚C 影響下，其表現量在處理後的前三小時增加，之後又降回基準值；在冷刺激(4˚C)下，熱休克蛋白 90 mRNA 表現量沒有明顯改變。此外，當海獸胃線蟲處理在熱刺激下，其熱休克蛋白 90 蛋白質表現量會隨處理時間增加呈正相關，藉此推測熱休克蛋白 90 mRNA 和蛋白質表現量會受熱刺激上調，尤其當海獸胃線蟲處於熱壓力下，熱休克蛋白 90 mRNA 和蛋白質表現會明顯增加。為了探討熱休克蛋白 70 和 90 在海獸胃線蟲

生活史中可能參與的角色，尤其是感染型第三期幼蟲和第四期幼蟲的生活史，分別寄生從冷血魚類轉移到恆溫海洋哺乳類動物或意外宿主人類體中，我們檢測熱休克蛋白 70 和 90 在不同發育階段的表現量變化。熱圖和演化樹結果顯示，海獸胃線蟲熱休克蛋白 70 和 90 推測的胺基酸序列和其他的線蟲熱休克蛋白 70 和 90 呈現高度同源性。海獸胃線蟲熱休克蛋白 70 和 90 蛋白質三維結構預測與其他生物具有高度保守性。定量即時聚合酶鏈鎖反應和西方點墨分析顯示這兩種蛋白質在第四期幼蟲表現量顯著高於第三期幼蟲。此外，海獸胃線蟲熱休克蛋白 70 轉錄表現量高於熱休克蛋白 90，推測在不同發育階段，這兩種蛋白可能有不同的調控系統。本研究推測海獸胃線蟲熱休克蛋白 70 和 90 蛋白質在生物體處於熱壓力環境下扮演重要角色，同時也可能參與海獸胃線蟲的發育過程。

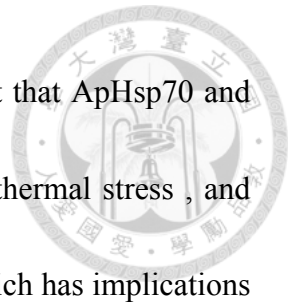
關鍵字：海獸胃線蟲；分子分類檢索表；熱休克蛋白 90；熱休克蛋白 70；壓力；發育



Abstract

Anisakis nematodes were found in a variety of marine fishes around the world and they are known to cause anisakiasis in human hosts. *Anisakis* third-stage larvae (L3, n = 502) were isolated from 250 spotted mackerel *Scomber australasicus* caught from the coastal waters of Yilan, in northeastern Taiwan (25◦ N, 121◦ E). *Anisakis* nematodes were pre-identified morphologically and later molecularly by PCR-RFLP. A simple molecular taxonomic key, utilizing RFLP by two restriction enzymes *Hinf*I and *Hha*I, enabled the differentiation of the genus *Anisakis*. We obtained six species of the genus *Anisakis*, *A. pegreffii*, *A. typica*, *A. paggiae*, *A. brevispiculata*, *A. physeteris*, and a recombinant genotype between *A. pegreffii* and *A. simplex* sensu stricto. Thereby we provide new host and locality records for *A. paggiae*, *A. brevispiculata* and *A. physeteris*. *A. pegreffii* was determined to be the dominant species (prevalence = 57.2%) and important agent of human anisakiasis. The phylogeny of the Maximum Likelihood and Neighbor Joining trees show two well supported clades: one includes the species of *A. pegreffii* and the other includes *A. paggiae*, *A. physeteris* and *A. brevispiculata*, while *A. typica* has basal position to all other *Anisakis* spp. analyzed. To analyze the cellular response to temperature stress in *Anisakis*, the heat shock protein 90 (Hsp90) was chosen in the study, as it plays a key role in many cellular processes and responds to stress conditions such as heat or cold shock. The Hsp90 genes of five species (except

the recombinant genotype) were cloned by rapid amplification of cDNA ends (RACE). Quantitative RT-PCR showed that Hsp90 transcript levels increased slightly under heat shock (50˚C) treatment, and increased gradually during the first 3 h, and thereafter, returned to its baseline value at 37˚C. Under cold shock (4˚C) treatment, the mRNA expression of Hsp90 did not change significantly. In addition, we found a clear time-dependent Hsp90 protein expression pattern of *A. pegreffii* exposed to high temperature. Our results suggest that the mRNA and protein expression patterns of Hsp90 are related to the temperature, and are especially significantly increased under heat stress. On the other hand, to elucidate the possible roles of Hsp70 and Hsp90 in the life cycle of the parasitic nematode *Anisakis*, particularly third- and fourth-stage larvae, which are transferred from cold-blooded fish to warm-blooded marine mammals or accidentally to human hosts, and we examined the expression profiles of Hsp70 and Hsp90 in different developmental stages of *A. pegreffii*. On heatmap and phylogenetic analysis, ApHsp70 and ApHsp90 shared the highest amino acid sequence identity with other nematodes and formed a monophyletic clade. The three-dimensional (3D) structure prediction of the newly characterized ApHsp70 and known ApHsp90 gene showed highly conserved motifs between *A. pegreffii* and other species. Quantitative real-time PCR and western blot analysis revealed higher mRNA and protein expression for ApHsp70 and ApHsp90 in fourth- than third-stage larvae, with higher mRNA and



protein expression for ApHsp70 than ApHsp90. Our results suggest that ApHsp70 and ApHsp90 may play important roles in *Anisakis* in response to the thermal stress , and might be important molecules in the development of *A. pegreffii*, which has implications for its control.

Keywords: *Anisakis* nematode; molecular taxonomic key; Hsp90; Hsp70; stress; development

Contents

List of Tables and Figures

Chapter 1 General introduction

1-1 *Anisakis* nematodes

1-1-1 The taxonomy of *Anisakis*

The phylum Nematoda, also known as the roundworms, is the most diverse species phylum after the phylum Arthropoda, they inhabit a very broad range of environments. They are ubiquitous in marine, freshwater and terrestrial environments, and from the polar regions to the tropics, as well as the highest to the lowest of elevations (Zhang, 2013). Many roundworm species are free-living in nature and feed on bacteria, fungi, protozoans and other nematode (40% of the described species); many parasitic forms include pathogens in animals (invertebrates and vertebrates (44% of the described species) and plants (15% of the described species) (Dropkin, 1989). Various authorities distinguish among 16 to 20 different orders within this phylum. The important nematodes belong to six orders: Rhabditida, Strongylida, Oxyurida, Ascaridida, Spirurida, and Enoplida. Rhabditida is an order of free-living, *Caenorhabditis elegans* is a popular model organism as it possesses all the characteristics mentioned, and shares many essential biological characteristics found in human biology. The most common of these is *Ascaris lumbricoides* (Ascaridida) with an estimated 807-1,221 million people in the world are infected, intestinal roundworm infections constitute the largest group of

helminthic diseases in humans. The nematodes infecting humans include several species of filarial worms, the most important of these are *Wuchereria bancrofti* and *Brugia malayi* (Spirurida) that cause elephantiasis and lymphatic filariasis, respectively. Other species are *Enterobius vermicularis* (Oxyurida) known as Pinworms, *Necator americanus* and *Ancylostoma duodenale* (Strongylida) causing Hookworm, and *Trichinella spiralis* causing Trichinosis (Enoplida) (Gutierrez, 2000).

Nematodes of the genus *Anisakis* are the most frequently reported parasitic species, which continuously attracts mankind health concerns relating to anisakiasis. The classification scheme of the species of *Anisakis* is given by Anderson *et al*., (1974).

Phylum Nematoda

Class Secernentea

Order Ascaridida

Family Anisakidae

Genus *Anisakis* Dujardin, 1845

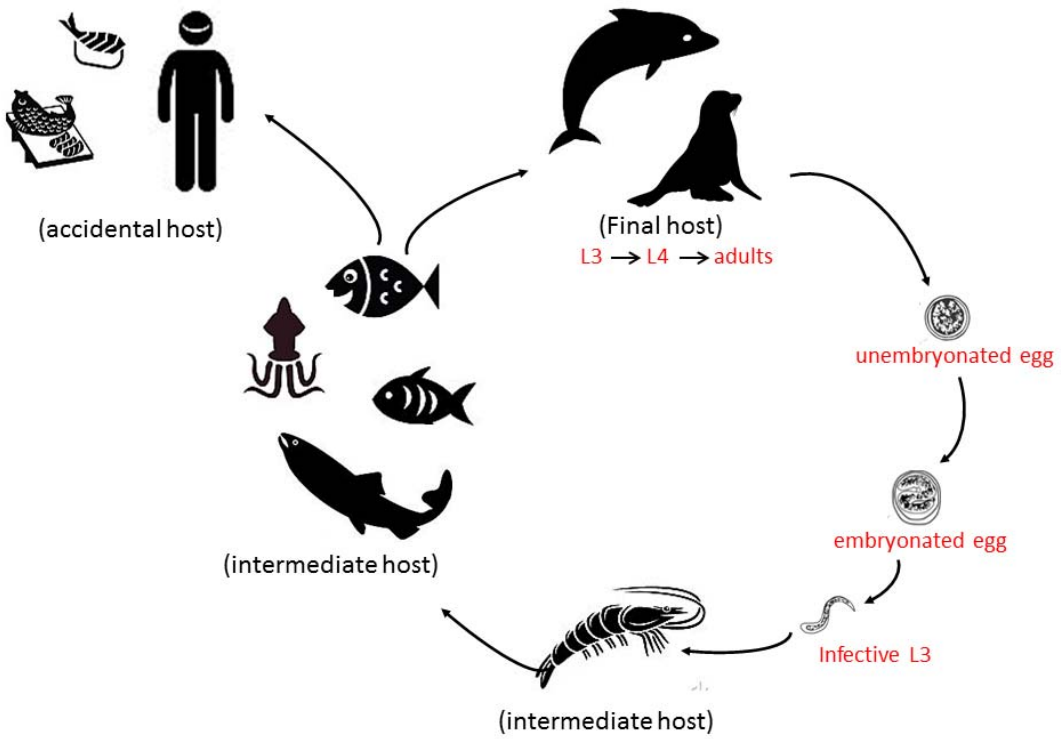
According to morphological characterization and molecular techniques revealed that the *Anisakis* genus consists of 10 valid species: *A. simplex* sensu stricto (s. s.), *A. pegreffii*, *A. simplex* C (3 sibling species of the *A. simplex* complex), *A. typica*, *A. ziphidarum*, *A. nascettii*, *A. physeteris*, *A. brevispiculata*, *A. paggiae* and *Anisakis* sp. (Davey, 1971; Paggi *et al*., 1998; D'Amelio *et al*., 2000; Abollo *et al*., 2003; Mattiucci

et al., 2005; Nadler *et al*., 2005; Valentini *et al*., 2006; Mattiucci *et al*., 2009).

1-1-2 Life cycle of *Anisakis* nematodes

The adults of the genus *Anisakis*, known as the 'herring worm' or the 'whale worm', live in the digestive tract of marine mammals, mainly whales (Young, 1972). Their life cycles are completed in aquatic ecosystems, and involve various invertebrates and fish hosts as intermediate/paratenic hosts and cetaceans as definitive hosts.

The life cycle of *Anisakis* nematodes follows the nematode life cycle model, including five stages separated by four molts. Eggs passed in the feces of marine mammals and embryonate in seawater. Then embryonated eggs hatch to third stage larvae, which are considered to be the infective stage. The larvae are eaten by euphausiaceans or crustaceans, which are subsequently eaten by marine fishes or cephalopods, and the larvae penetrate the wall of the gut, usually on the outside of the visceral organs, but occasionally in the muscle tissue. Fish or squid are intermediate/paratenic hosts, in which the larvae grow, but do not molt into adults. The life cycle is completed when infected fish or squid are consumed by marine mammals (whale, seal, or dolphin), in which two molts take place, the fifth stage becoming the adult worm. The adults live in the intestine, feeds, grows, mates and releases eggs into the seawater (Audicana *et al*., 2002; Klimpel *et al*., 2004).



1-1-3 Geographical distribution of *Anisakis* spp.

The existence of species-specific distribution of *Anisakis* spp. follows a variety of factors, that differ from (1) their characteristic lifecycle, (2) migration pathways of intermediate/paratenic hosts, (3) host preference for cetaceans, and (4) the final host distribution (Klimpel and Palm, 2011). A synopsis of geographical aspects of each species is presented below.

- *A. pegreffii* (Nascetti *et al*., 1986)

  Previously described as *A. simplex* A (Nascetti *et al*., 1986). *A. pegreffii* is the

dominant species of the genus *Anisakis*, and is widespread in many marine fish species. In the case of *A. pegreffii*, which is widely distributed between 55˚N and 55˚S, extends from Mediterranean Sea through the East Atlantic Ocean down to the Antarctic Peninsula, with additional records in Japan, Korea, China and Taiwan (Kuhn *et al*., 2011; Zhang *et al*., 2013), additionally, mainly in coastal and bank areas between 20 and 200 m (Moiseev, 1991). Many commercial fish species in the Taiwanese waters are infected by *A. pegreffii* (Shih *et al*., 2010; Chou *et al*., 2011; Chen *et al*., 2014).

- *A. simplex* (s. s.) (Nascetti *et al*., 1986)

Previously described as *A. simplex* B (Nascetti *et al*., 1986). *A. simplex* s. s. is widespread between 20˚N and the Arctic polar circle (80˚N); it is presented in the northern hemisphere range within the western and eastern Atlantic and Pacific Ocean (Mattiucci and Nascetti, 2006). The occurrence of a recombinant genotype of *A. simplex* s. s. and *A. pegreffii* was found in a sympatric geographic area. The recombinant individuals may be a product of interspecific hybridization (Abollo *et al*., 2003). A recombinant genotype has been identified in the Gilbratar strait (Cadiz and Alboran Sea), Iberian Peninsula waters, Galician coast, NW Spain, Korea, Japan, China and Taiwan.

- *A. simplex* C Mattiucci *et al*., 1997

A. simplex C is recorded a discontinuous range, including Chile, New Zealand waters, Pacific Canada and the Atlantic South African coast, that dispersed to the

northern and southern hemispheres (Mattiucci and Nascetti, 2006).

- *A. typica* (Diesing, 1860)

The species range of *A. typica* is widespread in warmer temperate and tropical waters between 45˚N and 30˚S. *A. typica* is a common parasite of various fish species, which have an epipelagic distribution in the Atlantic Ocean close to the coast of Brazil, Morocco, Portugal and Madeira. Furthermore, *A. typica* has been found in the Mediterranean Sea, Indian Ocean off Somalia, Australia, Japan, Taiwan, China, Thailand, Indonesia and Australia (Mattiucci *et al*., 2002; Koinari *et al*., 2013).

- *A. ziphidarum* Paggi *et al*., 1998

A. ziphidarum is distributed in the East Atlantic Ocean near Madeira and the Moroccan coast, the West Pacific between New Zealand and South Australia, and the southern top of Africa, extended range with other records in the West Atlantic Ocean and the Mediterranean Sea (Kuhn *et al*., 2011).

- *A. nascettii* Mattiucci *et al*, 2009

A. nascettii was described off the coast of New Zealand South Africa and West Atlantic Ocean (Mattiucci *et al*, 2009; Kuhn *et al*., 2011).

- *Anisakis* sp. Valentini *et al*., 2006

Anisakis sp. is distributed South African and New Zealand waters. This gene pool has been indicated reproductively isolated from the sympatric species *A. ziphidarum*

occurring in the same geographic location and hosts. It is considered more closely related to *A. ziphidarum* rather by genetic characterized (Mattiucci and Nascetti, 2006; Valentini *et al*., 2006).

- *A. physeteris* (Baylis, 1920)

A. physeteris is mainly distributed the Altanic Ocean and Mediterranean Sea between 40˚N and 20˚S, and few Asian countries such as Japan, Taiwan and China (Murata *et al*., 2011; Zhang *et al*., 2013).

- *A. paggiae* Mattiucci *et al*., 2005

The distribution of *A. paggiae* northwards has been reported so far within the North Atlantic, South African Atlantic coast, New Zealand, Japan and Taiwan (Chen *et al*., 2014; Kuhn *et al*., 2011). *A. paggiae* is a common parasite of deep-water fish as a second intermediate host, and its life cycle mainly takes place in the meso-/bathypelagic zone (Klimpel *et al*., 2011).

- *A. brevispiculata Dollfus, 1966*

A. brevispiculata is mainly distributed central Atlantic Ocean from Florida towards the Portugal coast, South African Atlantic coast, Japan and Taiwan (Mattiucci *et al*., 2007; Chen *et al*., 2014).

Their geographical range seems to be wide and related to their definitive hosts. *A. simplex* complex (*A. simplex* s. s., *A. pegreffii* and *A. simplex* C) exhibits low definitive

host specificity. In contrast, *A. physeteris* complex (*A. physeteris*, *A. brevispiculata* and *A. paggiae*) indicates high definitive host specificity. Furthermore, both complexes exhibit low specificity in the second intermediate hosts (Klimpel *et al*., 2008).

1-1-4 Morphological and genetic identification

According to a revision of the genus *Anisakis*, Davey (1971) gave the following generic description:

The third stage larvae of the genus of *Anisakis* are characterized by the presence of three bilobed lips, one dorsal and two ventrolateral, a boring tooth ventral to the mouth, an excretory pore between the ventrolateral lips, and a hung of ventriculus between the esophagus and the intestine. Distinction among the three genera depends on the presence or absence of an intestinal caecum and a ventricular appendage. The adult worms possess three protruding lips around its mouth opening. The *Anisakis* species were preliminary identification relied on number and distribution patterns of caudal papillae.

Traditionally, the taxonomic concepts of nematodes have relied heavily on a limited number of morphological characters. Anisakid larvae of species identification are usually very difficult due to the lack of unique characters. For this reason, the results of earlier classification systems were uncongruous with each other and the causes of the

problem produced many synonyms. According to the morphological descriptions, some very poor, of 21 species of the genus *Anisakis*, Davey (1971) rearranged the extent of individual variation in taxonomic characters, and he concluded that there are only three valid species (14 synonyms within them) as follows: *A. simplex* (with 10 synonyms), *A. typica* (with 1 synonym) and *A. physeteris* (with 3 synonyms). In the past three decades, the taxonomy of anisakid nematodes has been redefined based on multilocus enzyme electrophoretic analyses (Mattiucci *et al*., 1986). These analyses have revealed that many anisakid mophospecies including *A. simplex*, *Pseudoterranova decipiens*, and *Contracaecum osculatum*. For example, the mophospecies *A. simplex* consists of three sibling species, *A. simplex* s. s., *A. pegreffii* and *A. simplex* C. Sibling species are morphologically very similar or even identical, but genetically different, life cycle, host preferences, and zoogeographical distribution (Valentini *et al*., 2006; Klimpel *et al*., 2007). Furthermore, the third-stage larvae of *Anisakis* were categorized morphologically into *Anisakis* Type I larvae and *Anisakis* Type II larvae on the basis of their ventriculus length and the presence or absence of a mucron (Berland, 1961). *Anisakis* Type I larvae had a longer ventriculus and mucro while type II larvae had a shorter ventriculus and no mucro. *Anisakis* Type I larvae were found to include the species *A. simplex* s. s., *A. pegreffii*, *A. simplex* C, *A. typica*, *A. ziphidarum*, and *A. nascettii*, whereas *Anisakis* Type II larvae include the species *A. physeteris*, *A. brevispiculata*, and *A. paggiae*

(Berland, 1961). Morphological comparison of *A. simplex* s. s. and *A. pegreffii* larvae showed a significant difference in ventricular length (Quiazon *et al*., 2008), although comparative studies with other type I larvae have not been performed.

Although the identification of anisakid species by morphology is unreliable, specific identification can be achieved using molecular approaches. Recent molecular techniques, the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of the internal transcribed spacer (ITS) region (ITS-1 and ITS-2) and the 5.8 subunit ribosomal DNA gene have been successfully employed for the identification of these species because these spacers showed high levels of interspecies divergence in the presence of low level intraspecies variation (Shih *et al*., 2004; Umehara *et al*., 2006). The ITS region of the genus *Anisakis* was digested with 16 restriction enzymes (*Alu*I, *Dra*I, *Eco*RI, *Eco*RV, *Hae*III, *Hha*I, *Hinf*I, *Hpa*II, *Hsp*92II, *Mbo*I, *Nci*I, *Pst*I, *Pvu*II, *Rsa*I, *Sac*I and *Taq*I). 12 out of 16 endonucleases cut the target sequence in one or more of the species, while four endonucleases (*Dra*I, *Eco*RV, *Pst*I and *Sac*I) did not (D'Amelio *et al*., 2000). Many studies have been performed on the ITS region with *Hinf*I and *Hha*I for all species of Anisakid nematodes (Espiñeira *et al*., 2010; Shih *et al*., 2010).

On the other hand, micro-satellites, sequences analysis of the nuclear large subunit ribosomal DNA (LSU rDNA) and the mitochondrial cytochrome C oxidase subunit II

(cox2) have been used to distinguish the species among *Anisakis* spp. (Nadler *et al*., 2005; Valentini *et al*., 2006). Therefore, through a combination of morphological and molecular analyses, *Anisakis* Type I larvae include the species *A. simplex* s. s., *A. pegreffii*, *A. simplex* C, *A. typica*, *A. ziphidarum*, and *A. nascettii*, whereas Type II larvae include the species *A. physeteris*, *A. brevispiculata*, and *A. paggiae* (Mattiucci *et al*., 2002, 2005, 2009). On the contrary, Murata *et al*., (2011) reported that *Anisakis* nematodes were divided morphologically into 4 types. *Anisakis* Type II (*A. physeteris*), Type III (*A. brevispiculata*), and Type IV (*A. paggiae*) larvae all had a short ventriculus, but their tails were morphologically different. Type II larvae had a long, conical, and tapering tail without a mucron. Type III larvae were characterized by a short and rounded tail. Type IV larvae were characterized by a short, conical, and pointed tail without a mucron. Therefore, *A. physeteris*, *A. brevispiculata*, and *A. paggiae* can be readily differentiated not only by genetic analysis but also by morphological characteristics of L3 larvae. In addition, molecular approaches have the advantage that they allow securing species identification and providing data for phylogenetic relationships.

1-1-5 Anisakiasis and prevention

The first case of anisakiasis was described in the Netherlands in 1960 (Van Thiel *et*

al., 1960), and then many cases have been reported in Japan and Western Europe, where raw fish are consumed frequently. Over the past decade, it has been increasing in most regions of the world, probably due to the increased consumption of raw or undercooked fish and improved technological advances in *Anisakis* diagnosis (Audicana and Kennedy, 2008).

Anisakiasis is an important fishborne zoonosis provoked by the accidental ingestion of anisakid nematodes. Four species of the family Anisakidae are known to cause infection in humans: *A. simplex* s. s., *A. pegreffii*, *A. physeteris* and *P. decipiens* (Mercado *et al*., 2001; Quiazon *et al*., 2011). Human may become accidental host when they ingest raw or undercooked infected seafood such as Japanese sushi, sashimi, Hawaiian lomi-lomi, Latin American ceviche and Dutch salted or smoked herring. Even if seafood is frozen or well-cooked, it induces allergic reactions in sensitive patients to this parasite.

Human might suffer two distinct clinical syndromes: gastrointestinal anisakiasis and allergic anisakiasis. The digestive symptoms of anisakiasis included acute gastric, abdominal pain, nausea, vomiting, abscessing or eosinophilic granulomas and stomach ulcers. Besides, the excretory-secretory products of larvae and dead larvae may also induce severe allergic reactions such as rash, itching, anaphylaxis, acute urticarial and angioedema (Ishikura *et al*., 1993; Audicana *et al*., 2002). In vivo test, patients

sensitized to *Anisakis simplex*, and the latency of gastric symptoms was within 48h and the latency of allergic symptoms was within 24 h after consuming raw or uncooked fish (Daschner and Pascual, 2005). There have been twelve allergens of *A. simplex* described and only four are major allergens, which have been recognized by > 50% of the patients (Quiazon *et al*., 2013). To date, there are five (Ani s 1, 4, 5, 8 and 9) of these are heat-stable allergens, which have been found to be highly resistant to heat (Audicana and Kennedy, 2008).

The diagnosis and identification of human infections is difficult. This is due to the low specificity of the clinical symptoms related to human infections (Simonetta *et al*., 2011). The digestive and allergic symptoms are often confused with food poisoning (i.e. bacterial or viral infection) and may lead to misdiagnosis. Anisakiasis treatment is not always obligatory. Diagnosis of anisakiasis requires a history that the patient has consumed raw or uncooked seafood. The medical doctors can visualize the worms with endoscopy. Skin prick tests and determination of specific IgE to *Anisakis* are also routinely performed (Fæste *et al*., 2014). Another way to identify the infective worms is via histopathologic study of the gastric or intestinal tissue. When patient has a serious health condition, and the larvae must be removed surgically or during endoscopic examination. A drug called albendazole might also kill it (Arias-Diaz *et al*., 2006).

Food safety management can reduce the occurrence of foodborne pathogens and

improve public health protection. Much research has been published regarding the survival and resistance of anisakids under different treatment conditions. The survival tolerance of the third stage *Anisakis* larvae were observed in various conditions, such as in microwaving, freezing, heating, salting, anthelmintic drug and Condiments (Wang *et al*., 2010). To kill the larvae in fish, high and low temperature is commonly applied. The treatment of some seafood may involve methods by salting, curing, pickling and smoking at 40 ºC which are not enough to kill anisakids. Lanfranchi and Sardella (2010) found that *Anisakis* can survive to the maximum of 330 days in NaCl solution (0.85%) at 4 – 5.5°C. In high temperature condition, the movement of larvae was examined at 40 and 50°C for up to 30min. There are still a part of larvae alive under the heat at 60°C for 10min. (Vidaček *et al*., 2011). Svanevik *et al*., (2013) reported that *Anisakis* larvae, which migrate from the intestine of the fish into the flesh, as vector for bacteria contaminate resulting in the fish flesh. The U.S. Food and Drug Administration (FDA) recommended that fish is cooked reaching an internal temperature of 63°C (145°F) and stuffed fish increase internal temperature to 74°C (165°F). In some studies have reported on *Anisakis* species have been found to -20ºC for short periods and the FDA recommends freezing fish at -20ºC for at least 168 hours (7 days) and blast-freezing at -35ºC for at least 15 hours.

1-1-6 A comprehensive review of *Anisakis* biology

To data, many different types of studies with *Anisakis*, including (1) Ecology and genetic structure of *Anisakis* spp. from different countries or regions in the world (Mattiucci, 2004; Abdou and Dronen, 2007; Cavallero *et al*., 2012; Mladineo *et al*., 2012; Quiazon *et al*., 2013), (2) *Anisakis* species use as biological tags for the application of stock identification or indicator of heavy-metal pollution (Beverley-Burton, 1978; Chou *et al*., 2011), (3) Anisakiasis and allergy in human (Audicana and Kennedy, 2008; Zullo *et al*., 2010; Baird *et al*., 2014), (4) Food safety and hygiene of the *Anisakis* (Peñalver, 2010; Levsen and Karl, 2014), (5) Cloning and molecular characterization of the allergens or other genes from *A. simplex* (Arrieta *et al*., 2000; Kim *et al*., 2006; Yu *et al*., 2007; Tseng and Shih, 2007; Chen *et al*., 2014).

1-2 Heat shock proteins

1-2-1 Introduction

These proteins were named as heat shock proteins (Hsps) because they were first thought to be induced by heat and are conserved and ubiquitous in nature that help cell survival during energy stress. Hsps are highly conserved and present in almost all species, except for some archaea (Lindquist, 1986). Hsps are essential chaperones required for key cellular functions. They are involved in folding/unfolding of proteins,

cell-cycle control and signaling, differentiation and development, apoptosis and help modulate signals involved in the immune system and the inflammatory response (Devaney, 2006; Shiny *et al*., 2011). This family of proteins also plays key roles in protection of cells against temperature stress. Furthermore, Hsps respond not only to increased temperatures, but also chemicals, heavy metals, UV light, hypoxia and other stressors can induce their synthesis (Pirkkala *et al*., 2001). Hsps are classified into families on the basis of their approximate molecular weight on SDS-PAGE, such as small Hsp, Hsp 60, Hsp70, Hsp 90 and Hsp 100 (Lindquist and Craig, 1988).

1-2-2 Heat shock protein 90

Hsp 90 is highly conserved ubiquitous proteins, representing almost 1-2% of the total cellular protein in unstressed cells. It functions as a molecular chaperone that is important for growth and development, such as members of the steroid receptor family (Kimmins and MacRae, 2000), the tumor suppressor p53 and telomerase (Picard, 2002). Hsp90 is indispensable protein in many eukaryotes. It is essential for viability in the yeast *Saccharomyces cerevisiae* and its mutation causes defects in eye development in the fruit *Drosophila melanogaster*. Besides, Hsp90 function is crucial for cancer progression in human.

Hsp90 exists as a homodimer, and contains three domains: a highly conserved

N-terminal ‘ATPase domain’, a charged linker region, that connects the N-terminus with the middle domain followed by ‘middle region’ and the C-terminal dimerization domain, which were involved client proteins and co-chaperones binding (Johnson, 2012). Hsp90 undergoes conformational changes from an open to a closed conformation during its ATPase cycle. These changes are thought to be relevant for the stabilization and activation of the client proteins (Pearl and Prodromou, 2006).

Hsp90 is a relatively well-characterized Hsp in nematodes (Skantar and Carta, 2004; Him *et al*., 2009). Hsp90 sequences from *B. malayi* and *B. pahangi* are 99.9% identical. Hsp90 from *Brugia* spp. and *C. elegans* share 84% identity, while *Brugia* spp. and human are 77% identity. Despite they share a highly conserved but functionally diverse within the phylum. In common with most other parasitic nematodes undergo a heat shock as part of their life cycle. For instance, a mosquito carrying the third stage larvae of *B. pahangi* transfer to human by biting, and third stage larvae were shifted from 28˚C to 37˚C. The expression of Hsp90 is in all development stages (Devaney *et al*., 2005). However, the transcription levels of Hsp90 of parasitic nematode *Nippostrongylus brasiliensis* were higher in infective L3 larvae than in other larval stages (Arizono *et al*., 2011).

Specific inhibitors, geldanamycin (GA) a naturally occurring ansamycin compound produced by *Streptomyces hygroscopicus*, some nematodes Hsp90 are susceptible to

inhibition with GA. Exposure of *Brugia pahangi* to nanomolar concentrations of GA is lethal to microfilariae and to adult worms (Devaney *et al* ., 2005). Him *et al*., (2009) found that Hsp90 of free living and parasitic with free living larvae (i. e. *Anisakis simplex*) failed to bind to GA. However, Hsp90 of parasite with eggs (i. e. *Ascaris suum*) and obligate parasites (i. e. *B. pahangi*) can bind to GA. They suggested that differences in the ability of nematode Hsp90 to bind to GA are correlated with particular life histories. In addition, Hsp90 is a desirable candidate use in nematode phylogenetics studies because it is highly conserved and is featured gene not easily confused with related genes.

1-2-3 Heat shock protein 70

Members of the Hsp70 family were first shown to play an important role in stress, such as heat shock (Parsell and Lindquist, 1993). Hsp 70 is the most conserved protein family compared to other Hsp families. Prokaryotic Hsp70 (DnaK) proteins share about 50% amino acid identity with eukaryotic Hsp70s. All known Hsp70 proteins exhibit highly conserved amino acid sequences and these proteins consist of two domains. The highly conserved N-terminal ATPase domain has protease sensitive sites, while the more divergent C-terminal domain contains the peptide binding site (Zhu *et al*., 1996). In eukaryotes, four subfamilies can be distinguished according to their function and

localisation in different cellular compartments: cytosol, endoplasmic reticulum, mitochondria and chloroplasts (Boorstein *et al*., 1994). In all multicellular studies so far, multiple cytosol-type members show a constitutive expression under normal physiological conditions (Hsc70) while other are stress-inducible (Hsp70).

Hsp70 has been characterized from a range of parasitic nematodes, including *Brugia malayi* (Selkirk *et al*., 1989), *Trichinella spiralis* (Wang *et al*., 2009), *Nippostrongylus brasiliensis* (Arizono *et al*., 2011) and *Parastrongyloides trichosuri* (Newton-Howes *et al*., 2006). In both *B. malayi* and *P. trichosuri*, the Hsp70 mRNA appeared to be constitutively expressed in all life cycle stages examined and was further inducible by heat shock. However, the mRNA expression of Hsp70 from *N. brasiliensis* in third stage larvae was higher than in other larval stages. Hsps are induced by cold exposure in some nematodes. A temperature shift from 37°C to 4°C increases the expression of Hsp70 in the parasitic first stage larvae of *T. spiralis*, *T. nativa* and *T. nelsoni* , but level of Hsp90 decline or do not change (Martinez *et al* ., 2001). However, the exact function of the Hsp70 gene family in parasitic nematodes remains unclear.

Due to the high degree of sequence conservation of Hsp70 has been widely used as a suitable phylogenetic marker. It has been applied to phylogenetic relationships (such as between animals, plants and fungi) or to clarify the monophyletic group of the Metazoa (Borchiellini *et al*., 1998). Hsp70 genes and proteins have also been used for

phylogenetic analysis of protozoan parasites such as *Trypanosoma* and *Leishmania* (Simpson *et al*., 2004; Fraga *et al*., 2010).

1-3 Aims of this dissertation

The aims of this study are (1) to identify and evaluate the prevalence in spotted mackerel (*Scomber australasicus*) from the northeast coast of Taiwan, and creating a simple molecular taxonomic key assisted rapid diagnosis of *Anisakis* species. Much less is known about the role of *Anisakis* Hsp90 in stress responses, (2) to understand gene and protein expression profiles of *A. pegreffii* Hsp90 under heat stress. Except in response to stress, other functions of Hsp in different stages of *A. pegreffii* remain unclear, (3) to interest in understanding the possible role of *Anisakis* nematodes Hsp90 and Hsp70 in the developmental stages.

To achieve these aims, the following studies were conducted to explore:

(1) Occurrence and prevalence of fish-borne *Anisakis* larvae in the spotted mackerel *Scomber australasicus* from Taiwanese waters. This study identified anisakid nematodes at the species level in spotted mackerel caught from the coastal waters of Yilan in 2012-2013. All the collected *Anisakis* larvae were identified by morphological and molecular approaches. Additionally, a molecular taxonomic key was developed according to the restriction fragments that would be useful for immediate identification

of *Anisakis* spp.. Phylogenetic relationships between the *Anisakis* nematodes were discussed.

(2) Effect of different temperatures on the expression of the newly characterized heat shock protein 90 (Hsp90) in L3 of *Anisakis* spp. isolated from *Scomber australasicus*. We cloned, characterized and compared the full-length cDNA of Hsp90 of five *Anisakis* spp. In addition, we investigated the expression levels of Hsp90 after cold and heat shock. For that, we used the third-stage larvae of *A. pegreffii* as a model, which is dominant species detected in our experiment and causes human anisakiasis.

(3) The structural conservation and expression patterns of Hsp70 and Hsp90 in different developmental stages of *A. pegreffii*. We cloned, characterized and compared the Hsp70 cDNA sequence of *A. pegreffii*. We have used homology modeling to derive a putative three-dimensional structure for Hsp70 and Hsp90 based on known crystal structure of yeast Hsp90 and Hsp70. The evolutionary relationships of Hsp70 and Hsp90 among protozoa, helminthes and human were discussed.

Chapter 2 Occurrence and prevalence of fish-borne *Anisakis* larvae in the spotted mackerel *Scomber australasicus* from Taiwanese waters

2-1 Introduction

The spotted mackerel *Scomber australasicus* is a small- to medium-sized pelagic fish, which is mainly distributed in the tropics and subtropics between 43°N and 50°S, and from 32°E to 110°W. The known geographical ranges are from the Indo-West Pacific, the Red Sea, China and eastwards to Hawaii and Mexico, extending northwards to the Pacific coast of Japan and southwards to Australia and New Zealand (Collette and Nauen, 1983). Mackerel is an important food fish that is consumed worldwide. According to the statistics of the United Nations Food and Agriculture Organization, mackerel had reached the peak of its development between 2005 and 2007, with landings of around 200-260 thousand metric tons. Since 2008 the catch of mackerel has been on a declining trend, with an average of approximately 150-160 thousand metric tons per year (http://www.fao.org/). Mackerel is harvested all year round and is an economically important fish species in Taiwan (Tzeng, 2004). According to official Taiwan statistics, the annual yield and output value of mackerel in the past five years was approximately 50-90 thousand metric tons and 33-40 million US dollars

(http://www.fa.gov.tw/). Over 80% of the spotted mackerel is industrially produced as salted, frozen or canned fish and is exported worldwide. It is an important issue that treatment in the fish processing and monitoring of zoonotic infections ensure food safety for consumers.

Anisakids are fish-borne parasites and are reported to be widely distributed in marine fish, cephalopods and whales throughout the world's oceans and seas (Kuhn et al., 2011). Anisakiasis is a zoonotic disease caused by the consumption of infectious third stage larvae (L3) in raw or undercooked seafood products. Human can become accidental hosts and might suffer from two distinct clinical entities, namely gastrointestinal anisakiasis and gastroallergic anisakiasis. Up to now, the cases of human anisakiasis are caused by *Anisakis simplex* sensu stricto, *A. pegreffii*, *A. physeteris*, and *Pseudoteranova decipiens* (Hochberg and Hamer, 2010). Out of the totally approximately 20,000 cases of anisakiasis reported worldwide up to today, most were primarily reported in Japan, European and United States. However, there have been increasing case reports from Canada, Brazil, Chile, New Zealand, China, Korea and Egypt as well (Choi et al., 2009; Qin et al., 2013).

Fish are one of the most important food sources in the seas surrounding Taiwan. Although the first case of intestinal anisakiasis in Taiwan was described in 2014, there is increasing consumption of raw or salted fish with a high risk of consumer exposure to

this zoonotic infection. Unfortunately, Anisakid nematode larvae have appeared within canned mackerel in fish processed in Taiwan (Shih, 2004). The presence of larvae in canned fish might induce allergic reactions in those previously sensitized to thermally stable *A. simplex* allergens. Further investigation of the parasite fauna of a variety of fish species and the molecular epidemiology of human anisakiasis in Taiwan is required. The aim of the present study was to revise the morphological characterization of *Anisakis* L3 larvae, in comparison with molecular diagnosis by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Additionally, a molecular taxonomic key was developed according to the restriction fragments that would be useful for immediate identification of *Anisakis* spp.. Phylogenetic relationships among taxa were based on sequencing of the internal transcribed spacer (ITS) regions of ribosomal DNA.

2-2 Material and methods

2-2-1 Parasite collection

A total of 250 fresh specimens of spotted mackerel *Scomber australasicus* were caught in the coastal waters of Yilan, northeastern Taiwan (25° N, 121° E) from July 2012 to June 2013. All specimens were placed on flake ice and immediately transported to the laboratory. 502 live third-stage larvae of *Anisakis* were obtained from the body

cavity and visceral organs. The larvae were isolated and washed in physiological saline. They were examined microscopically and identified based on the morphological characteristics described previously (Smith, 1983). The morphometric data of *Anisakis* larval specimens (identified by PCR-RFLP; see subsection 2.3.) were recorded and images were taken of the head, tail and ventriculus. Anisakid larvae were preserved at -20˚C until processing for DNA extraction.

2-2-2 DNA extraction and PCR

Genomic DNA was extracted and purified from individual L3 according to a standard CTAB phenol-chloroform protocol (Rogers and Bendich, 1985) with slight modifications. DNA concentration and quality were measured by a NanoDrop® TC1-E20 spectrophotometer. For amplification of the rDNA region containing ITS-1, the 5.8S gene and ITS-2, the previously described primers NC5 5′-GTA GGT GAA CCT GCG GAA GGA TCA T-3′ and NC2 5′-TTA GTT TCT TTT CCT CCG CT-3′ (Zhu et al., 1998) were used. Each reaction mixture contained 5 μL 10X Ex Taq Buffer, 4 μL dNTPs (2.5 mM each), 2 μL of each primer (10 mM), genomic DNA (25-50 ng in 2 μL) and 0.25 μL TaKaRa Ex Taq (5 U/μL; Takara, Kyoto, Japan) in a total volume of 50 μL. PCR was performed in a thermocycler (Quanta Biotech, UK) under the following conditions: an initial denaturation at 95℃ for 10 min was followed by 35 cycles of 95

℃ for 30 s, 55℃ for 30 s, and 72℃ for 75 s with a final extension of 72℃ for 7 min. PCR products were separated by electrophoresis on 1.5% agarose gels, stained with ethidium bromide and photographed upon transillumination with a Kodak digital camera (DC290 Zoom).

2-2-3 DNA sequencing and PCR-RFLP

Single DNA band containing PCR products were isolated and purified from agarose gel by the Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taiwan). The sequencing was carried out using ABI prism 3730 automated DNA sequencer. PCR-RFLP patterns of ITS rDNA of larvae in the genus *Anisakis* were digested with two endonucleases *Hha*I and *Hinf*I. Individual PCR products (5 μL) were digested with 10 U/μL of these enzymes in a final volume of 20 μL, and activated at 37℃ for 4 h. Finally, digested products were analyzed by electrophoresis in a 3% agarose gel and visualized as described above.

2-2-4 Data analysis

he standard infection parameters (prevalence, mean intensity and mean abundance) were used to quantify parasite populations and the density of parasitic infection in host population (Bush et al., 1997). The analysis of sequence similarity of the ITS region

was carried out by using the BLAST program at NCBI (http://blast.ncbi.nlm.nlh.gov/). Additional known ITS nucleotide sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank) for phylogenetic analysis. A phylogenetic tree was computed using MEGA5.2 software according to the maximum likelihood (ML) and neighbor joining (NJ) based on the nucleotide sequence of ITS-1, 5.8S and ITS-2, and drawn using nearest-neighbor-interchange (NNI) with Kimura 2-parameter model. The relative support for clades in the ML and NJ analyses was determined following 1000 bootstrap replicates with partial deletion. *Ascaris suum* was used as an outgroup to root the *Anisakis* phylogenetic tree.

2-3 Results

2-3-1 Identification of *Anisakis* nematodes

502 *Anisakis* larvae were collected from 250 spotted mackerel in Taiwanese waters. The parasites were mainly found in the viscera, including the stomach, gut, liver, spleen, gonads (Fig. 2. 1). The prevalence, mean intensity and mean abundance recorded for the total specimens were 72.8%, 2.8 (1-15) and 2.0 (0-15) separately. *Anisakis* L3 larvae were divided morphologically into two types followed by molecular approaches. *Anisakis* type I larvae had a longer ventriculus and mucro while type II larvae had a shorter ventriculus and no mucro. We obtained six species of the genus *Anisakis*, *A.*

pegreffii, *A. typica* belonging to type I, *A. physeteris*, *A. brevispiculata*, *A. paggiae* belonging to type II and a recombinant genotype between *A. simplex* sensu stricto and *A. pegreffii* (Fig. 2. 2).

2-3-2 Molecular genotypes based on PCR-RFLP

A total of 502 individual worms were examined. Restriction of the ITS-1, the 5.8S gene and ITS-2 (approximately 960 bp in length) with *Hha*I produced two bands in *A. pegreffii* and recombinant genotype (ca. 550 and 430 bp), in *A. physeteris* (ca. 520 and 390 bp) and in *A. paggiae* (ca. 520 and 410 bp), *A. brevispiculata* showed three bands (ca. 390, 330 and 190 bp), and *A. typica* showed four bands (ca. 320, 240, 180 and 160 bp). Digestion with *Hinf*I yielded only one band in *A. paggiae* (ca. 920 bp) and in *A. brevispiculata* (ca. 900 bp), *A. typica* exhibited two bands (ca. 620 and 350 bp), three bands in *A. pegreffii* (ca. 370, 330 and 250 bp) and in *A. physeteris* (ca. 380, 270, 250 bp), and recombinant genotype showed a unique pattern characterized by four bands (ca. 620, 370, 300 and 250 bp) (Fig. 2. 3). According to the restriction results, we developed a molecular taxonomic key, which is based on a dichotomic method, for species differentiation among the *Anisakis* spp. studied (Fig. 2. 4).

2-3-3 Prevalence of *Anisakis* larvae

According to the results of the restriction profile, 287 out of 502 (57.2%) *Anisakis* larvae were identified as *A. pegreffii*, which was by far the most abundant species. A recombinant genotype was identified in 127 out of 502 (25.3%), and was the subdominant species in all *Anisakis* larvae. The prevalences of *A. typica*, *A. physeteris* and *A. paggiae* in total *Anisakis* nematodes were 50 individuals (10%), 20 (4.0%), and 15 (3.0%), respectively. On the other hand, only three larvae (0.5%) were identified as *A. brevispiculata* (Fig. 2. 5).

2-3-4 Genetic analysis

Genetic characterization of five species of the genus *Anisakis* with PCR for rDNA region were carried out by DNA sequencing. The sequences of ITS1–5.8S–ITS2 from *A. pegreffii* (accession number, KM658328), *A. paggiae* (KM658329) and *A. physeteris* (KM658331) were 100% identical to the published sequences (*A. pegreffii* [JN968606], *A. paggiae* [AB592795] and *A. physeteris* [JN968636]). The alignment of *A. brevispiculata* (KM658332) and *A. typica* (KM658330) with published sequence, resulted in a maximum of 99% and 96% similarity with sequences in GenBank (*A. brevispiculata* [AB592794] and *A. typica* [JN968962]). A phylogenetic tree using maximum likelihood and neighbor-joining analyses based on ITS sequences produced trees with similar topology. The topology of the ML and NJ trees showed two well

supported sister clades, one including *A. pegreffii* and the other *A. paggiae*, *A. physeteris* and *A. brevispiculata*. *A. typica* is the basal group to these two clades (Fig. 2. 6).

2-4 Discussion

Our previous research work has examined three species of the genus *Anisakis*, *A. pegreffii*, *A. typica* and a recombinant genotype of *A. pegreffii* and *A. simplex* s.s. from *S. australasicus* in Taiwanese waters (Chou et al., 2011). In the present study, based on morphological and molecular genetic analysis, performed on larvae in the same fish hosts, we have confirmed the presence of *A. pegreffii*, *A. typica*, *A. paggiae*, *A. physeteris*, *A. brevispiculata* and a recombinant genotype. The total prevalence and mean intensity of *Anisakis* L3 larvae were considerably lower (72.8% and 2.8) than in our previous study (93.6% and 17.1). *A. pegreffii* was the most frequently recovered species (97%) in our previous study, higher than in this study (57.2%). The frequency of the recombinant genotype of *Anisakis* species was 3% in our previous study, a lower percentage than we have identified (25.3%) in this study. However, an increase in prevalence was found for three species (*A. paggiae*, *A. physeteris*, *A. brevispiculata*) compared to our previous study. The climate change is altering the world oceans, and consequences affect the marine ecosystems, and change the species distribution and

biodiversity in aquatic habitats. The impact of climate change on increasing water temperature leads to a large number of pelagic fish species following warm currents northwards, resulting in increasing *Anisakis* spp. infection of fish (Marcogliese, 2008). The distribution of parasite species will be directly affected by climate change, but also indirectly effected through altering the range and abundance of their intermediate and final host (Marcogliese, 2008). The ENSO (El Nino Southern Oscillation) phenomenon causes a drastic fluctuation in the temperature of the seawater and ocean currents. The phenomenon has been suggested to alternate *Anisakis* distribution and prevalence by bringing new sylvatic mammals to the region, such as fish and cephalopods adapted to warm currents (Arriaza et al., 2010). A study by Liu and Zhang (2013) revealed that, over the past 100 years, the sea surface temperature (SST) of waters around Taiwan increased by more than 2.7 ˚C. The increased SST resulted in an annual increase in the total number of fish species, while the cumulative percentages of the top 10 captured fish species, including spotted mackerel, decreased annually in Taiwanese waters (Lu and Lee, 2014). At present, there is no study that has evaluated the fluctuation in water temperature related to parasite population dynamics in this region. We suggest that the changing infection levels of *Anisakis* larvae are involved not only in biotic factors (life cycle biology, host ranges and prevalence rates of the zoonotic species in definitive, intermediate and paratenic hosts), but also in anthropogenic influence (climate change

and human activities) (Klimpel and Palm, 2011).

The morphological characterization is unreliable for the identification of anisakid larvae to the species level; however the application of a biomolecular method, such as PCR-RFLP analysis of the ITS region of ribosomal DNA yielded a correct species identification. The ITS region of the genus Anisakis (*A. simplex* s.s., *A. pegreffii*, *A. simplex* C, *A. typica*, *A. ziphidarum*, *A. physeteris*, and *A. schupakovi*) was digested with 16 restriction enzymes (*Alu*I, *Dra*I, *EcoR*I, *EcoR*V, *Hae*III, *Hha*I, *Hinf*I, *Hpa*II, *Hsp92*II, *Mbo*I, *Nci*I, *Pst*I, *Pvu*II, *Rsa*I, *Sac*I and *Taq*I). 12 out of 16 endonucleases cut the target sequence in one or more of the species, while four endonucleases (*Dra*I, *EcoR*V, *Pst*I and *Sac*I) did not (D'Amelio et al., 2000). Many studies have been performed on the ITS region with *Hinf*I and *Hha*I for all species of Anisakid nematodes (Espiñeira et al., 2010; Shih et al., 2010). In this study, the restriction profiles of *Anisakis* spp. are consistent with previous studies. These reports indicate that the two endonucleases *Hinf*I and *Hha*I have enough ability to discriminate between different species of the genus *Anisakis* by fragment lengths and patterns.

The tree topologies derived from ML and NJ analyses were congruent and showed that *A. physeteris*, *A. brevispiculata* and *A. paggiae* were analyzed as a sister group to the remaining anisakids analyzed (*A. pegreffii* and *A. typica*). Clustering of the *A. physeteris* complex within a single clade according to the ITS phylogeny, is consistent

with previously reported phylogenetic trees using mitochondrial cox2 and allozyme data (Koinari et al., 2013; Murata et al., 2011). In addition, *A. physeteris* seems to share a common ancestor with *A. brevispiculata*. The phylogenetic position of the *A. physeteris* complex (*A. physeteris*, *A. paggiae* and *A. brevispiculata*) is also supported by a relatively homogeneous distribution throughout the central Atlantic Ocean and host-parasite association were host specific for the sperm whales, which may be a derived characteristic reflecting their evolutionary history (*Physeteris catodon*, *Kogia breviceps*, and *K. sima*) (Klimpel et al., 2011). *A. pegreffii* and *A.typica* had high genetic heterogeneity, although they belonged to type I by morphology. According to cox2 and ITS analysis, *A. typica* was closely related to *A. ziphidarum* and *A. nascettii*, whereas *A. pegreffii* indicated close genetic relationships among the species of the *A. simplex* complex (*A. simplex* s.s., *A. pegreffii* and *A. simplex* C) (Anshary et al., 2014). Our results are consistent with these findings, suggesting that *A. typica* forms a separate group to *A. pegreffii* and *A. physeteris* complex, and *A. typica* represents a distinct lineage.

Mackerel is a common name used for a number of different species of fish from the family Scombridae. *Scomber* is a genus of ocean-dwelling mackerels, and the four species of *Scomber* are commonly infected with Anisakid nematodes. Fish with a high recorded variability of species of the genus *Anisakis* infection was the spotted mackerel,

S. australasicus (*A. pegreffii*, *A. simplex* s.s., *A. typica*, *A. paggiae*, *A. physeteris*, *A. brevispiculata* and a recombinant genotype), followed by chub mackerel, *S. japonicas* (*A. pegreffii*, *A. simplex* s.s., *A. typica*, *A. physeteris*, *A. ziphidarum* and a recombinant genotype), atlantic chub mackerel, *S. colias* (*A. pegreffii*, *A. physeteris*, *A. nascettii* and *A. typica*) , and atlantic mackerel, *S. scombrus* (*A. pegreffii*, *A. simplex* s.s. and *A. physeteris*) (Abollo et al., 2001; Bak et al., 2014; Chen et al., 2014; Costa et al., 2011; Pontes et al., 2005). *A. simplex* s.s. or *A. pegreffii* were found in four species of *Scomber*, and they are the most common cause of human anisakiasis. We did not find *A. simplex* s.s. in *S. australasicus*; however, high prevalence and infection burden of *A. pegreffii* was identified in this study. *A. simplex* s.s. is the major etiological agent of anisakiasis and the infection rate of *A. simplex* s.s. was significantly higher than *A. pegreffii* (Umehara et al., 2007). Suzuki et al., 2010 showed that *A. simplex* s.s. larvae in *S. japonicus* were found in fish muscle more frequently than in the body cavity, whereas *A. pegreffii* was found mainly in the body cavity. They suggest that anisakiasis is caused primarily by *A. simplex* s.s. because it has a higher rate of penetration in the fish muscle than *A. pegreffii*. The olive flounder and rainbow trout were orally challenged with L3 larvae of *A. simplex* complex, and their results indicated that *A. simplex* s.s. larvae were infected in both the body cavity and body muscle, while *A. pegreffii* larvae were recovered only in the body cavity (Quiazon et al., 2011). Arizono et al., 2012 suggested

that *A. simplex* s.s. had a significantly higher rate of penetration into the solid agar than *A. pegreffii*, and they showed that *A. simplex* s.s. tolerated the acidic artificial gastric juice better than *A. pegreffii*. These reports indicate that *A. simplex* s.s. larvae have the potential ability to invade the human body and were the main causative agent of anisakiasis. Besides, a few cases of infection with *A. physeteris* have been reported from the southern islands of Japan (Asato et al., 1991). To data, the well-known world distribution of anisakid nematodes, which have been reported from all major oceans and various fish species. However, there is much less information on the cellular and molecular function in *Anisakis* nematodes. In the next chapter, we will introduce cellular and molecular regulation of *Anisakis* under thermal stress.

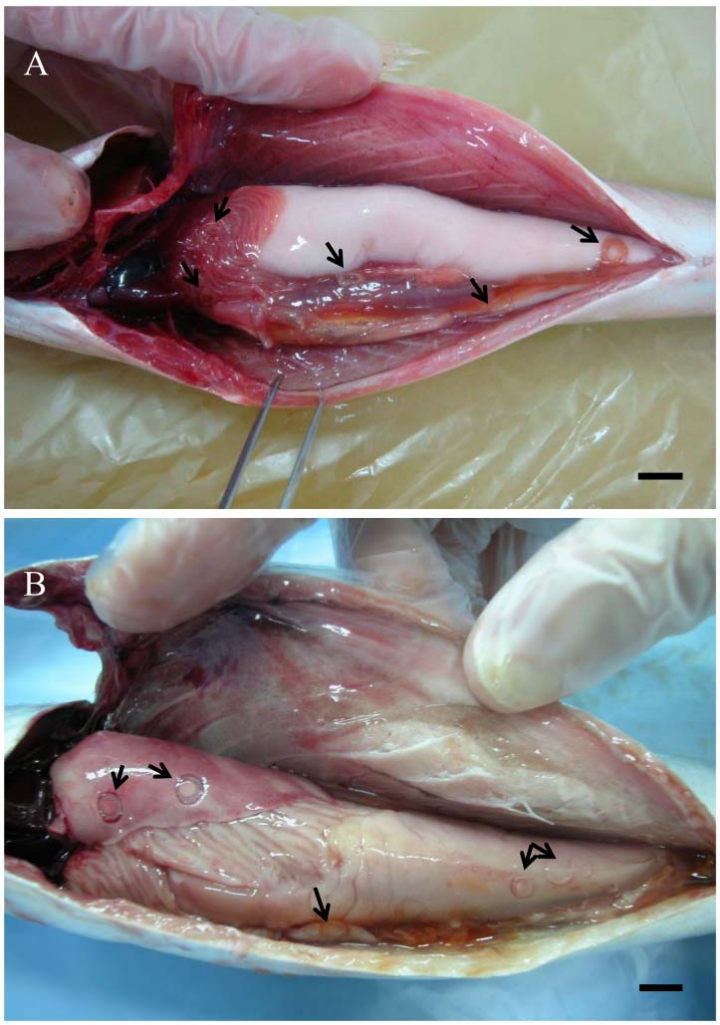


Fig. 2. 1. ***Scomber australasicus*** **heavily infected with** ***Anisakis*** **spp. third-stage larvae (L3).**

(A) The larvae were found encysted in the pyloric appendage, mesenteries, visceral cavity and male gonad. L3 encapsulated in mesentery tissue (arrows). (B) *Anisakis* larvae penetrating the liver and white adipose tissue. Encysted L3 in a typically spiral shape (arrow) was located mainly in the visceral organs. Scale Bar: 1cm.

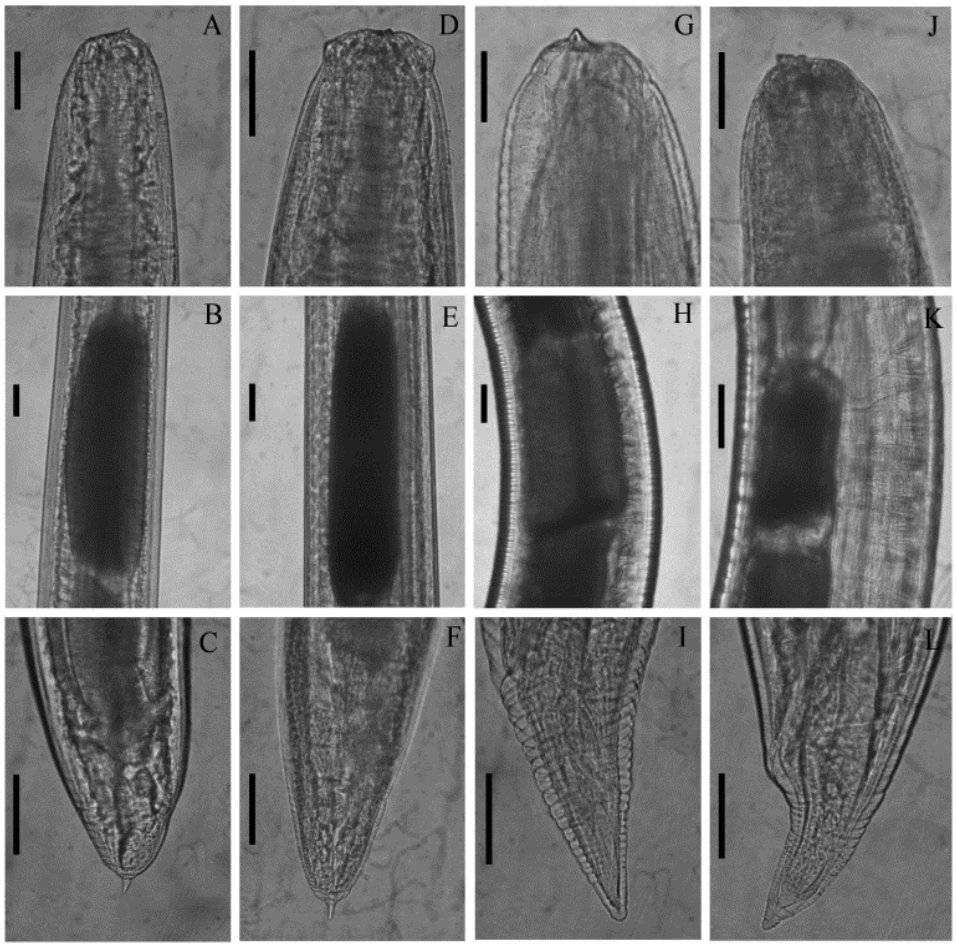


Fig. 2. 2. Morphology of third-stage larvae of the genus *Anisakis* from *S. australasicus*. (A-C) *A. pegreffii*; (D-F) *A. typica*; (G-I) *A. physeteris*; (J-L) *A. paggiae*. (A, D, G, J) cephalic end; (B, E, H, K) ventricular part; (C, F, G, L) caudal end. Bar: 100 μm.

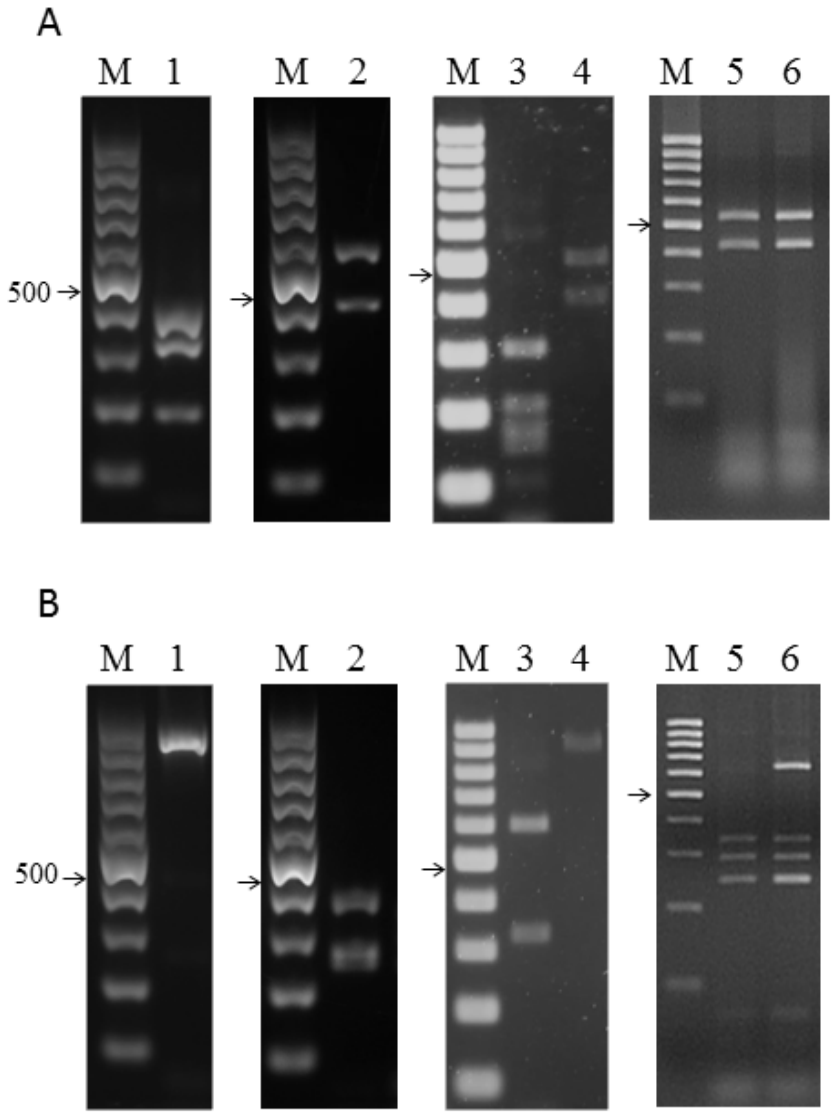


Fig. 2. 3. PCR-RFLP analysis for the identification of the genus *Anisakis*.

RFLP patterns digested by the endonucleases (A) *Hha*I and (B) *Hinf*I. Lane 1: *A. brevispiculata*; Lane 2: *A. physeteris*; Lane 3: *A. typica*; Lane 4: *A. paggiae*; Lane 5: *A. pegreffii*; Lane 6: Recombinant genotype of *A. pegreffii* and *A. simplex* s. s.. M: DNA marker (100-bp ladder).

1. a. The ITS regions digested with *Hha*I, and produced more than two bands.....2
   b. The ITS regions digested with *Hha*I, and produced two bands.....3
2. a. The result produced three bands and fragment sizes are ca. 390, 330, and 190 bp.....*A. brevispiculata*
   b. The result produced four bands and fragment sizes are ca. 320, 240, 180 and 160 bp.....*A. typica*
3. a. The ITS regions digested with *Hinf*I, and produced three or more bands.....4
   b. The ITS regions digested with *Hinf*I, and produced one band and fragment size is ca. 920 bp.....*A. paggiae*
4. a. The result produced three bands.....5
   b. The result produced four bands and fragment sizes are ca. 620, 370, 300 and 250 bp.....Recombinant genotypes
5. a. The fragment sizes are ca. 370, 300, and 250 bp.....*A. pegreffii*
   b. The fragment sizes are ca. 380, 270, and 250 bp.....*A. physeteris*

Fig. 2. 4. Molecular taxonomic key for six species of the genus *Anisakis* based on PCR-RFLP of the ITS region of rDNA.

Restriction enzymes *Hha*I and *Hinf*I were used for species differentiation.

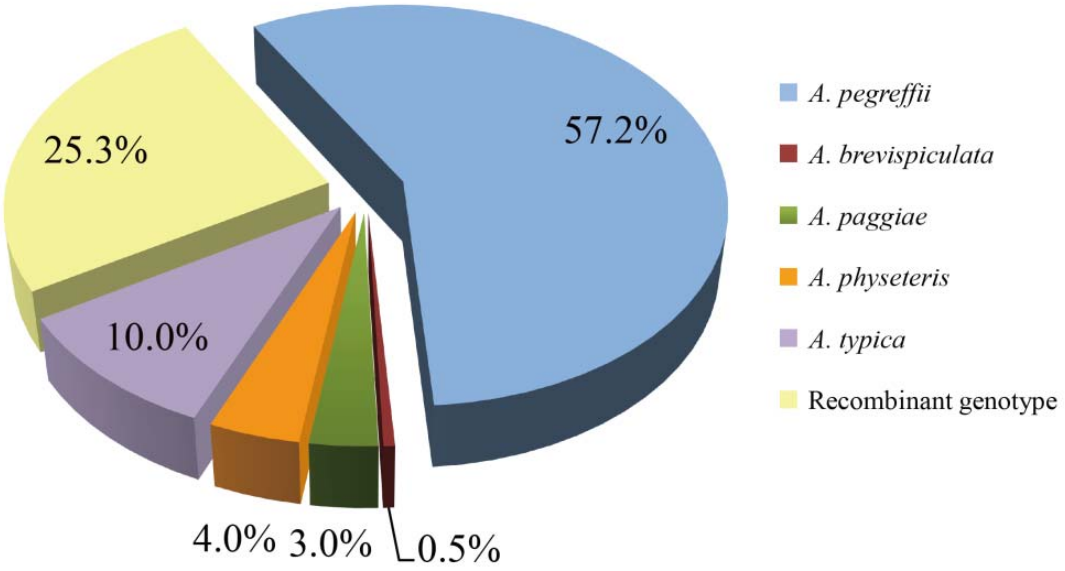


Fig. 2. 5. The percentage and distribution of *Anisakis* larvae found in *S. australasicus* from Taiwanese waters.

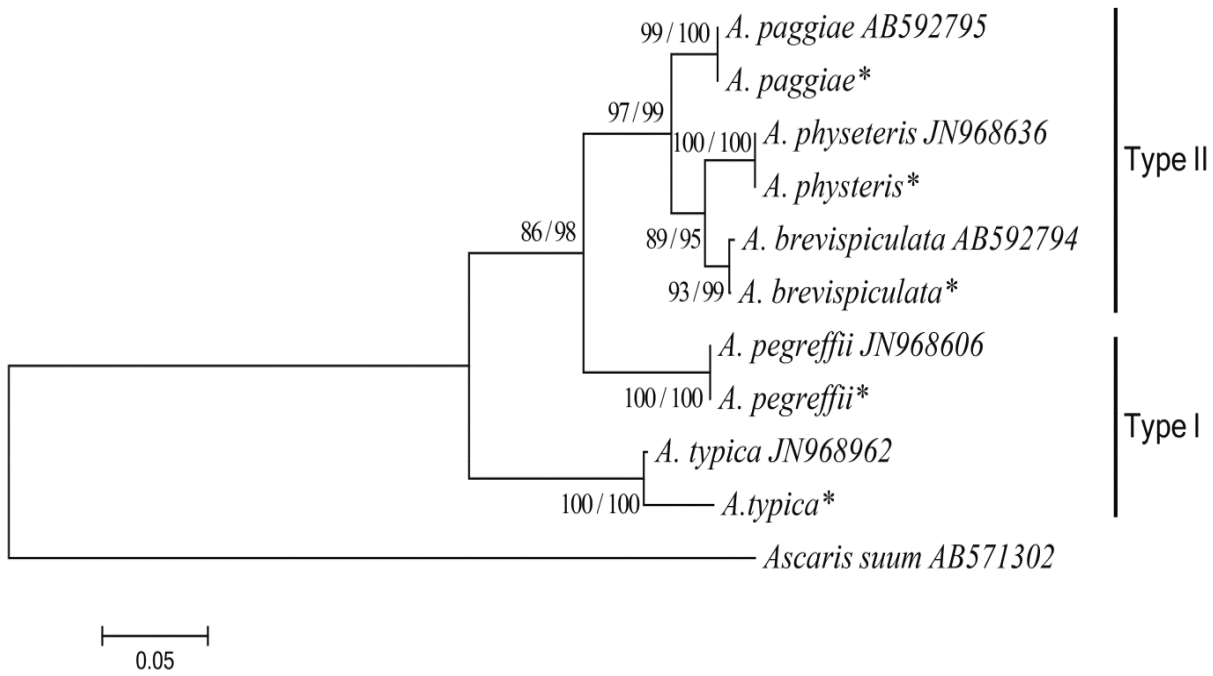


Fig. 2. 6. Phylogenetic relationships of six species of the genus *Anisakis* in this study (*) and sequence of *Anisakis* species with accession numbers from the GenBank based on ITS1-5.8S-ITS2 gene sequence.

Significant bootstrap supports from 1000 replicates of maximum likelihood (ML) and neighbor joining (NJ) are shown at each node in the order ML/NJ and percentages ≥85% are shown at the internal nodes. *Ascaris suum* was used to as an outgroup.

Chapter 3 Effect of different temperatures on the expression of the newly characterized heat shock protein 90 (Hsp90) in L3 of *Anisakis* spp. isolated from *Scomber australasicus*

3-1 Introduction

Marine nematodes of the genus *Anisakis* are food-borne parasites that can infect over 200 pelagic fish species and cephalopods such as squid. Humans may become accidental host if they ingest raw or undercooked infected seafood such as Japanese sushi, sashimi, Hawaiian lomi-lomi, Latin American ceviche and Dutch salted or smoked herring (Bouree *et al*., 1995; Alonso-Gómez *et al*., 2004). Even dead parasites in frozen or well-cooked seafood can induce allergic reactions in sensitive patients to this parasite (Rodríguez-Mahillo *et al*., 2010; Tejada *et al*., 2013). Three species of the family Anisakidae have been found to cause infections in humans: *A. simplex* sensu stricto, *A. pegreffii* and *Pseudoterranova decipiens* (Quiazon *et al*., 2011; Arizono *et al*., 2012; Mattiucci *et al*., 2013). The digestive symptoms of anisakiasis include acute gastric, abdominal pain, nausea, vomiting, abscessing or eosinophilic granulomas and stomach ulcers. The excretory-*secretory* products of larvae and dead larvae may also induce severe allergic reactions such as rash, itching, anaphylaxis, acute urticaria and angioedema (Audicana *et al*., 2002; Baird *et al*., 2014; Sharp and Lopata, 2014). To date,

12 allergens of *A. simplex* are described and 5 of those (Ani s 1, 4, 5, 8 and 9) are highly resistant to heat (Audicana and Kennedy, 2008; Rodriguez-Perez *et al*., 2008; Quiazon *et al*., 2013).

Several papers have been published concerning the survival and resistance of anisakids under different treatment conditions (Brutti *et al*., 2010; Tejada *et al*., 2014). The survival capability of the third-stage *Anisakis* larvae was tested in various conditions, such as in microwaving, freezing, heating, salting, as well as application of anthelmintic drugs and condiments (Wang *et al*., 2010). The common method to kill the larvae in fish is application of high or low temperature. Lanfranchi *et al*. (2010) found that *Anisakis* can survive up to 330 days in NaCl solution (0.85%) at 4 – 5.5°C. Vidaček *et al*. (2010) reported that larvae were immobile and dead after heating at 60°C for ≥3 min, 70°C for ≥1 min and 80°C for ≥30 s. According to the U.S. Food and Drug Administration (FDA) recommended that fish should be cooked assuring an internal temperature of at least 63°C and 74°C for stuffed fish. *Anisakis* species have been found to be alive after freezing at -20ºC for short periods and the FDA recommends freezing fish at -20ºC for at least 168 hours or blast-freezing at -35ºC for at least 15 hours.

Heat shock proteins (Hsps) have been found in almost all living organisms, from bacteria to humans. These proteins are involved in development, growth, signal transduction, cellular metabolism and innate immune response. They play critical roles

in responses to stress such as low or high temperature, heavy metal exposure or parasite infections (Asea *et al*., 2000; Devaney, 2006; Saunders and Verdin, 2009; Shiny *et al*., 2011; Yang *et al*., 2013; De Jong H *et al*., 2014). According to their molecular weight, Hsps have been grouped into several families: Hsp100 (100 kDa), Hsp90 (83-90 kDa), Hsp70 (67-70 kDa), Hsp60 (60 kDa), Hsp40 (40 kDa) and small heat shock proteins (16-25 kDa).

Hsp90 is an essential molecular chaperone in eukaryotic organisms and abundant in unstressed cells, constituting 1-2% of the cytosolic proteins (Pratt, 1998). Hsp90 exists as a homodimer consisting of a highly conserved N-terminal 'ATPase domain', a charged linker region, that connects the N-terminus with the middle domain followed by ‘middle region’ and C-terminal dimerization domain, which were involved client proteins and cochaperones binding (Johnson, 2012; Eckl and Richter, 2013). Its functions are related to cell growth and differentiation, apoptosis, signal transduction, oncogenic transformation, antigen presentation and cancer (Graefe *et al*., 2002, Tsan and Gao, 2004, Péroval *et al*., 2006; Kumar *et al*., 2007; Tsutsumi and Neckers, 2007; Lamoureux *et al*., 2013). Hsp90 also plays an important role in response to stress condition such as heat or cold shock and induces stress proteins to protect cells. On the other hand, Hsp90 is a useful marker for phylogenetic analysis in nematodes (Skantar and Carta, 2004; Him *et al*., 2009). To date, no Hsp90 gene of *Anisakis* spp. has been

characterized, therefore, we cloned, characterized and compared the full-length cDNA of Hsp90 of five *Anisakis* spp.. Additionally, we investigated the expression levels of Hsp90 after cold and heat shock. For that, we used the third-stage larvae of *A. pegreffii* as a model, which is commonly found in many marine fishes and causes human anisakiasis.

3-2 Material and methods

3-2-1 Sampling and experimental design

Two hundred and fifty specimens spotted mackerel (*Scomber australasicus*) were obtained from professional fisherman in the coastal waters of Yilan, north-eastern Taiwan (25°N, 121°E) during 2012- 2013. Fresh specimens were stored on ice and transported to the laboratory immediately. Live *Anisakis* spp. third-stage larvae (L3) were recovered from visceral cavity and intestine of fish. After washing in physiological saline, species and developmental stages of Anisakid nematodes were pre-identified based on the morphological characteristics described previously (Smith, 1983; Shih, 2004; Murata *et al*., 2011) and later characterized genetically to species level by PCR restriction fragment length polymorphism.

The present study is divided into two major parts: molecular characteristics of the Hsp90 gene and the effect of different temperatures on expression of Hsp90. For the

molecular studies, DNA and RNA were extracted from single *Anisakis* individuals. The DNA was used for subsequent species identification by PCR-RFLP and the RNA extraction was reverse transcribed into complementary DNA (cDNA) for molecular cloning of the Hsp90 gene.

To examine the expression levels of Hsp90 of anisakid nematodes in response to different temperature and time treatments, we selected *A. pegreffii* as study object. Freshly isolated larvae were exposed to either 4°C, 37°C and 50°C. Thereby we imitated three conditions: the fish intermediate hosts that are stored in the range 0-4°C in fish markets, larvae invading the gastrointestinal tract of humans (37°C); and lightly cooked infected fish at 50°C. Five live larvae each were added to 1 ml of distilled water in 3.5 cm Petri Dishes, and incubated at either 4°C for 1, 2, 3, 6, 12 and 24 h in the refrigerator, or at 37°C in 5% CO_2 for 1, 2, 3, 6, 12 and 24 h. For the 50°C incubation, five larvae were added to 1 ml of distilled water in 1.5 ml reaction tubes, and were incubated for 1, 2, 3, 5 and 7 min in a water bath. After each treatment, the larvae were examined immediately under a dissecting microscope. Spontaneous and stimulated (touching the larvae with a needle without damaging them) movements of the larvae were recorded. Every individual was put in a cryovial and stored in liquid nitrogen until use. Freshly isolated worms from the fish were frozen immediately as control groups. A total of 204 *A. pegreffii* larvae were used for both the expression of Hsp90 mRNA and

protein expression, and twelve *A. pegreffii* larvae were used as controls.

3-2-2 DNA, RNA and protein extraction

Both DNA and RNA were extracted from individual L3 using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions with some modifications. Briefly, the frozen larva was homogenized using a motorized pestle and homogenized tissue in 1 ml of Trizol with the addition of 0.2 ml of chloroform. The mixture was vortexed and centrifuged (13,000 rpm, 15 min, 4°C) resulting in an aqueous (containing RNA) and organic phase (containing DNA and proteins). The aqueous layer was transferred to a separated tube and RNA was precipitated with 600 μl isopropanol overnight at -80°C and pelleted by centrifugation (13,000 rpm, 10 min, 4°C), washed in 1 ml of 75% ethanol, centrifuged again, and dried for 10 min. The pellet was resuspended in 30 μl nuclease and RNase-free water at 60°C for 15 min, centrifuged again, and the clear upper aqueous layer was transferred to a new reaction tube.

DNA was precipitated from the remaining interphase/organic layer with 300μl absolute ethanol. The mixture was centrifuged (4,800 rpm, 10 min, 4°C), and the upper aqueous layer (containing proteins) was transferred to a new tube. The pellet was washed in 1 ml of 0.1 M sodium citrate in 10% ethanol and centrifuged (10,000 rpm, 10

min, 4°C). The DNA sample was dried for 10 min at room temperature and was resuspended in 20 µl TE buffer (Tris-EDTA buffer) at 65°C for 10 min, followed by centrifugation. The clear supernatant was transferred to a new reaction tube.

Proteins were precipitated by 800µl isopropanol followed by centrifugation (11,000 rpm, 10 min, 4°C). The protein pellet was washed with 2 ml of 0.3 M guanidine hydrochloride in 95% ethanol, and incubated for 20 min followed by addition of 2 ml of 100% ethanol, and centrifugation at 6,000 rpm for 5 min at 4°C. The pellet was dried for 10 min at room temperature, dissolved in 25 µl 1% SDS (1:1; v/v; protein fraction), and incubated at 50°C in a water bath. This solution was centrifuged (10,000 rpm, 10 min, 4°C), and the supernatant containing the proteins was transferred to a new tube. RNA, DNA and protein concentration and quality were measured by a NanoDrop® TC1-E20 spectrophotometer. DNA was stored at –20°C, RNA and protein was stored at –80°C until use.

3-2-3 PCR-RFLP

Larvae of *Anisakis* spp. were identified by a PCR-RFLP analysis as previously described (Cavallero *et al*., 2011). The same protocols and conditions for the PCR-RFLP analysis of the ITS region (ITS1-5.8S-ITS2) were followed in this study. The PCR products of the ITS region were digested with two endonucleases *Hinf*I and

*Hha*I. The digested products were separated by electrophoresis on 2% agarose gel and visualized by staining with ethidium bromide.

3-2-4 RT-PCR, cloning of Hsp90 and amplification of Hsp90 cDNA

To remove the traces of genomic DNA from the Trizol extracted RNA, 1 µl DNase I (1 U/ µl, Invitrogen) was added to 1 µg of RNA sample. cDNA was synthesized using 1 µg of RNA, 0.5 µg $dT_{12\text{-}18}$, 1.25 µl dNTP (10 mM), 1 µl RNase inhibitor (25U/ µl, Promega, USA), and 1 µl M-MLV reverse transcriptase (200U/ µl, Promega), topped up to 25 µl with nuclease-free water. The mixture was incubated for 60 min at 37˚C and subsequently transferred on ice. Amplification of Hsp90 of *Anisakis* spp. were performed by PCR in a total volume of 25 µl containing 2.5 µl 10X PCR buffer, 2 µl $MgSO_4$ (25 mM), 2.5 µl dNTPs (2 mM each), 1 µl of each primer (10 mM) (Table 3. 1), 3µl cDNA, and 1 µl KOD Hot Start DNA Polymerase (1 U/µl, TOYOBO, Japan). PCR was performed as follows: 95˚C for 3 min followed by 30 cycles of 30 s at 95˚C, 30 s at 60˚C, 2 min 30s at 72˚C with a final extension at 72˚C for 15 min. The PCR products were separated using agarose gel electrophoresis, and band of the expected size were gel purified with the QIAquick Gel Extraction kit (Qiagen, Germany). The purified PCR products were dA-tailed, cloned into pGEM®-T Easy Vector (Promega) and sequenced.

The 5' and 3' ends of Hsp90 cDNA were amplified using a 5'/ 3' RACE system for

rapid amplification of cDNA ends kit (Invitrogen) as specified by the manufacturer. To obtain 3' RACE, two forward primers, GSP4_3race and GSP5_3race, were designed according to the above known full-length cDNA sequence of Hsp90 (Table 3. 1). The three adapter primers were provided in the kit. First-strand cDNA was synthesized by SuperScript™ II Reverse Transcriptase at 42˚C for 50 min using an adapter primer on RNA sample. Fist PCR amplification was performed using a universal amplification primer and GSP4_3race. 5 min at 95˚C were followed by 35 cycles, which were performed as follows: 30 s at 95˚C, 30 s at 63˚C, 1min 30 s at 72˚C, with a final extension at 72˚C for 7 min. The 10-fold dilution of first PCR amplification product was prepared for nested PCR. Nested PCR were performed using an abridged universal amplification primer and GSP5_3race. After an initial 5 min at 95˚C, 35 cycles were performed as follows: 30 s at 95˚C, 30 s at 55˚C, 45 s at 72˚C with a final extension at 72˚C for 7 min.

For 5' RACE, two reverse primers, Hsp90_37r_5race and Hsp90_38r_5race, were designed according to the above known full-length cDNA sequence of Hsp90 (Table 3. 1). First-Strand cDNA Synthesis of 5' RACE was synthesized by SuperScript™ II reverse transcriptase at 42˚C for 50 min using the Hsp90_37r_5race. The obtained cDNA was modified by DNA tailing by TdT, and PCR amplification was performed using Hsp90_38r_5race and an abridged anchor primer provided in the kit. After an

initial 5 min at 95˚C, 35 cycles were performed as follow: 30 s at 95˚C, 30 s at 50˚C, 1 min at 72˚C with a final extension at 72˚C for 7 min. Amplified products were separated on a 1.5% agarose gel, and the DNA was purified, cloned into pGEM-T Easy Vector. Following transfection into competent *Escherichia coli* DH5α cells, recombinants were identified by blue and white selection and the positive clones were sequenced. All primers designed are shown in Table 3. 1.

3-2-5 Real-time PCR analysis

A. pegreffii was selected and used for real-time PCR. The cDNA synthesized in the above reaction was used as a template with the target gene Hsp90 and the house-keeping gene GAPDH as a reference. Each cDNA sample was transcribed from a RNA pool from three larvae of *A. pegreffii* to increase efficiency of normalization and maximize the number for which quantity data were obtained. For each temperature and time point four pools were used as replicates. The fluorescent real-time PCR assay was carried out in a CFX384 real-time PCR system (BioRad, USA). The amplification were performed in a 10μl reaction volume containing 5 μl of 2X iQ™ SYBR® Green Supermix (BioRad), 1 μl of each primer (10 mM), 1 μl of 10-fold diluted cDNA, and 3.6 μl dH2O. Cycling conditions involved an initial 5 min at 95˚C, 40 cycles were performed as follows: 30 s at 95˚C, 30 s at 55˚C, and 30 s at 72˚C. After the reaction, a

melting curve analysis from 65˚C to 95˚C was applied to ensure consistency and specificity of the amplified product. All reactions were carried out in triplicates, including a control with no template. The data was expressed as mean ± SD (n = 4). Analysis of the real-time PCR data was conducted with the Bio-Rad CFX Manager™ Software (Hellemans *et al*., 2007; Pfaffl, 2001). The baseline was set automatically by the software. The concentration of the target gene and control gene were determined based on the threshold cycle number (Ct). The qPCR experiment was designed and performed according to the MIQE guide recommendation (Bustin *et al*., 2009). Primers are shown in Table 3. 1.

3-2-6 Western blot analysis

Western blot analysis was conducted for *A. pegreffii*. Like for the qPCR, each protein sample was pooled from three larvae of *A. pegreffii* to increase efficiency of normalization and maximize the number for which quantity data were obtained and four replicates of these pooled samples were tested. For each sample, 15 μg of total protein were run in 10% SDS-PAGE gels, and transferred by Western blot to nitrocellulose membranes. Incubation with primary polyclonal rabbit anti-Hsp90 alpha (GTX109753, Genetex Inc, CA) at a 1: 1,000 dilution, and was performed overnight at 4°C. After washing with TBS-T, the blots were incubated in the secondary antibody conjugated

horseradish peroxide (GTX213110-01, Genetex Inc, CA) at a 1: 5,000 dilution for 1 h at 37°C. Polyclonal rabbit anti-GAPDH (GTX100118, GeneTex Inc, CA) at a 1: 2,000 dilution, and was used as a control screen to normalize protein loading. Protein bands were visualized with western ECL kit (Bioman, Taiwan), and quantified using an Auto-Chemi system (UVP; Bio-Imaging Systems) and the image acquisition analysis software Labworks (version 4.6). The protein data were expressed as the mean ± SD (n = 4). Differences between the control and exposed treatments were determined using one-way ANOVA followed by Dunnett's Multiple Comparison Test. Differences were considered statistically significant at $p < 0.05$.

3-2-7 Bioinformatic analysis

Multiple alignments of deduced amino acid sequence of Hsp90 and 3′-UTR sequences were performed with ClustalW2. The results were displayed using BioEdit v7.2.0. The molecular weight and theoretical isoelectric points were calculated using the Compute pI/Mw tool (ExPASy server). The motifs of protein sequences were searched using the MyHits (http://myhits.isb-sib.ch/).

3-3 Results

3-3-1 Morphological and PCR-RFLP analysis of *Anisakis* spp.

The third-stage of *Anisakis* larvae were obtained from *S. australasicus* collected in Taiwanese waters. Through a combination of morphological and molecular analyses the species *A. typica*, *A. pegreffii* belonging to Type I and *A. paggiae*, *A. brevispiculata*, *A. physeteris* belonging to Type II were detected. Additionally, the molecular analysis revealed a recombinant genotype of *A. simplex* s. s. and *A. pegreffii*. *S. australasicus* is a new host record for *A. paggiae*, *A. brevispiculata*, *A. physeteris* and these species were described for the first time in the coastal waters of northeastern Taiwan. The larvae exhibited the following RFLP patterns: Digestion with *Hinf* I produced three bands (ca. 370, 300, and 250 bp) in *A. pegreffii*, two bands (ca. 620 and 350 bp) in *A. typica*, three bands (ca. 360, 270, and 250 bp) in *A. physeteris*, only one band (ca. 920 bp) in *A. paggiae*, one band (ca. 900 bp) in *A. brevispiculata* and four bands of (ca. 620, 370, 300 and 250 bp) in the recombinant genotype. Digestion with *Hha*I produced two major bands (ca. 550 and 430 bp) in *A. pegreffii*, four bands (ca. 320, 240, 180 and 160 bp) in *A. typica*, two bands (ca. 520 and 390 bp) in *A. physeteris*, 2 bands (ca. 520 and 410 bp) in *A. paggiae*, 3 bands (ca. 390, 330, and 190 bp) in *A. brevispiculata* and 2 bands (ca. 550 and 430 bp) in the recombinant genotype (Fig. 3. 1; recombinant genotype data not shown).

3-3-2 Molecular characterization of Hsp90

The complete sequences of ApeHsp90, AphHsp90, ApaHsp90, AtHsp90, and AbHsp90 were 2457, 2649, 2609, 2730, and 2683 bp in length, respectively. The lengths of the open reading frame (ORF) deduced amino acid of the five genes were 2163 bp/ 721 aa (AphHsp90), 2163 bp/ 721 aa (ApaHsp90), 2163 bp/ 721 aa (AtHsp90), 2157 bp/ 719 aa (ApeHsp90), and 2166 bp/ 722 aa (AbHsp90). Each of these ORF sequences contained a 5′-terminal untranslated region (UTR) with 22-nt spliced leader SL1, which is present in all nematodes, and a 3′-terminal UTR with a polyadenylation signal sequence and a poly (A) tail (Table 3. 2). The calculated molecular weights were 83.01, 83.17, 83.21, 83.21, and 83.18 kDa. The theoretical isoelectric points (pI) were 4.95, 5.01, 4.97, 4.99, and 4.99, respectively. The deduced peptide sequence contained five highly conserved segments (I-V) that are common to all eukaryotic Hsp90 proteins (Fig. 3. 2). The cytoplasmic Hsp90 C-terminal region of MEEVD motif was highly conserved in the five *Anisakis* species. A Lysine-rich motif and a conserved domain of the histidine kinase-, DNA gyrase B-, and Hsp90-like ATPase were also discovered by using MyHits. In the 3′-UTR sequences of five *Anisakis* spp. with variable lengths and 57.6- 92.7% sequence identity between the five species (Fig. 3. 3).

3-3-3 Expression of Hsp90 mRNA in response to different temperatures

Quantitative RT-PCR was performed to examine the mRNA expression pattern of the Hsp90 gene in *A. pegreffii*. The mRNA expression of Hsp90 at 50˚C was increased gradually ($R^2 = 0.8856$), and the expression of Hsp90 increased after 1 min and peaked at 7 min (Fig. 3. 4A). The result indicated that after thermal stress treatment at 37°C, the mRNA expression of Hsp90 increased gradually (1 h, 2 h, and 3 h), and recovered afterwards (6 h, 12 h, and 24 h) (Fig. 3. 4B). Nematodes were exposed to cold stress at 4°C, and the mRNA expression of Hsp90 did not change significantly (Fig. 3. 4C). There is no statistically significant difference between the groups in our study.

3-3-4 Protein expression of Hsp90 by heat and cold stress treatments

Western blot analysis demonstrated a different expression of Hsp90 after heat (50 or 37°C) and cold (4°C) stress treatment in *A. pegreffii*. At high temperature (50°C) condition, protein expression of Hsp90 increased markedly; the expression levels were significantly higher at 5 and 7 min compared to control ($p < 0.05$). After the heat shock (37°C) treatment, the expression level was lowest at 6 h ($p < 0.05$), and expression raised significantly at 12 and 24 h compared to control ($p < 0.05$). At low temperature (4°C), the expression level was higher at 1 h ($p < 0.05$, compared to control); however, it decreased gradually afterwards (Fig. 3. 5).

3-4 Discussion

In our previous study, we found three *Anisakis* nematodes in *S. australasicus* collected from Taiwanese waters: *A. pegreffii*, *A. typica* or a recombinant genotype of *A. pegreffii* and *A. simplex* sensu stricto (Chou *et al*., 2011). In addition, *A. simplex* s. s. and *A. pegreffii* had previously been recovered from this species of fish in Japanese waters. (Quiazon *et al*., 2008). In the present study, *A. paggiae*, *A. physeteris* and *A. brevispiculata* are reported for the first time from this host, providing new host and locality records. We determined the full-length cDNA sequence of the Hsp90 gene from *A. pegreffii*, *A. typica*, *A. paggiae*, *A. physeteris* and *A. brevispiculata*. The trans-splicing of 22-nt spliced leader (SL) to the mature 5′ UTR of the mRNA is a common feature present in diverse eukaryotes, including trypanosomatid protozoa, urochordates, cnidarians, rotifers, platyhelminths, and nematodes (Guiliano and Blaxter, 2006). These conserved SL were also found in five species of the genus *Anisakis* showing 100% sequence identity (Table 3. 1). The 3'-UTR has a conserved AATAAA or ATTAAA polyadenylation signal, which is important for the transport out of the nucleus and to stabilize the mRNA (van Oers *et al*., 1999). *A. pegreffii*, *A. typica* and *A. paggiae* contained a AATAAA or ATTAAA motif present in the 3'-UTR while *A. physeteris* and *A. brevispiculata* represented a CATAAA motif. The extremely conserved C-terminal pentapeptide MEEVD, which is responsible for the interaction with tetratricopeptide

repeat (TPR) domain-containing co-chaperones and is found in many eukaryotes (Prasad *et al*., 2010). These specific characters were recognized in five species of the genus *Anisakis*. Overall, the amino acid sequences of Hsp90 from *Anisakis* was highly conserved with only few gaps in the sequence and some variable amino acids.

The sequence of Hsp90 isolated in the present study might be used as a highly conserved and ubiquitous phylogenetic marker (Stechmann and Cavalier-Smith, 2003) for future studies of relationships among nematodes or for eukaryotic global phylogeny.

Temperature is one of the most important parameters affecting the abundance and distribution of nematode species around the world (Basáñez *et al*., 2012). In general, the heat tolerance of nematodes is low. Previous studies have shown that *A. simplex* L3 that were exposure to 50°C for 5 min showed a decrease in mobility by 50%. The larval cuticle ruptured, shrinked and became more susceptible by higher temperatures and longer heating times (Tejada *et al*., 2006; Vidaček *et al*., 2010; 2011). In heat stress experiment, our study reveals same results as previous research. The larvae were heated at 50°C for 1, 2, 3, 5 and 7 min in a water bath and they stayed alive within 7 min. However, the larvae were heated at 50°C for 10 min in a water bath and we examined these larvae under a dissecting microscope and we found that they changed their appearance from bright translucent to opaque white, the bodies were ruptured and the gut broke through the mouth opening. *Anisakis* larvae survived in a domestic

refrigerator (4 -5.5°C) in Petri dishes with NaCl solution (0.85%) and without nutrients or antibiotic. They survived almost a year under these conditions (Lanfranchi *et al*., 2010). In cold stress experiment, the larvae were incubated at 4°C within 24 h, all stayed alive, but they were less active. On the contrary, the larvae were incubated at 37°C for 24 h, in air atmosphere with 5% CO_2 were increasingly active in movement.

The central dogma of molecular biology states that DNA makes RNA and RNA makes proteins, but there is uncertainty regarding the general correlation between levels of RNA and proteins. This correlation is found between mRNA and protein expression changes in different genes, different developmental stages or different physiological conditions within the same species (Mattick, 2003; Fu *et al*., 2007). The use of mRNA expression patterns by themselves, however, is deficient in understanding the complicated biological processes of proteins, such as transcriptional splicing, post-transcriptional mechanisms, translational regulation and degradation, which may affect the relative quantities of a protein present in a cell or individuals. Proteins are the major direct executors of life processes and might reflect gene function more directly than mRNA (Guo *et al*., 2008). In our experiments, the time-dependent protein expression patterns of Hsp90 were observed under high temperature. Both RNA and protein expression levels went up and reached the highest level at 7 min. The Hsp90 protein expression was lowest at 6 h ($p < 0.05$), and expression was significantly

increased at 12 and 24 h compared to the control ($p < 0.05$) at 37°C. The result is quite opposite to what RNA expression suggested at 37°C. The protein expression significantly increased two-fold after 1 h of treatment and it decreased gradually at cold temperatures (4°C); however, the RNA expression of Hsp90 did not change significantly. These results indicated that the positive correlation between mRNA and protein expression at 50°C. Conversely, there was no relation between mRNA and protein expression at 4°C and 37°C. Zhu *et al*. (2012) reported that Hsp90 overexpression under heat stress condition is higher than under cold stress condition in root-knot nematode, *Meloidogyne incognita*. They found that Hsp90 is more conducive to protect *M. incognita* under high temperature stress. Expression of Hsp90 considerably increased under heat stress (Prodromou *et al*., 2000). Dangi *et al*. (2014) proposed that increased Hsp expression during short-term heat stress may be owing to the fact that heat stress stimulated and quickly initiated the transcription of Hsp mRNA and translation of Hsp protein to protect cells from heat stress. Feng *et al*. (2010) reported that the Hsp90 mRNA levels of the carmine spider mite, *Tetranychus cinnabarinus* increased significantly after being induced at 40°C; moreover, TcHsp90 was also induced at 4°C. These research results suggest that Hsp90 might be responsible for the different intensity of heat and cold stresses due to its ability to protect the individual. Previous research suggested that *A. simplex* larvae exposed to 50°C for 5 min still had a survival

rate of 50% (Vidaček *et al*., 2010). In the present study, *A. pegreffii* larvae were exposed to 50°C for 7 min and they stayed alive. Our findings suggest that larvae have the ability to tolerate short-term heat stress, and that this may be related to Hsp90 expressions to protect cells. However, it has to be further verified whether the Hsp90 gene plays a vital role in heat tolerance. In addition, our results indicated a stronger response of the expression of Hsp90 after heat stress compared to cold stress in *A. pegreffii*.

Table 3. 1 Primers designed for Hsp90 gene cloning and real time RT-PCR analysis.

Primer name	Sequence (5'-3')	Amplicon length (bp)
*A. pegreffii*_Hsp90	F: ATGTCTGATCAGAAAGGAGAAGGCGAAACGTTCGC	2157
	R: TCAATCGACTTCTTCCATCCGTGATGC	
*A. typica*_Hsp90	F: ATGTCTGATCAGAAAGGAGAAGGCGAAACGTTCGC	2163
	R: TTAATCGACTTCTTCCATTCGTGACGC	
*A. paggiae*_Hsp90	F: ATGTCTGAGCAGAAAGGAGAAGGCGAAACTTTCGC	2163
	R: TTAATCGACCTCCTCCATCCGCGACGC	
*A. physeteris*_Hsp90	F: ATGTCTGAGCAAAAAGGAGAAGGCGAAACTTTCGC	2163
	R: TCAGTCAACTTCTTCCATCCGCGACGC	
*A. brevispiculata*_Hsp90	F: ATGTCTGAGCAAAAAGGAGAAGGCGGCGAGACTTT	2166
	R: TTAGTCAACTTCTTCCATCCGCGATGC	
Hsp90_37r_5'race	R: CAGTCGTCATCGTTGTGCTTC	550-551
Hsp90_38r_5'race	R: AGAGCCTCCATGAATGCTTTC	439-442
GSP4_3'race	F: TGGTCAGCCAACATGGAACGTATCATGAAGGC	771-913
GSP5_3'race	F: CGGGTTGAGGCCGATAAGAATGA	642-784
As_hsp90_RT	F: CAATAAGGAGGACAAGACAATG	183
	R: AGGAACGCAGAATAGAAACC	
GAPDH_RT	F: CCCCTTCATCAACATCGACT	152
	R: TCAGCTCCCCATTTGATTTC	

Table 3. 2 5' UTR and 3' UTR nucleotide sequence of Hsp90 in five *Anisakis* species and the full-length Hsp90 with the corresponding GenBank accession numbers.

Species	nematode spliced leader SL1 in 5′ UTR	Length of 5′ UTR (bp)	Polyadenylation signal sequence in 3′ UTR	Length of 3′ UTR (bp)	Accession numbers in GenBank
A. pegreffii	GGTTTAATTAC CCAAGTTTGAG	66	ATTAAA	234	KF840393
A. typica	GGTTTAATTAC CCAAGTTTGAG	67	AATAAA	500	KF840394
A. paggiae	GGTTTAATTAC CCAAGTTTGAG	64	AATAAA	382	KF840395
A. physeteris	GGTTTAATTAC CCAAGTTTGAG	67	CATAAA	419	KF840396
A. brevispiculata	GGTTTAATTAC CCAAGTTTGAG	67	CATAAA	450	KF840397

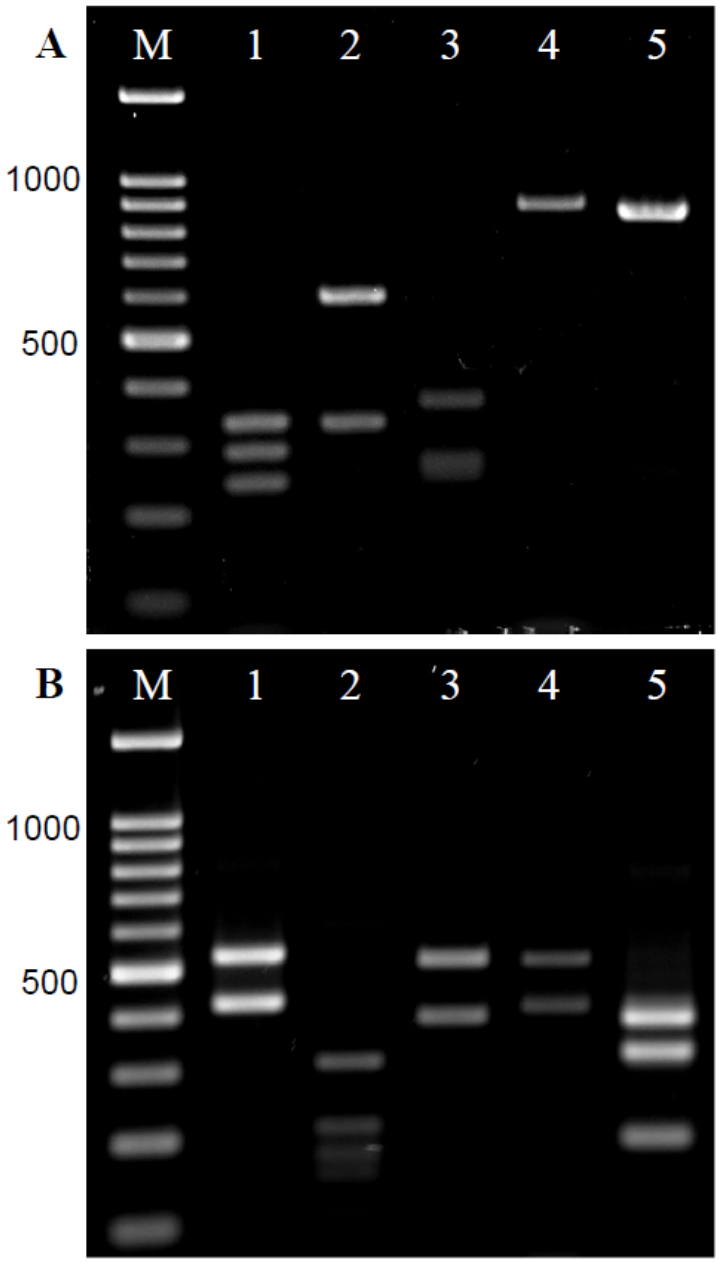


Fig. 3. 1. PCR-RFLP analysis for the identification of five *Anisakis* species shows two different fragment patterns by digestion of the ITS region (ITS1-5.8S-ITS2) with the restriction enzymes *Hinf*I (A) and *Hha*I (B).

Land 1- *A. pegreffii*. Land 2- *A. typica*. Land 3- *A. physeteris*. Land 4- *A. paggiae*. Land 5- *A. brevispiculata*. M- 100 bp marker.

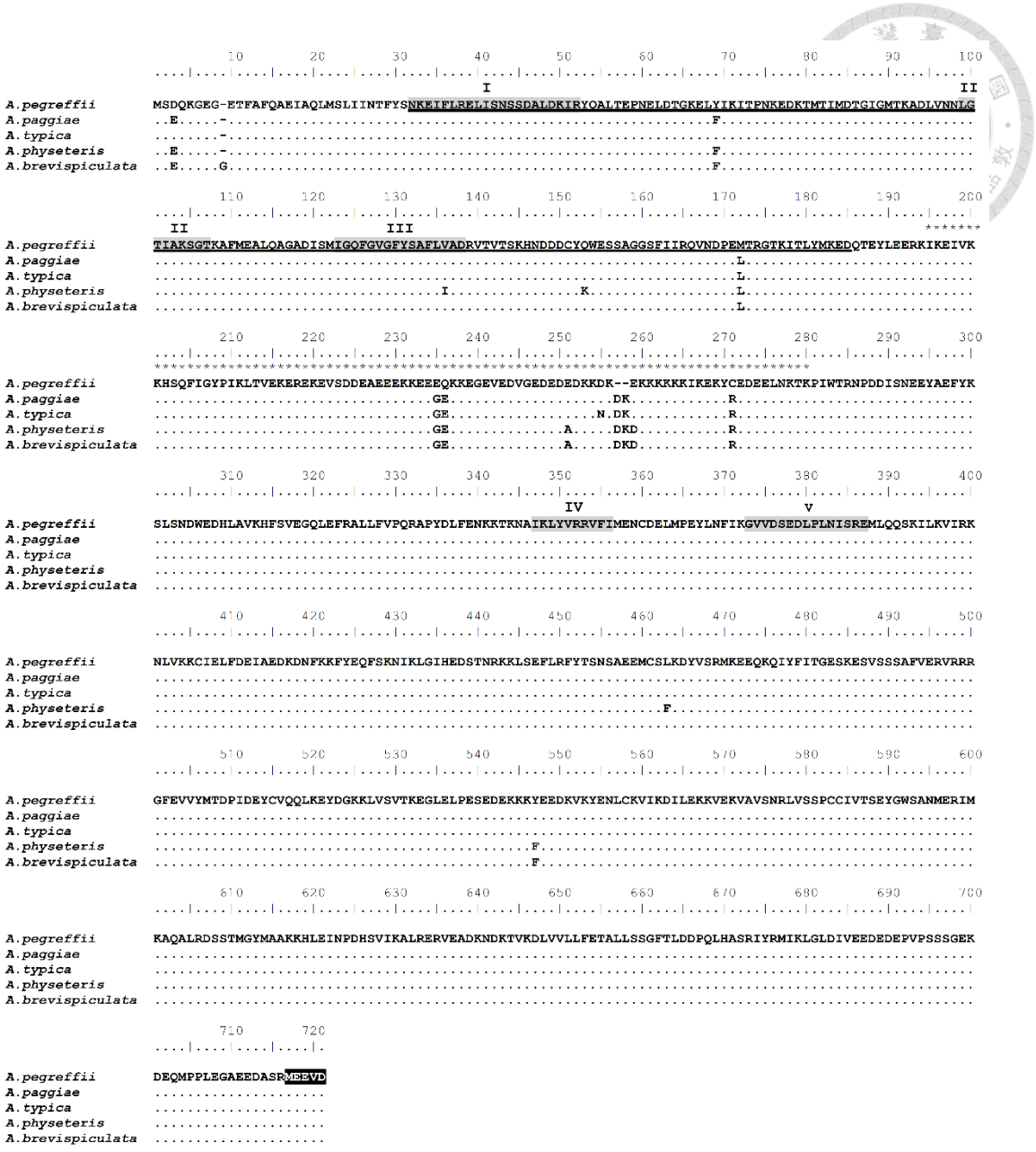


Fig. 3. 2. Multiple sequence alignment of the deduced peptide sequences of Hsp90 from the five *Anisakis* species.

The dots in the alignment represent identical residues, and gaps are indicated by hyphens. Five Hsp90 signature sequences (I-V) and cytoplasmic C-terminal region MEEVD are shadowed. The histidine kinase-, DNA gyrase B-, and Hsp90-like ATPase domain is underlined. Lysine-rich motif is indicated by asterisk.

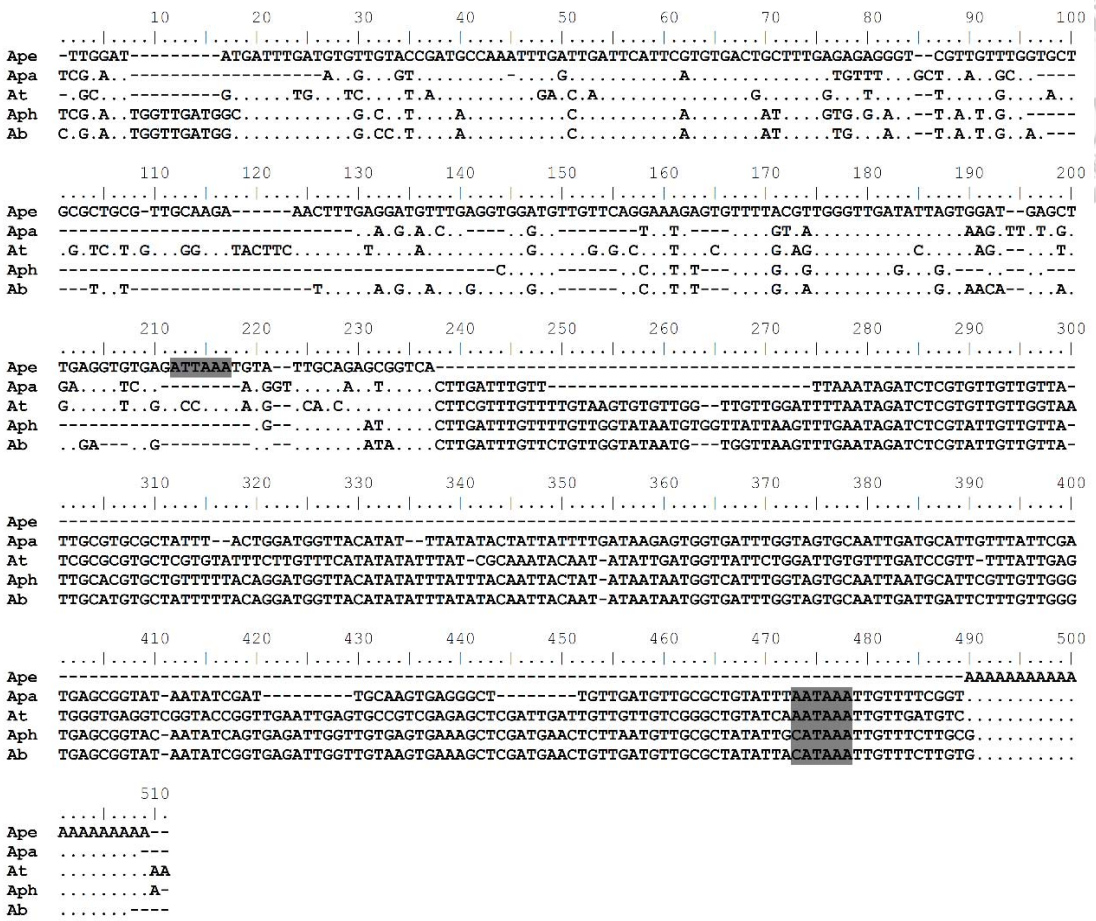


Fig. 3. 3. Comparative sequence of 3′-UTR of Hsp90 gene in 5 *Anisakis* species. The identical sequence and INDELs are represented by dots and dashes, respectively. The polyadenylation signal sequences are shadowed. Ape- *A. pegreffii*; Apa- *A. paggiae*; At- *A. typica*; Aph- *A. physeteris*; Ab- *A. brevispiculata*.

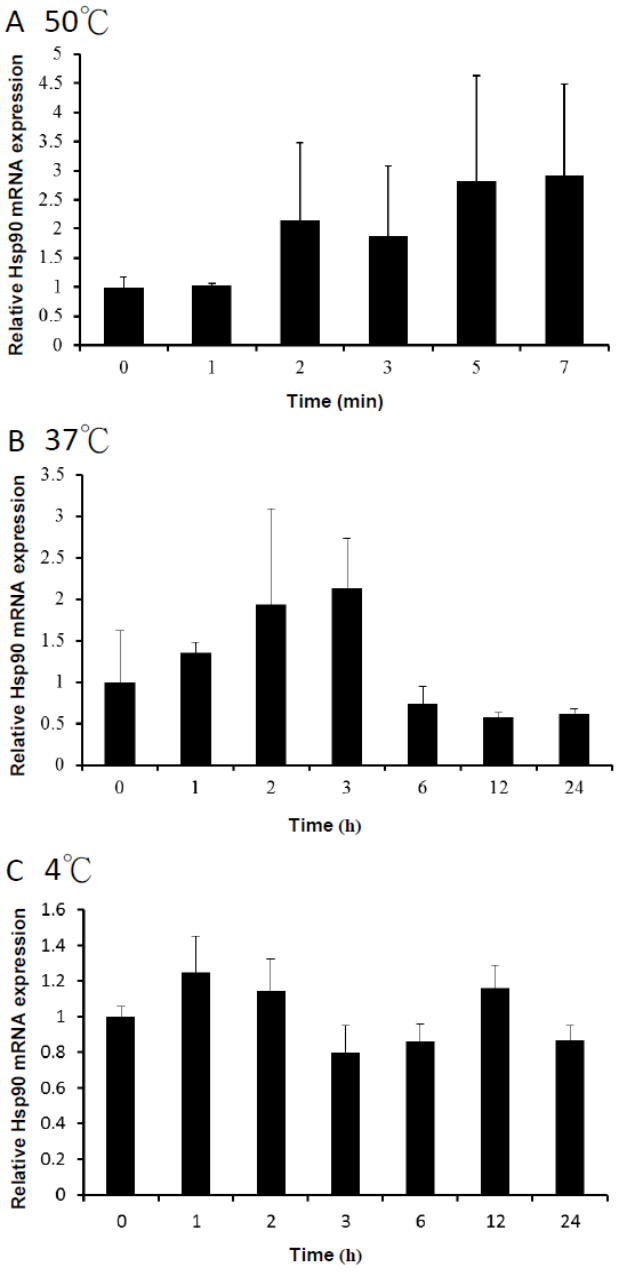


Fig. 3. 4. The mRNA expression level of Hsp90 in *A. pegreffii* at three different temperatures at different time points by quantitative real-time PCR with SYBR Green. (A) 50℃. (B) 37℃. (C) 4℃.

The value is expressed as the mean ± SD (n = 4). No statistically significant difference between control (exposure time = 0) and exposed treatments.

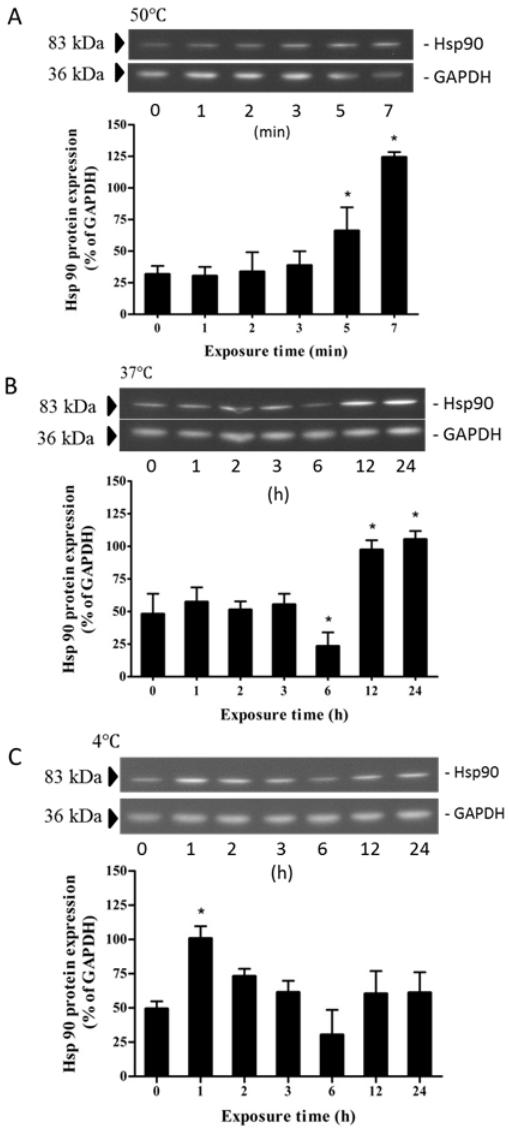


Fig. 3. 5. Western blotting analysis of Hsp 90 and GAPDH expression.

Relative expression of proteins were expressed as a percentage in the third-stage of *A. prgreffii* treated with different temperatures across time points. Western blot (top) and the corresponding histogram (bottom) represent the expression dynamics in three treatments. (A) 50℃. (B) 37℃. (C) 4℃. *$p < 0.05$ compared with control ($n = 4$).

Chapter 4 The structural conservation and expression patterns of Hsp70 and Hsp90 in different developmental stages of *Anisakis pegreffii*

4-1 Introduction

In most cases, the third larval stage (L3) of parasitic nematodes is an infective stage, when the larvae are transferred from cold-blooded animals (mosquitoes, insects or fish) to homoiothermic animals. When infective L3 larvae invade another host with change of external environments, including temperature, osmotic pressure and host immunity, they have to switch several molecular and physiological features to respond to changes in the surrounding environment (Perry and Wharton, 2011).

Heat shock proteins (Hsps) have been identified in organisms from bacteria to eukaryotes and show protein sequence conservation across species. They are important in many cellular processes, including development, growth, protein folding, signal transduction and apoptosis. They play key roles in response to stress conditions such as thermal stress, parasite infections and heavy-metal exposure, and protect organisms against environmentally induced cellular damage (Devaney, 2006; Grabner et al., 2014). Inhibitors of Hsp90 and Hsp70 were investigated as targets for treatment of cancer and parasitic diseases (Edkins and Blatch, 2012). Ubiquitous in all species and highly

conserved, Hsps provide a useful model for evolutionary studies (Gupta, 1995). They are classified and named on the basis of their molecular size (e.g., Hsp100, Hsp90, Hsp70, Hsp60, Hsp40) and low molecular mass.

Hsp70 is one of the most conserved Hsps and is a multigenic family. Generally, Hsp70 proteins are induced in response to stress, called inducible Hsp70, although some Hsp70s constitutively expressed in cells are called cognate Hsc70 (Lindquist and Craig, 1988). Hsp70s have three functional domains. ATP-dependent chaperones have a conserved ~44-kDa N-terminal ATPase domain, a ~18-kDa substrate binding domain (SBD) and a ~10-kDa variable C-terminal region. The C-terminus EEVD motif of cytosolic Hsp70s interacts with co-chaperones such as Hsp70/Hsp90 organising protein (Hop) (James et al., 1997). Hsp70 has been characterized in a wide range of organisms, including *Pomacea canaliculata* (Song et al., 2014), *Brugia malayi* (Selkirk et al., 1989), *Parastrongyloides trichosuri* (Newton-Howes et al., 2006), and *Homo sapiens* (Hunt and Morimoto, 1985). In addition, Hsp70 is developmentally regulated in many fungi, plant, protozoa, trematode, nematode and animal species (Wang and Lindquist, 1998; Yeh and Hsu, 2002; Kaiser et al., 2003; Bennuru et al., 2011; Yang et al, 2012), with overexpression in certain developmental stages (Mahroof et al., 2005).

Hsp90 is the most abundant molecular chaperone in eukaryotic cells and is responsible for the folding of other proteins. Hsp90 has multiple roles in stress

adaptation and development and participates in the conformational regulation of signal transduction molecules such as tyrosine kinases and steroid hormone receptors (Dittmar and Pratt, 1997). In addition, Hsp90 has a role in potentiating drug resistance for use in cancer and parasitic infections (Him et al., 2009). Hsp90 exists as a homodimer, with each monomer containing three highly conserved functional domains, namely an N-terminal domain (25 kDa), a middle domain (35 kDa) and a C-terminal dimerization domain (12 kDa) (Nemoto et al., 1997). The N-terminal and middle domain are connected by a charged linker region that varies in amino acid composition and length depending on the species. The C-terminal domain contains the MEEVD motif that provides the binding site for a set of co-chaperone molecules (Pearl and Prodromou, 2006). Hsp90 has been cloned and characterized from a variety of prokaryotes and eukaryotes and is essential for parasite viability, stage-specific expression, and heat shock response as part of the life cycle (Banumathy et al., 2003; Gillan and Devaney, 2014).

Nematodes of the genus *Anisakis* are parasites of the alimentary tract of marine mammals, although the infective phase (L3) is found in a large variety of fish, cephalopods, and crustaceans worldwide (Mattiucci and Nascetti, 2006). The life cycle is completed in aquatic ecosystems. Cetaceans (final host) and humans (accidental host) become infected by consuming hosts containing L3 larvae. The transfer of L3 between

fish and mammals is associated with temperature change from cold-blooded to 37˚C. *In vitro* cultivation of *Anisakis* has shown that temperature and CO_2 are required for molting and survival in the digestive tract of mammals (Dávila et al., 2006). However, relatively little is known about the expression of Hsps at different developmental stages of *Anisakis* nematodes.

Our previous work identified Hsp90 in *Anisakis* species (Chen et al., 2014). Here we aimed to characterize Hsp70 and Hsp90 in *A. pegreffii* and provide gene and protein expression profiles at different stages. We cloned, characterized and compared the full-length Hsp70 cDNA sequence of *A. pegreffii* and used homology modeling to derive a 3D structure for Hsp70 and Hsp90 based on the crystal structure of yeast Hsp90 (PDB id: 2CG9) and bovine Hsp70 (PDB id: 1YUW). We discuss the evolutionary relationship of Hsp70 and Hsp90 among protozoa, helminthes and human.

4-2 Materials and methods

4-2-1 Parasites collection and cultivation

L3 larvae of *Anisakis* species were collected from the spotted mackerel (*Scomber australasicus*) caught in the coastal waters of Yilan, northeastern Taiwan (25˚ N, 121˚ E). *In vitro* culture of L3 to L4 larvae was previously described (Iglesias et al., 2001).

Briefly, the larvae were washed and immersed in an antibiotic-antimycotic solution for 30 min, then individually inoculated into RPMI-1640 medium with 20% heat-inactivated fetal bovine serum (IFBS) and 1% commercial pepsin, adjusted to pH 4.0. *In vitro* culture was performed at 37˚C with 5% CO_2 in atmosphere. L4 larvae were sampled at 3 to 4 days, and the exact stage of each larva was determined daily. After washing L3 and L4 larvae with saline solution (NaCl, 0.9%), samples were frozen at -80˚C.

4-2-2 RNA, DNA and protein extractions from single larva

Total genomic DNA and RNA from individuals was extracted by use of TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and the following procedure was previously described (Chen et al., 2014). The larvae were homogenized with TRIzol and chloroform, then centrifuged. The aqueous layer containing RNA was transferred to a separate tube and precipitated with isopropanol. The pellet was washed in 75% ethanol, centrifuged again, and dried at room temperature, then resuspended in nuclease and RNase-free water. DNA was precipitated from the remaining organic layer with 100% ethanol. The mixture was centrifuged, and the upper aqueous layer containing protein was transferred to a new tube. The pellet was washed in 0.1 M sodium citrate in 10% ethanol, centrifuged and dried. DNA was resuspended in Tris-EDTA buffer. Protein was

precipitated by adding iso-propanol and centrifugation. The pellet was washed with 0.3 M guanidine hydrochloride in 95% ethanol. Protein was added to 100% ethanol, and centrifuged again. The pellet was dried and dissolved in 1% SDS (1:1 (v/v); protein fraction). RNA, DNA and protein concentration and quality were measured by spectrophotometry. DNA was stored at −20˚C, and RNA and protein were stored at −80 ˚C.

4-2-3 PCR-RFLP

Molecular genotypes of *Anisakis* larvae were determined by PCR-RFLP as described (Cavallero et al., 2011). The restriction enzymes used for the PCR products of the ITS region (ITS1-5.8S-ITS2) were *Hinf*I and *Hha*I. Digested products were analyzed by electrophoresis in a 2% agarose gel containing ethidium bromide and visualized by illumination with shortwave ultraviolet light.

4-2-4 RT-PCR and full-length Hsp70 cDNA cloning

Each RNA sample was treated with DNase I (1 U/l, Invitrogen) to remove genomic DNA contamination. Single-stranded cDNA was synthesized from 1 µg total RNA, with 0.5 µg $dT_{12–18}$, 1.25 µl dNTP (10 mM), 1 µl RNase inhibitor (25 U/µl, Promega, USA), and 1 µl M-MLV reverse transcriptase (200 U/µl, Promega), topped up to 25 µl with

nuclease-free water. The reaction was incubated at 37˚C for 60 min and transferred to ice.

To clone the Hsp70 cDNA, we designed a primer pair of *A. pegreffii*_Hsp70 (Table 1) on the basis of the conserved nucleotide sequence of the known Hsp70 from other nematode species. Reactions were performed in a total volume of 25 μl containing 2.5 μl 10×PCR buffer, 2 μl $MgSO_4$ (25 mM), 2.5 μl dNTPs (2 mM each), 1 μl of *A. pegreffii*_Hsp70 primer (10 mM), 3 μl cDNA, and 1 μl KOD Hot Start DNA Polymerase (1 U/μl, TOYOBO, Japan). PCR was performed at 95˚C for 3 min followed by 30 cycles of 30 s at 95˚C, 30 s at 63˚C, 2 min 30 s at 72˚C with a final extension at 72˚C for 7 min. The PCR products were separated by electrophoresis, and the desired DNA band was excised and purified with use of the QIAquick Gel Extraction kit (Qiagen, Germany). The purified PCR products were subcloned into the pGEM-T Easy Vector (Promega). After transfection into *Escherichia coli* DH5α-competent cells, recombinants were identified by blue and white spot selection, and positive clones were sequenced.

4-2-5 Quantitative analysis of Hsp70 and Hsp90 mRNA expression

The mRNA levels of *A. pegreffii* Hsp70 (ApHsp70) and Hsp90 (ApHsp90) at L3 and L4 were evaluated by fluorescent real-time PCR. cDNA synthesis was used as a

template with the target gene Hsp90 or Hsp70 and the housekeeping gene GAPDH as a reference. Each cDNA sample was transcribed from a RNA pool from three larvae of *A. pegreffii* to increase efficiency of normalization and maximize the data obtained. For each developmental stage, four pools were used as replicates. Reactions were performed in a 10-µl reaction volume including 5 µl of 2× iQTM SYBR Green Supermix (Bio-Rad), 1 µl each primer (10 mM) (Table 1), 1 µl of 10-fold diluted cDNA, and 3.6 µl dH_2O. PCR was performed at 5 min at 95˚C, 40 cycles of 30 s at 95˚C, 30 s at 55˚C, and 30 s at 72˚C, then melting curve analysis from 65˚C to 95˚C was used to ensure consistency and specificity of the amplified product. All samples were carried out in triplicate, including a control with no template. Data are expressed as mean ± SD (n = 4). Analysis of real-time PCR data involved use of Bio-Rad CFX Manager (Hellemans et al., 2007). The level of the control and target gene were determined by the threshold cycle number (Ct). The qPCR experiment was designed and performed according to the MIQE guide recommendation (Bustin et al., 2009).

4-2-6 Western blot analysis of Hsp70 and Hsp90 expression

Each protein sample was pooled from three larvae of *A. pegreffii* to increase efficiency of normalization, and four replicates of pooled samples were analyzed. An amount of 15 µg total protein was applied to 10% SDS-PAGE gels, transferred to

nitrocellulose membranes, screened with the polyclonal rabbit antibodies anti-Hsp90-α (GTX109753; 1:1000 dilution), anti-Hsp70 alpha (GTX111088; 1:4000) or anti-GAPDH (GTX100118, all Genetex Inc., CA; 1:2000) overnight at 4˚C, washed, then incubated with horseradish peroxide-conjugated secondary antibody (GTX213110-01, Genetex, CA; 1:5000) for 1 h at 37˚C, then visualized by enhanced chemiluminescence (Bioman, Taiwan). Quantification was by use of an Auto-Chemi system (UVP; Bio-Imaging Systems) and the image acquisition software Labworks 4.6.

4-2-7 Bioinformatic analysis

Protein sequence alignment involved use of ClustalW2, with results displayed by use of BioEdit v7.2.0. The motifs of protein sequences were searched by use of MyHits. The sequence identities of Hsp90 and Hsp70 from different organisms were analyzed by use of the BioEdit sequence alignment editor and data were analyzed as a heatmap by use of MeV software. Phylogenetic analyses of amino acid sequences involved the Maximum Likelihood (ML) method within MEGA6 (Tamura et al., 2013). The phylogenetic tree was computed by using the LG model and Gamma distribution. 3D models of *A. pegreffii* Hsp70 and Hsp90 proteins were constructed by homology modelling. First, the protein sequences underwent an NCBI BLAST search for template structures. Two template structures, Hsp70 from bovine (PDB id: 1YUW) and Hsp90

from yeast (PDB id: 2CG9), were selected. Model building involved use of MODELLER v9.14 with the graphic user interface program EASYMODELLER 4.0. The target models of Hsp70 and Hsp90 were generated and refined according to the loop database in MODELLER v9.14. All modelled protein structures were analyzed by use of Swiss-PDB. The modelled protein structures were generated by use of PyMOL v1.3. Accession numbers for amino acid sequences from these organisms are in Table 2.

4-3 Results

4-3-1 cDNA cloning and characterization of Hsp70 from *A. pegreffii*

With the known Hsp70 sequence, we designed a specific primer pair to clone the open reading frame (ORF) of ApHsp70, and obtained a fragment of 1950 bp encoding a protein of 649 amino acids. The deduced amino acid sequence was 70.7 kDa with isoelectric point 5.4. Three Hsp70 family signatures (IDLGTTYS, IFDLGGGTFDVSIL and IVLVGGSTRIPKVQ) of ApHsp70 are located at positions 9-16, 198-211 and 335-349. The putative cAMP- and cGTP–dependent protein kinase phosphorylation sites (KKSN and KRNT, respectively), amidation site (IGRR) and tyrosine kinase phosphorylation site (RMVQEAEKY) are located at positions 188-191, 413-419, 74-77 and 518-526, respectively. In addition, a cytoplasmic Hsp70 carboxyl-terminal region of

EEVD is at position 646-649.

4-3-2 Mapping and sequence comparison of Hsp70 and Hsp90

We performed sequence alignment of ApHsp70 and ApHsp90 sequences and representative sequences from the filarial nematode *Wuchereria bancrofti*, the protozoan parasite *Plasmodium falciparum*, and human and yeast. ApHsp70 showed high amino acid sequence similarity with other known Hsp70s, especially in the regions of Hsp70 family signatures (I-III), highly conserved regions present in all Hsp70 family members. The Hsp70 C-terminal region of EEVD and the potential non-organelle eukaryotic consensus motif (RARFEEL) were commonly found in the five species. Although regions of homology extended throughout the amino acid sequences of the Hsp70s for the five species, the C-terminal region showed greater divergence than the N-terminal region (Fig. 1). Multiple sequence alignment of Hsp90 for the five species revealed five highly conserved segments (I-V) common to all eukaryotic Hsp90 proteins. The MEEVD motif, a highly conserved sequence, was at the C-terminus of the cytoplasmic Hsp90 proteins. The extreme N-terminus was highly variable in all organisms, especially human, displaying a PEETQ that is unique to vertebrate Hsp90 sequences. We found 27 residues that were especially long in PfHsp90 at positions 227-253 and 16 residues (glutamate and lysine) not present in the other four species (Fig. 2).

The sequence identities of HSP70 and Hsp90 of *A. pegreffii* and other organisms were presented as a heatmap (Fig. 3). The identity between ApHsp70 and ApHsp90 and other organisms was 90.7% and 92.3%, respectively, for *Wuchereria bancrofti*; 86.8% and 87.3% for *B. malayi*; 89% and 90.2% for *L. loa*; 90.7% and 84.6% for *N. americanus*; 90.9% and 86.6% for *H. contortus*; 90.7% and 86.7% for *A. ceylanicum*; 78.4% and 67.4% for *S. mansoni*; 67.9% and 59% for *P. falciparum*; 71.6% and 62.6% for *T. gondii*; 71.8% and 63.4% for *N. caninum*; 71.6% and 60.6% for *L. amazonensis*; 71.1% and 61% for *T. brucei*; 74.6% and 58.7% for yeast; and 79.8% and 76.7% for human.

4-3-3 Phylogenetic relationships

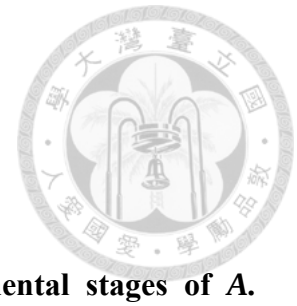
Using the amino acid sequences for Hsp70 and Hsp90, we constructed a rooted phylogenetic tree (with yeast *S. cerevisiae* as an outgroup) showing the relationships among 13 important parasitic species including seven nematode species, one trematode species, five protozoan species and one vertebrate. The trees showed similar topologies for Hsp70 and Hsp90, with only minor differences in the branching order of some species clusters. Hsp70 proteins formed two clades: one grouped all nematodes, blood-fluke and human, and a monophyletic clade grouped all protozoans. The Ascaridida (*A. pegreffii*) and Spirurida (*W. bancrofti*, *B. malayi* and *L. loa*) formed a

monophyletic group, and its sister cluster Strongylida (*A. ceylanicum*, *N. americanus* and *H. contortus*) represented the phylum Nematoda (Fig. 4). For the Hsp90 tree, *A. pegreffii* and other nematodes were clustered together and formed a sister group with human, clustered to blood-fluke in all bootstraps, and finally formed a group independent of protozoans. Protozoan and metazoan Hsp90 were separated and formed two distinct branches.

4-3-4 Structural modeling of Hsp70 and Hsp90

To reveal the protein structure of Hsp70 and Hsp90 of *A. pegreffii*, we used the protein sequences for a search of template structures against the Protein Data Bank. The Hsp70 from bovine (PDB id: 1YUW) showed 87% sequence identity and 93% similarity with ApHsp70 from residues 1 to 554. The model of ApHsp70 (Fig. 6A) showed a Y-shaped structure and was superimposed onto the template structure of bovine Hsp70, with root-mean-square deviation (RMSD) 0.27 Å; the structure should be highly conserved from residues 1 to 554, except for the C-termini from residues 555 to 661. The Hsp90 from yeast (PDB id: 2CG9) showed 61% identity and 79% similarity with ApHsp90 from residues 8 to 680. The model of ApHsp90 (Fig. 6B) showed a dimer structure and was superimposed onto the template structure of yeast Hsp90, with RMSD 0.89 Å. A long loop of ApHsp90 from residues 216 to 291 could be modelled,

because the region in yeast Hsp90 contained no defined structure.



4-3-5 Expression of Hsp70 and Hsp90 at different developmental stages of *A. pegreffii*

We determined the ApHsp70 and ApHsp90 expression profiles in *A. pegreffii* at L3 and L4. The mRNA levels of ApHsp70 and ApHsp90 at maximal induction were higher at L4 than L3, by 6- and 2-fold, respectively (P <0.05) (Fig. 7A and 7B). As well, protein levels of ApHsp70 and ApHsp90 were higher at L4 than L3, by 3- and 2-fold (Fig. 7C and 7D).

4-4 Discussion

HSPs are molecular chaperones that are highly conserved across organisms. They have a pivotal function in response to thermal stress and are responsible for many cellular functions. Here, we elucidated the roles of Hsp70 and Hsp90 in the life cycle of the parasitic nematode *Anisakis*, particularly at L3 and L4, the stages of transfer from cold-blooded fish to warm-blooded marine mammals or accidentally to human hosts. The deduced amino acids of Hsp70 of *A. pegreffii* showed high amino acid homology with other nematodes. ApHsp70 and ApHsp90 shared the highest deduced amino acid

sequence identity with other nematodes and formed a monophyletic clade. 3D structure prediction of the newly characterized ApHsp70 and known ApHsp90 gene showed highly conserved motifs between *A. pegreffii* and other species. ApHsp70 and ApHsp90 mRNA and protein levels were higher at L4 than L3, with higher mRNA and protein expression for ApHsp70 than ApHsp90. ApHsp70 and ApHsp90 may play important roles in response to thermal stress in *Anisakis* and might be important molecules in its development.

The greatest molting and survival percentages of *Anisakis* were previously found with 5% CO_2, pH and pepsin, regardless of medium used (Iglesias et al., 2001; Dávila et al., 2006), with maximal development and survival of larvae obtained in nutritive medium with 5% CO_2 at 37˚C, as expected (Iglesias et al., 1997; 2001). We found survival of 50 L3 *Anisakis* cultured in RPMI-1640 supplemented with 20% heat-inactivated FBS and 1% pepsin, pH 4.0 and 37˚C, and in air with 5% CO_2, and 100% of L3 reached L4 stage between days 3 and 4.

We found high overall identity of AtHsp70 and AtHsp90 sequences with those from a parasitic nematode species, *W. bancrofti*, and lower identity with three other organisms. The typical signature sequences of the Hsp family (five for Hsp90 and three for Hsp70) were found in the five species. The conserved C-terminal motif EEVD, found in ApHsp70, is suggested to bind to many co-chaperones with small helical

tetratricopeptide repeat (TPR) domains, which indicates that ApHsp70 is a cytoplasmic Hsp (Freeman et al., 1995). ApHsp90 is a cytosolic Hsp90 because of its highly conserved regulatory motif MEEVD and upstream of MEEVD revealing several residues of GAEE that appear to be unique to nematodes. As well, the extended GAEE/DASRMEEVD motifs interacted with nematode Hsp90 co-chaperones (Skantar and Carta, 2004). One of the unique features of PfHsp90 is the presence of a long charge acidic linker region, 27-29 amino acids longer than in yeast, human and two nematodes. The linker region may not function as a structural connector between the N-terminal and middle domain region and form a surface extrusion in the N-terminal (Kumar et al., 2007).

We performed heatmap and phylogenetic tree analyses of seven nematodes species, one trematode species, and five protozoan species that are important parasites of humans and domestic animals and are responsible for considerable economic losses and public health problems worldwide (Blaxter, 2003; Innes et al., 2011). The sequence identities ranged from 86.5% to 99.5% for Hsp70 and 80.2% to 98.3% for Hsp90 in nematodes. Protozoa are a diverse group of mostly motile unicellular eukaryotic organisms, and the identity range is 65.8% to 98.5% for Hsp70 and 59.1% to 96% for Hsp90. However, the identity for the blood-fluke *S. mansoni* and human are close to the phylum Nematoda -- 76.7% to 80.9% and 76.7% to 80.7%, respectively, for Hsp70 and

62.9% to 67.7% and 73.8% to 77.6% for Hsp90. This overall identity reflects the highly conserved sequence pattern of nematodes, trematodes and human as compared with the diverse protozoan parasites.

Hsp70 is highly conserved in both sequence and structure alignment as compared with Hsp90. Our modeling of ApHsp70 showed 28 beta sheets and 13 helices in the bovine homology model. The Hsp70 homologue from other organisms contains approximately 28 sheets and 13 helices. Hsp70 may have a major role in providing stability to proteins and maintaining homeostasis in the cell under stress conditions (Desai et al., 2010). Structural modeling of ApHsp90 showed a long loop of ApHsp90 from residues 216 to 291. Sequence diversity of loop regions between ApHsp90 and Hsp90s from other species might indicate a biomarker of distinction between *Anisakis* and other species. We selected EKKEEEQKKEGEVEDVGEDE as a biomarker, with sequence identity between ApHsp90 and that for other nematode species ranging from 70% to 76% and those for human and yeast 52% and 55%, repectively.

Both Hsp70 and Hsp90 are considered useful in phylogenetic analysis because they are highly conserved throughout prokaryotes and eukaryotes (Fast et al. 2002; Chen et al. 2006). Hsp70 is widely used as a phylogenetic marker because of highly conserved sequences and the molecular chaperone function, combined with an ancient conserved function, for investigating deep evolutionary relationships (Gupta and Golding, 1993;

Borchiellini et al., 1998). It has been used with all monophyletic groups of the Metazoa, with available sequences consistently recovered for some species (Budin and Philippe, 1998). Hsp70 has also been used for phylogenetic analysis of different protozoan parasites (Fraga et al., 2010) with caution, because of paralogy distorting phylogenetic relationships (Krenek et al., 2013). Hsp90 is a proper phylogenetic marker analyzed in a variety of organisms and has a good balance of sequence conservation and diversity. It has distinct domain and signature sequences that allow for differentiation between Hsp90 and other family members. As well, some potential problems that arise from use of paralogous genes such as Hsp70 are not an issue with Hsp90 (Skantar and Carta, 2004). Much research has involved phylogenetic evaluation of Hsp90 sequences from several nematodes (Skantar and Carta, 2004; Him et al., 2009). We found that when comparing the phylogenies of 13 important parasitic species and human and yeast with Hsp70 and Hsp90, tree topologies were highly concordant between species and clusters. The Hsp90 tree topology supported groupings of nematodes with human and protozoan, with nematode species separated and forming two distinct branches (Skantar and Carta, 2004).

Petronijevic and Rogers (1987) described the infective stages of nematodes as being in hypometabolic dormancy and staying in this state until exposure to a suitable response to continue the cycle. In common with most infective L3 larvae, lymphatic

filarial worms and infectious sporozoite of *Plasmodium* undergo heat shock as part of their life cycle. Hsp expression is inducible through several stages of development in some parasites, which suggests that they play unique biological roles in certain stages of the life cycle. The transcription levels of NbHsp70 and NbHsp90 of the parasitic nematode *Nippostrongylus brasiliensis* were higher in infective L3 larvae than in other larval stages. NbHsp70 and NbHsp90 were upregulated in old infective L3 larvae and under heat stress, which suggested their involvement in survival of L3 larvae in harsh environments (Arizono et al., 2011), a phenomenon similar to findings in the *Caenorhabditis elegans* dauer stage (Cherkasova et al., 2000). NbHsp70 and NbHsp90 were detected throughout the nematode's life cycle and their levels were closely associated with development (Arizono et al., 2011). Tsdaf21/Hsp90 is highly and consistently expressed in all stages of *Trichinella spiralis* as is the protein level (Yang et al., 2013). The SjHsp70 of *Schistosoma japonicum* is widely expressed in all development stages. SjHsp70 expression is higher in eggs, which are trapped in host organs and interact directly with the host (Yang et al., 2012).

We found the mRNA and protein levels of ApHsp70 and ApHsp90 higher in L4 than L3 larvae. Hsp70 protein level was higher than Hsp90 level in L4 larvae. These differences may be due to the different regulatory systems of Hsp70 and Hsp90 at different development stages. Among the Hsp family, Hsp70 is one the most important

Hsps with expression rapidly increased on exposure to heat or other stress (Lindquist and Craig, 1988). *B. malayi* is transmitted by mosquitoes, which introduces L3 filarial larvae onto the skin of the human host for development into adults. The protein level of BmHsp70 was higher in microfilariae and adults than L3 larvae (Bennuru et al., 2011). In *Meloidogyne artiellia*, Hsp90 is constitutively expressed in all stages but was at higher levels in eggs and L4 larvae (De Luca et al., 2009). Malaria is caused by a parasite called *Plasmodium*, and transmission occurs when infected mosquitoes inoculate sporozoites into the human body. Parasites first take up residence in hepatic cells as exoerythrocytic forms (EEFs). Hsp70 protein is highly expressed in EEFs of *Plasmodium* and is barely detectable in sporozoites. Hsp70 expression could be used to follow the transformation of sporozoites into hepatic stages (Kaiser et al., 2003). In addition, Hsp90 has a role in *P. falciparum* during the parasite growth (Banumathy et al., 2003). Hsps have a role at the parasite–host interface as a survival strategy (Feder and Hofmann, 1999): parasites induce the chaperones as a survival strategy against temperature and physiological changes (Sharma, 1992). Our results suggest that ApHsp70 and ApHsp90 have a relationship with thermal stress, and L4 larvae of *A. pegreffii* may have important roles in resisting high temperature. The expression of ApHsp70 and ApHsp90 was higher at L4 than L3, which suggests that they might participate in developmental events. Further studies are needed to confirm this

assumption.

In conclusion, we investigated the molecular characterization and structure of ApHsp70 and ApHsp90 for sequencing variants and phylogenetics of *A. pegreffii* and other parasites. In addition, we examined the expression patterns of Hsp70 and Hsp90 at different developmental stages of *A. pegreffii*. ApHsp70 and ApHsp90 may be important components responding to heat stress during infection, and their rapid transcriptional upregulation in L4 larvae might be important for the survival and development of *A. pegreffii*.

Table 4. 1 Primers used in cloning Hsp70 gene and real-time PCR analysis of Anisakis *pegreffii*

Primer	Sequence (5' to 3')	Amplicon length (bp)	Reference
*A. pegreffii*_Hsp70	F: ATGGTGAAACAAAACGCGGTGGGTATC R: TTAGTCAACCTCTTCTATTGTCGGTCCAC	1950	This study
*A. pegreffii*_hsp70_RT	F: TGCCGTTATGGAGGGTA R: CCAATCAATCGTTTCGTC	177	This study
*A. pegreffii*_hsp90_RT	F: CAATAAGGAGGACAAGACAATG R: AGGAACGCAGAATAGAAACC	183	Chen *et al*., 2014
GAPDH_RT	F: CCCCTTCATCAACATCGACT R: TCAGCTCCCCATTTGATTTC	152	Chen *et al*., 2014

Table 4.2 GenBank accession numbers of amino acid sequences of Hsp70 and Hsp90 analyzed in this study [a]

Species name	Hsp70 sequences	Hsp90 sequence
Plasmodium falciparum	AAA29626	AAA66178
Toxoplasma gondii	AAC72002	AAQ24837
Neospora caninum	XP_003883640	XP_003881046
Leishmania amazonensis	AAA53690	P27741
Trypanosoma brucei	AAA30204	CAA32377
Schistosoma mansoni	AAA29898	CCD76276
Anisakis pegreffii	KF840392 [b]	KF840393
Brugia malayi	XP_001900197	XP_001901767
Wuchereria bancrofti	EJW86287	EJW88125
Loa Loa	XP_003144936	XP_003135662
Necator americanus	ENT71512	ETN83529
Haemonchus contortus	AEO14648	ACU00668
Ancylostoma ceylanicum	EYC11428	EYC13285
Saccharomyces cerevisiae	AET14830	P02829
Homo sapiens	ABC88476	BAF83804

a Unless noted otherwise, sequence obtained from another study

b This study

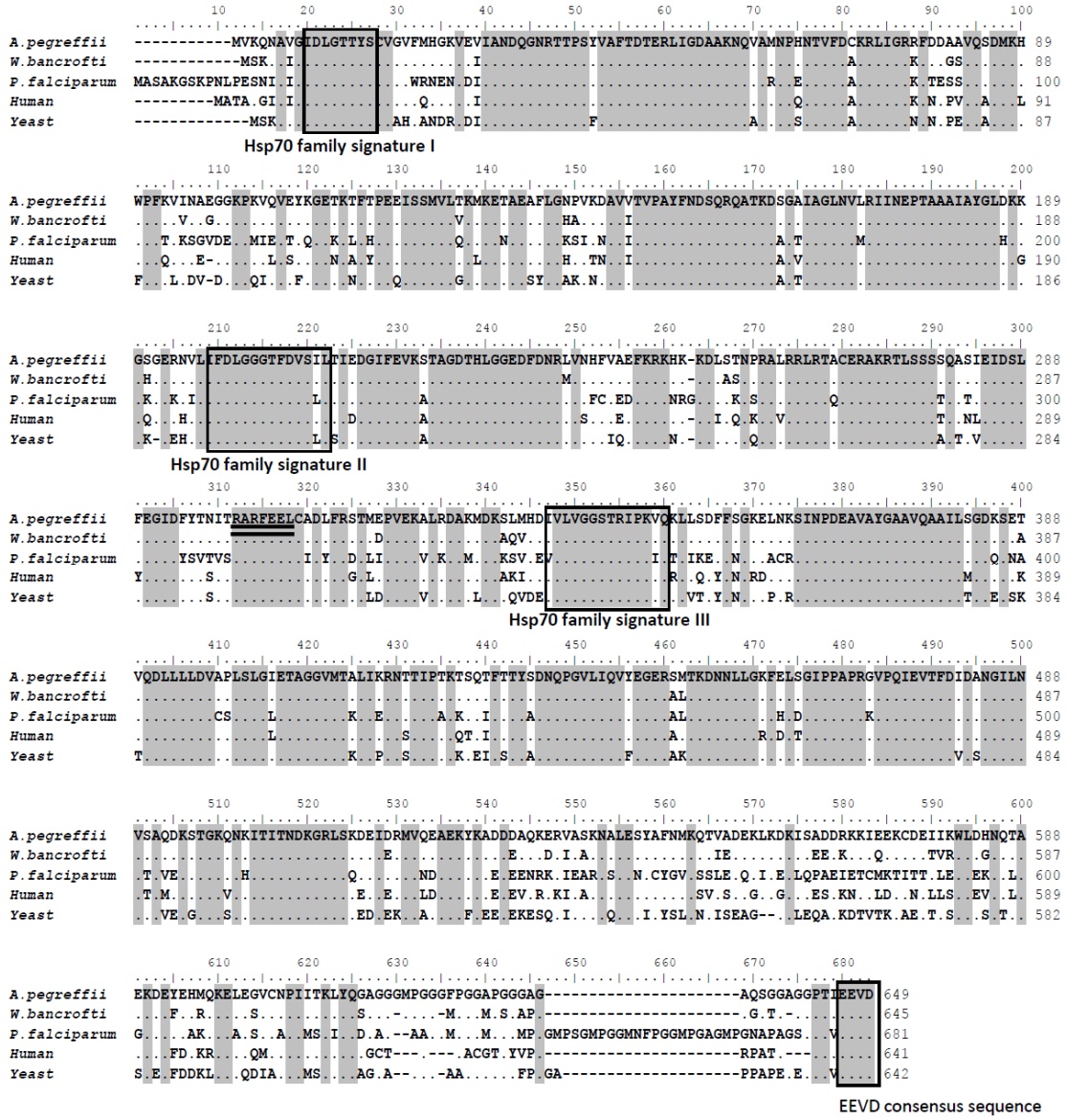


Fig. 4. 1. Multiple sequence alignment of Hsp70 from *Anisakis pegreffii* with four different organisms. Hsp70 family signature sequences (I-III) and the consensus sequence EEVD are in boxes. The dots and gray background represent identical residues, and gaps are indicated by hyphens.

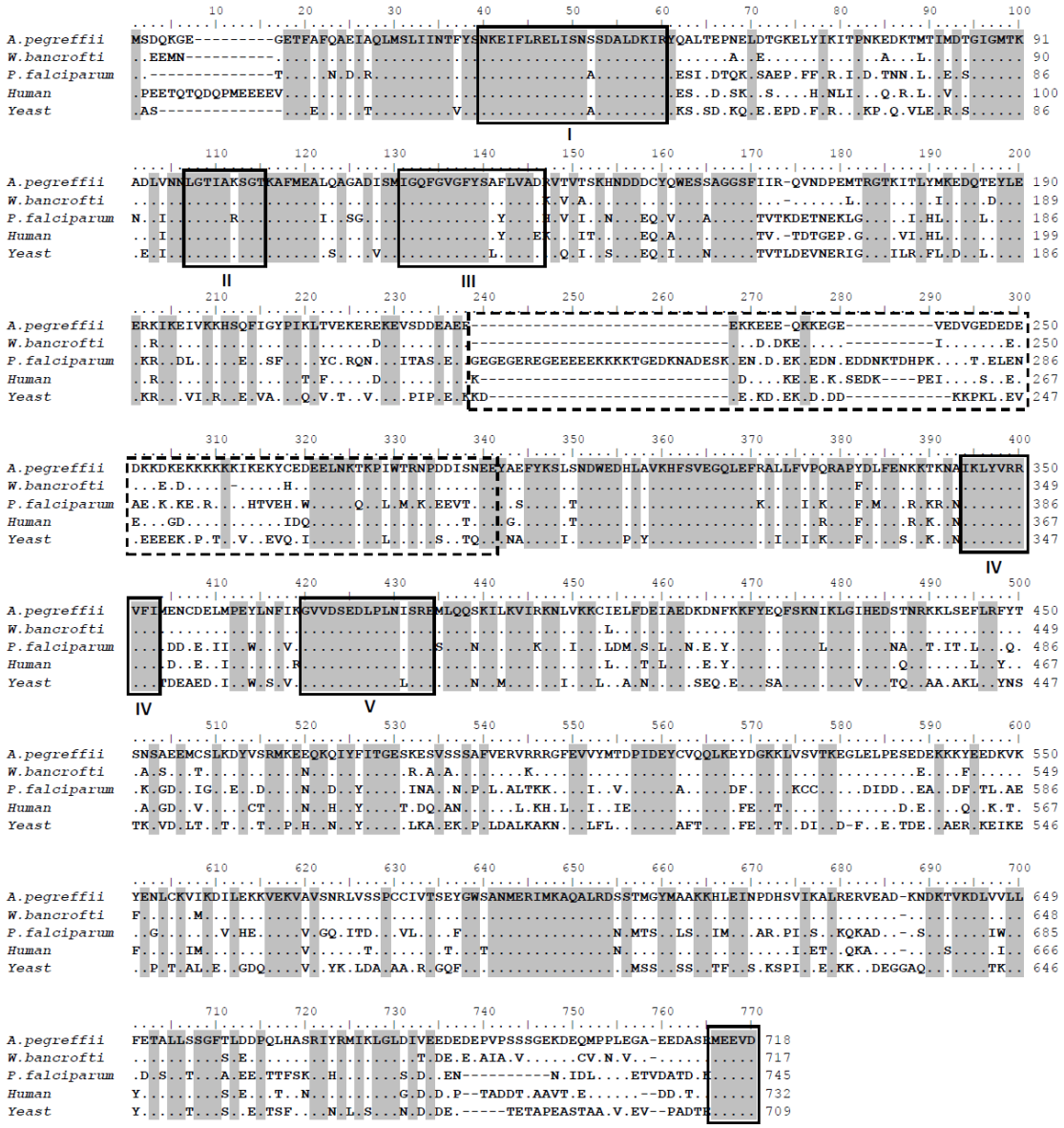


Fig. 4. 2. Multiple sequence alignment of Hsp90 proteins from *A. pegreffii* with four different organisms. Hsp90 family signature sequences (I-V) and the consensus sequence MEEVD are in boxes. The long loop indicated by a dashed box is from residues 216-291. The dots and gray background represent identical residues, and gaps are indicated by hyphens.

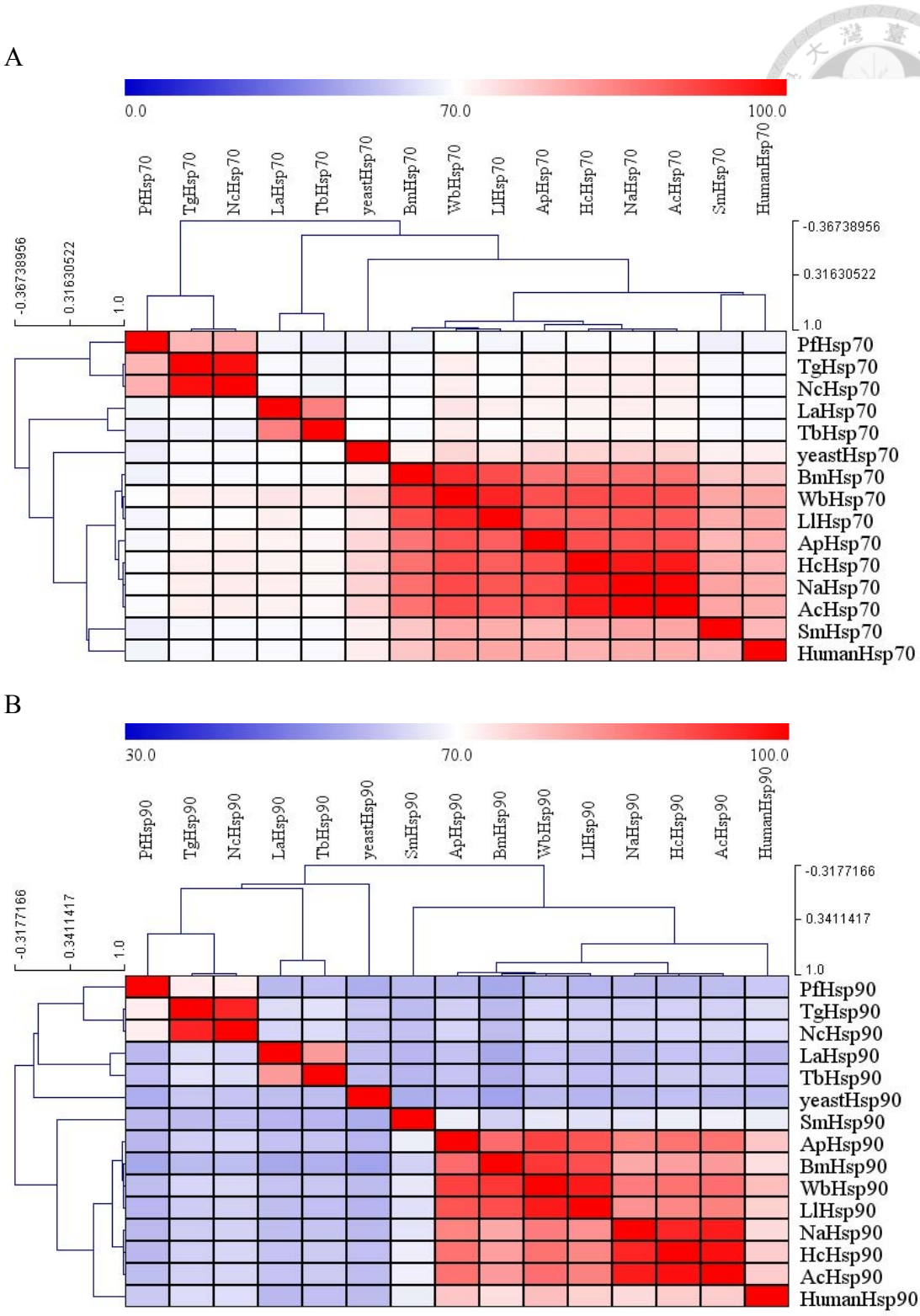


Fig. 4. 3. Sequence identity of Hsp70 and Hsp90 in different organisms. Data are presented as a heatmap. Hierarchical clustering is shown alongside the heatmap to illustrate how the inter-sequence identities relate to the similar sequence patterns. (A) Hsp70 and (B) Hsp90. Pf: *Plasmodium falciparum*; Tg: *Toxoplasma gondii*; Nc:

Neospora caninum; La: *Leishmania amazonensish*; Tb: *Trypanosoma brucei*; Sm: *Schistosoma mansoni*; Ap: *Anisakis pegreffii*; Bm: *Brugia malayi*; Wb: *Wuchereria bancrofti*; Ll: *Loa Loa*; Na: *Necator americanus*; Hc: *Haemonchus contortus*; Ac: *Ancylostoma ceylanicum*; yeast: *Saccharomyces cerevisiae*; Human: *Homo sapiens*.

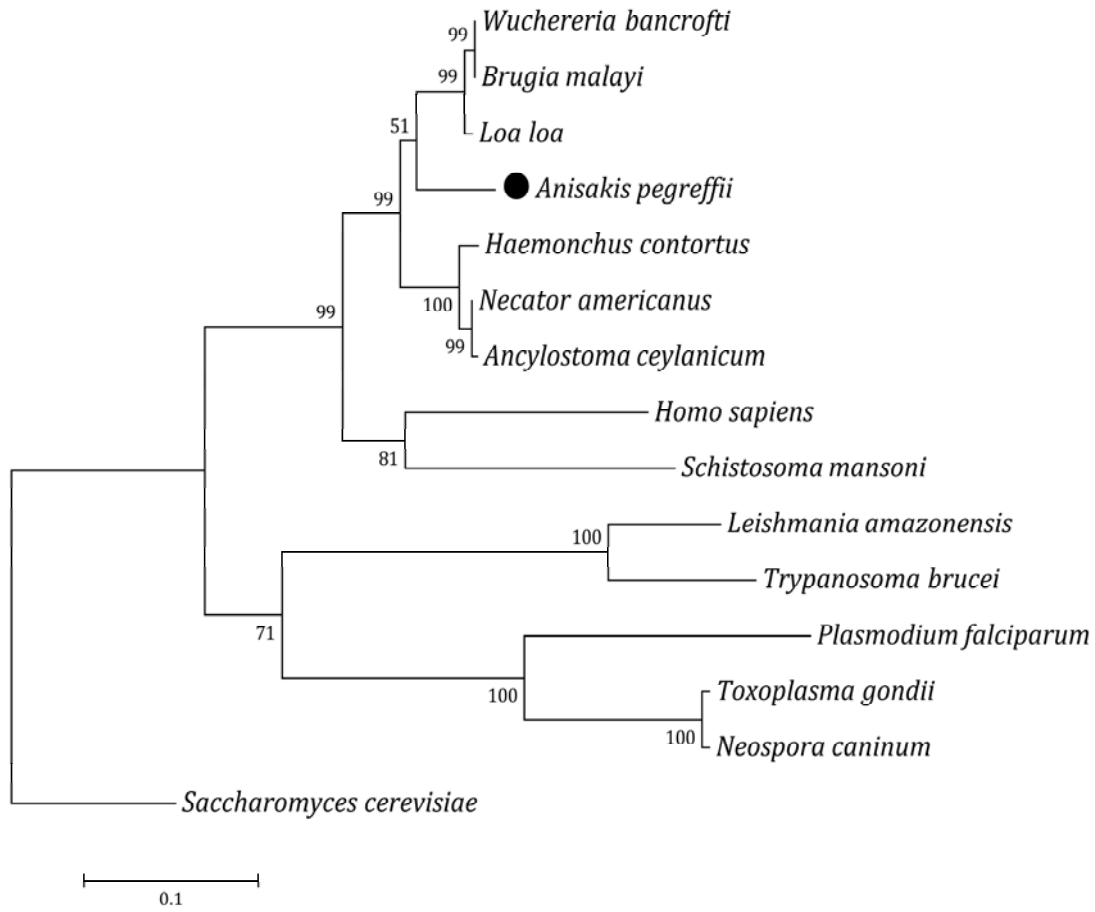


Fig. 4. 4. Phylogenetic relationships based on amino acid sequences for Hsp70 for *A. pegreffii* and 13 important parasitic species from six other nematodes, one trematode, five other protozoans, and one vertebrate, with yeast *S. cerevisiae* as an outgroup. Numbers at each branch indicate the node percentage supported by 1,000 bootstrap replicates by maximum likelihood.

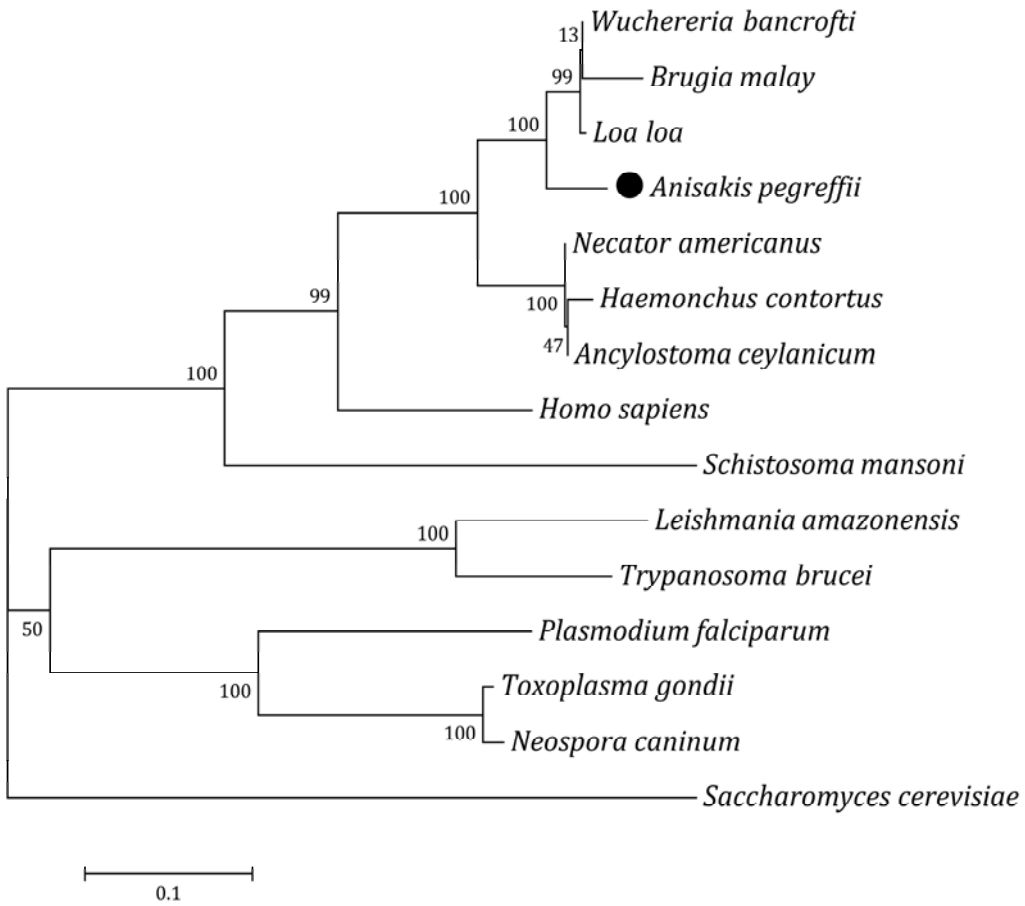


Fig. 4. 5. Phylogenetic relationships based on the amino acid sequences for Hsp90 for *A. pegreffii* and 13 important parasitic species from six other nematodes, one trematode, five other protozoans, and one vertebrate, with yeast *S. cerevisiae* as an outgroup. Numbers at each branch indicate the node percentage supported by 1,000 bootstrap replicates by maximum likelihood.

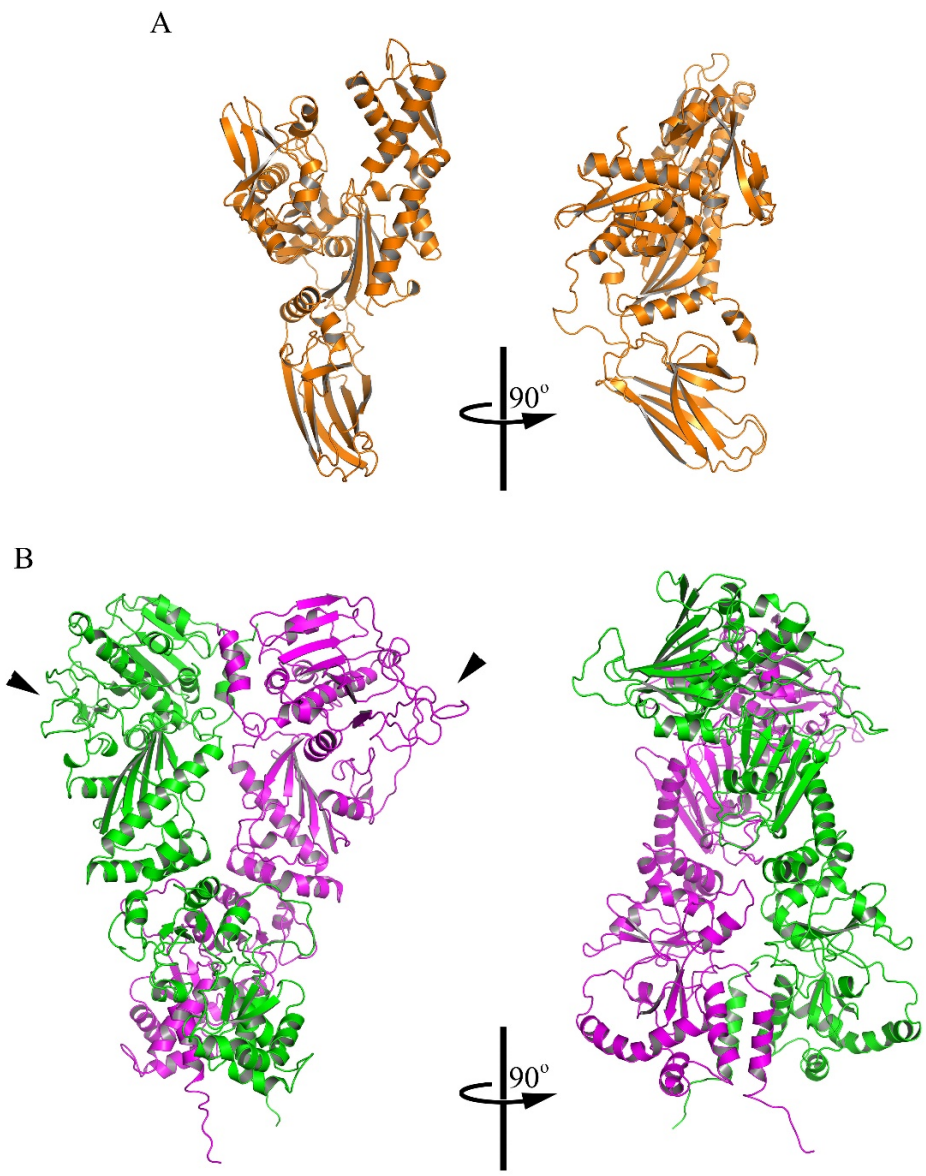


Fig. 4. 6. Ribbon diagrams of the modelled structures for Hsp70 and Hsp90. (A) Structure of Hsp70 based on the template structure of bovine Hsp70 (PDB id: 1YUW) shows a Y-shape in a monomer form. (B) Modelled structure of Hsp90 was based on the template structure of yeast Hsp90 (PDB id: 2CG9) and represents a dimer form. The long loops indicated by arrowheads are from residues 216-291. The diagrams were generated by use of PyMOL.

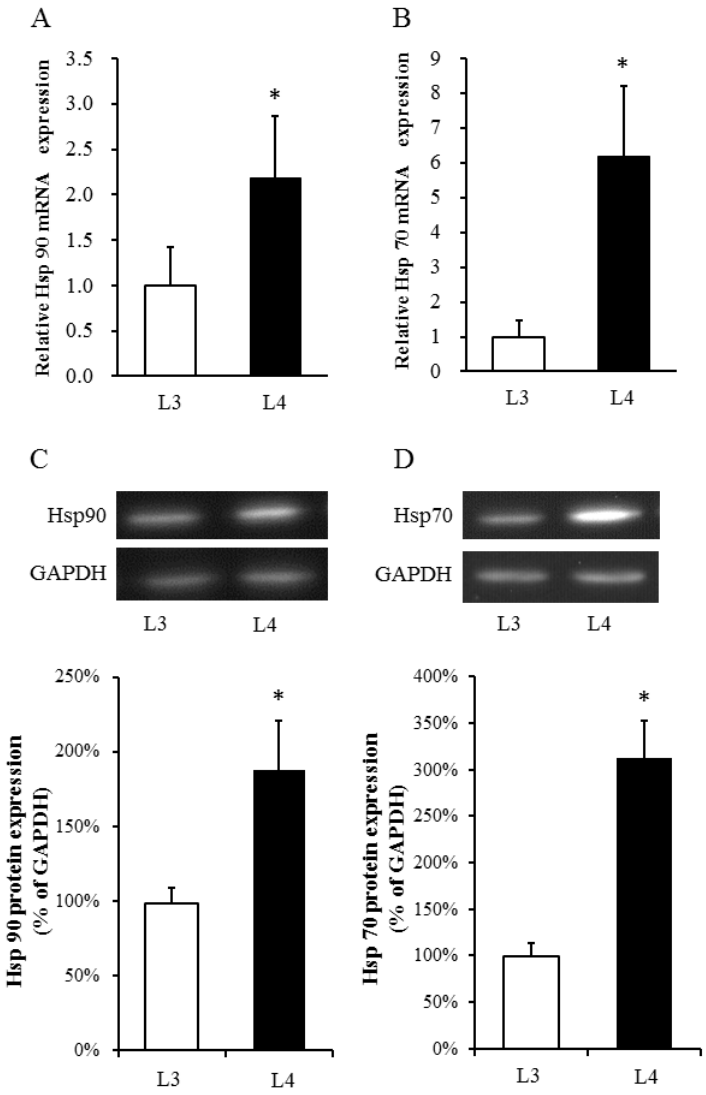
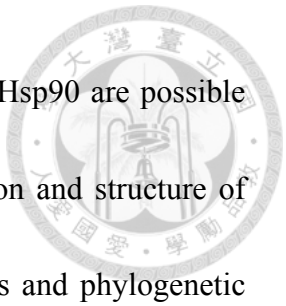


Fig. 4. 7. RT-PCR and western blot analysis of mRNA (A, C) and protein levels (B, D) of Hsp90 and Hsp70 at different stages (third- and fourth-stage larvae; L3, L4, respectively) of *A. pegreffii*. Data are mean ± SD (n = 4). *, p <0.05 compared to L3.

Chapter 5 Conclusion

The present study shows six species of *Anisakis* nematodes obtained from spotted mackerel *S. australasicus* in coastal waters of northeastern Taiwan, and new host and locality records were provided for *A. paggiae*, *A. physeteris* and *A. brevispiculata*. The presence of *A. pegreffii* was the most prevalent species in examined fish, and human could have zoonotic disease 'anisakiasis' by eating raw or undercooked infected fish. The morphological characterization is unreliable for the identification of anisakid larvae to species. However, the application of PCR-RFLP markers (endonucleases *Hinf*I and *Hha*I) proved to be an essential tool in correct identification of the species level and rapid diagnosis of human anisakiasis. We report for the first time the full-length cDNA sequence of Hsp90 and the molecular characteristics of this gene in five species of the genus *Anisakis*. In an experimental approach, we have examined the expression patterns of Hsp90 at different time-temperature combinations in *A. pegreffii*. Our findings suggest that the expression of Hsp90 in *Anisakis* larvae might respond to heat stress. On the contrary, the expression levels of Hsp90 did not change significantly at low temperature. Relative protein expression profiles of Hsp90 during exposure to heat stress showed similar trend as observed in the relative mRNA level expression. Therefore, expression of Hsp90 in *Anisakis* nematodes was found to be sensitive to temperature variation and might enhance their tolerance to environmental stress. We



extended our studies to other Hsp and have shown that Hsp70 and Hsp90 are possible roles in development. We investigated the molecular characterization and structure of ApHsp70 and ApHsp90, and would be helpful to sequence variants and phylogenetic evaluation between *A. pegreffii* and other parasites. In addition, we have examined the expression patterns of Hsp70 and Hsp90 in different developmental stages of *A. pegreffii*. Our results observed that rapid transcriptional up-regulation of ApHsp70 and ApHsp90 in response to fourth-stage larvae than infective third stage larvae. Our findings suggest that ApHsp70 and ApHsp90 are not only important component against heat stress but also might be important for survival and development of *A. pegreffii*.

In the future, it has to be verified whether the Hsp70 gene respond to different temperature tress, such as cold stress. We further hope to establish a life cycle of *Anisakis*, and can easily obtain different developmental stages including larval stage (L1-L4), female and male adult worms, allowing further studies to detect the expression patterns of Hsp70 and Hsp90 in all stages. The potential roles in sustaining the developmental regulation of Hsp70 and Hsp90 gene need to be further confirmed using additional technique, such as RNA interference. It has yet to be investigated whether Hsp70 and Hsp90 participate in the infection process.

References

Abdou, N.S., Dronen, N.O., 2007. Studies on the juveniles of a species of *Anisakis* (Nematoda: Anisakidae) from the orangespotted trevally, *Carangoides bayad* (Carangidae), from the Red Sea, Egypt. J. Egypt. Soc. Parasitol. 37, 1055-1064.

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* ss and *Anisakis pegreffii* (Nematoda: Anisakidae) in an area of sympatry. Infect. Genet. Evol. 3, 175-181.

Alonso-Gómez, A., Moreno-Ancillo, A., López-Serrano, M.C., Suarez-de-Parga, J.M., Daschner, A., Caballero, M.T., Barranco, P., Cabañas, R., 2004. *Anisakis simplex* only provokes allergic symptoms when the worm parasitises the gastrointestinal tract. Parasitol. Res. 93, 378-384.

Anderson, R.C., Chabaud, A.G., Willmont, S., 1974. General introduction. CIH keys to the nematode parasites of vertebrates. London, UK, CAB International, 17 pp.

Anshary, H., Freeman, M.A., Ogawa, K., 2014. Occurrence and molecular identification of *Anisakis* Dujardin, 1845 from marine fish in southern Makassar Strait, Indonesia. Korean J Parasitol. 52, 9-19.

Arias-Diaz, J., Zuloaga, J., Vara, E., Balibrea, J., Balibrea, J. L., 2006. Efficacy of

albendazole against *Anisakis simplex* larvae in vitro. Digest. Liver. Dis. 38, 24-26.

Arizono, N., Yamada, M., Tegoshi, T., Takaoka, Y., Ohta, M., Sakaeda, T., 2011. Hsp12. 6 expression is inducible by host immunity in adult worms of the parasitic nematode *Nippostrongylus brasiliensis*. PloS one. 6, e18141.

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.

Arriaza, B.T., Reinhard, K.J., Araújo, A.G., Orellana, N.C., Standen, V.G., 2010. Possible influence of the ENSO phenomenon on the pathoecology of diphyllobothriasis and anisakiasis in ancient Chinchorro populations. Mem Inst Oswaldo Cruz. 105, 66-72.

Arrieta, I., del Barrio, M., Vidarte, L., del Pozo, V., Pastor, C., Gonzalez-Cabrero, J., Cárdaba, B., Rojo, M., Mínguez, A., Cortegano, I., Gallardo, S., Aceituno, E., Palomino, P., Vivanco, F., Lahoz, C., 2000. Molecular cloning and characterization of an IgE-reactive protein from *Anisakis simplex*: Ani s 1. Mol. Biochem. Parasitol. 107, 263-268.

Asea, A., Kraeft, S.K., Kurt-Jones, E.A., Stevenson, M.A., Chen, L.B., Finberg, R.W., Koo, G.C., Calderwood, S.K., 2000. HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. Nat. Med. 6, 435-442.

Audicana, M.T., Ansotegui, I.J., de Corres, L.F., Kennedy, M.W., 2002. *Anisakis simplex*: dangerous--dead and alive? Trends Parasitol. 18, 20-25.

Audicana, M.T., Kennedy, M.W., 2008. *Anisakis simplex*: from obscure infectious worm to inducer of immune hypersensitivity. Clin. Microbiol. Rev. 21, 360-379.

Asato, R., Wakura, M., Sueyoshi, T., 1991. A case of human infection with *Anisakis physeteris* larvae in Okinawa, Japan. Jan J Parasito. 40, 181-183.

Audicana, M.T., Ansotegui, I.J., de Corres, L.F., Kennedy, M.W., 2002. *Anisakis simplex*: dangerous—dead and alive? Trends Parasitol. 18, 20-25.

Audicana, M.T., Kennedy, M.W., 2008. *Anisakis simplex*: from obscure infectious worm to inducer of immune hypersensitivity. Clin. Microbial. Rev. 21, 360-379.

Baird F.J., Gasser R.B., Jabbar A., Lopata A.L., 2014. Foodborne anisakiasis and allergy. Mol Cell Probes. 28, 167-174.

Bak, T.J., Jeon, C.H., Kim, J.H., 2014. Occurrence of anisakid nematode larvae in chub mackerel (*Scomber japonicus*) caught off Korea. Int J Food Microbiol. 191, 49-156.

Baird, F.J., Gasser, R.B., Jabbar, A., Lopata, A.L., 2014. Foodborne anisakiasis and allergy. Mol. Cell Probe. 28, 167-174.

Banumathy, G., Singh, V., Pavithra, S.R., Tatu, U., 2003. Heat shock protein 90 function is essential for *Plasmodium falciparum* growth in human erythrocytes. J. Biol. Chem. 278, 18336-18345.

Basáñez, M.G., McCarthy, J.S., French, M.D., Yang, G.J., Walker, M., Gambhir, M., Prichard, R.K., Churcher, T.S., 2012. A research agenda for helminth diseases of humans: modelling for control and elimination. PLoS Negl. Trop. Dis.6, e1548.

Baylis, H.A., 1920. On the Classification of the Ascabidae. I.—The Systematic value of certain characters of the alimentary canal. Parasitol. 12, 253-264.

Berland B., 1961. Nematodes from some Norwegian marine fishes. Sarsia. 2, 1–50.

Beverley-Burton, M., 1978. Population genetics of *Anisakis simplex* (Nematoda: Ascaridoidea) in Atlantic salmon (*Salmo salar*) and their use as biological indicators of host stocks. Environ. Biol. Fish. 3, 369–377.

Bennuru, S., Meng, Z., Ribeiro, J.M.C., Semnani, R.T., Ghedin, E., Chan, K., Lucas, D.A., Veenstra, T.D. & Nutman, T.B., 2011. Stage-specific proteomic expression patterns of the human filarial parasite *Brugia malayi* and its endosymbiont Wolbachia. Proc. Natl. Acad. Sci. USA. 108, 9649–9654.

Blaxter, M.L., 2003. Nematoda: genes, genomes and the evolution of parasitism. Adv. parasit. 54, 101-195.

Boorstein, W.R., Ziegelhoffer, T., Craig, E.A., 1994. Molecular evolution of the HSP70 multigene family. J. Mol. Evol. 38, 1-17.

Borchiellini, C., Boury-Esnault, N., Vacelet, J., Le Parco, Y., 1998. Phylogenetic analysis of the Hsp70 sequences reveals the monophyly of the Metazoa and specific

phylogenetic relationships between animals and fungi. Mol. Biol. Evol. 15, 647-655.

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.

Brutti A., Rovere P., Cavallero S., D'Amelio S., Danesi P., Arcangeli G., 2010. Inactivation of *Anisakis simplex* larvae in raw fish using high hydrostatic pressure treatments. Food Control 21, 331-333.

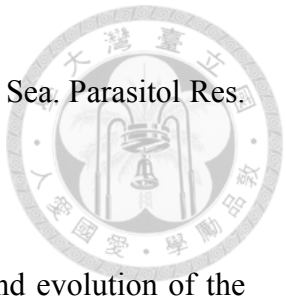
Budin, K., Philippe, H., 1998. New insights into the phylogeny of eukaryotes based on ciliate Hsp70 sequences. Mol. Biol. Evol. 15, 943-956.

Bush, A.O., Lafferty, K.D., Lotz, J.M., Shostak, A.W. 1997. Parasitology meets ecology on its own terms: Margolis *et al*. revisited. J Parasitol. 83, 575-583.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611-622.

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.

Cavallero, S., Nadler, S.A., Paggi, L., Barros, N.B., D'Amelio, S., 2011. Molecular characterization and phylogeny of anisakid nematodes from cetaceans from



southeastern Atlantic coasts of USA, Gulf of Mexico, and Caribbean Sea. Parasitol Res. 108, 781-792.

Chen, B., Zhong, D., Monteiro, A., 2006. Comparative genomics and evolution of the HSP90 family of genes across all kingdoms of organisms. BMC Genomics. 7, 156.

Chen, H.Y., Cheng, Y.S., Grabner, D.S., Chang, S.H., Shih, H.H., 2014. Effect of different temperatures on the expression of the newly characterized heat shock protein 90 (Hsp90) in L3 of *Anisakis* spp. isolated from *Scomber australasicus*. Vet. Parasitol. 205, 540-550.

Cherkasova, V., Ayyadevara, S., Egilmez, N., Shmookler, Reis, R.S., 2000. Diverse *Caenorhabditis elegans* genes that are upregulated in dauer larvae also show elevated transcript levels in long-lived, aged, or starved adults. J. Mol. Biol. 300, 433–448.

Chibale, K., Davies-Coleman, M., Masimirembwa, C. (2012). Drug discovery in Africa.

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. Vet. Parasitol. 177, 324-331.

Collette, B.B., Nauen, C.E., 1983. An annotated and illustrated catalogue of tunas,

mackerels, bonitos and related species known to date. FAO Species Catalogue. Scombrids of the world. FAO Fish.Synop. 125, pp. 1- 137.

Costa, G., Cavallero, S., D'Amelio, S., Paggi, L., Garcia Santamaria, M.T., Borges Perera, C., João Santos, M., Khadem, M., 2011. Helminth parasites of the Atlantic chub mackerel, *Scomber colias* Gmelin, 1789 from Canary Islands, Central North Atlantic, with comments on their relations with other Atlantic regions. Acta Parasitol. 56, 98-104.

Daschner, A., Pascual, C.Y., 2005. *Anisakis simplex*: sensitization and clinical allergy. Curr. Opin. Allergy. Clin. Immunol. 5, 281-285.

Davey, J. T., 1971. A revision of the genus *Anisakis* Dujardin, 1845 (Nematoda: Ascaridata). J. Helminthol. 45, 51-72.

D'Amelio, S., Mathiopoulos, K.D., Santos, C.P., Pugachev, O.N., Webb, S.C., Picanço, 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.

Dangi, S. S., Gupta, M., Nagar, V., Yadav, V.P., Dangi, S.K., Shankar, O., Chouhan, V.S., Kumar, P., Singh, G., Sarkar, M., 2014. Impact of short-term heat stress on physiological responses and expression profile of HSPs in Barbari goats. Int J Biometeorol. 58, 2085–2093.

Dávila, C., Malagón, D., Valero, A., Benítez, R., Adroher, F. J., 2006. *Anisakis simplex*: CO_2-fixing enzymes and development throughout the in vitro cultivation from third larval stage to adult. Exp. Parasitol. 114, 10-15.

De Jong, H., Koffeman, E.C., Meerding, J.M., Scholman, R.C., Wieten, L., de Jager, W., Klein, M., Otten, H., van Wijk, F., van der Zee, R., Bijlsma, J.W., Broere, F., van Eden, W., Prakken B.J., 2014. T cell recognition of naturally presented epitopes of self-heat shock protein 70. Cell Stress Chaperones. 19, 569-578.

De Luca, F., Di Vito, M., Fanelli, E., Reyes, A., Greco, N., De Giorgi, C., 2009. Characterization of the heat shock protein 90 gene in the plant parasitic nematode *Meloidogyne artiellia* and its expression as related to different developmental stages and temperature. Gene. 440, 16-22.

Desai, N.S., Agarwal, A.A., & Uplap, S. S., 2010. HSP: evolved and conserved proteins, structure and sequence studies. Int. J. Bioinform. Res. 2, 67-87.

Devaney, E., 2006. Thermoregulation in the life cycle of nematodes. Int. J. Parasitol. 36, 641-649.

Devaney, E., O'Neill, K., Harnett, W., Whitesell, L., Kinnaird, J.H., 2005. Hsp90 is essential in the filarial nematode *Brugia pahangi*. Int. J. Parasitol. 35, 627-636.

Diesing, K.M., 1860. Revision der Nematoden. Akademie der Wissenschaften zu Wien, Sitzungsberichte, Mathematischnaturwissenchaftlische Klasse, 42, 595–763.

Dittmar, K.D., Pratt, W.B., 1997. Folding of the glucocorticoid receptor by the reconstituted hsp90-based chaperone machinery. The initial hsp90.p60.hsp70-dependent step is sufficient for creating the steroid binding conformation. J. Biol. Chem. 272, 13047-13054.

Dollfus R.P.H., 1966. Helminthofauna de Kogia breviceps (Blainville, 1838) cetace odontocete. Annales de la Société de Science Naturelle de Charete-Maritime. 4, 3-6.

Dropkin, V.H., 1989. Introduction to plant nematology (Ed 2nd). John Wiley and Sons Inc. Dujardin, 1845. (Nematoda: Anisakidae). Syst. Parasitol. 51, 159-170.

Eckl, J.M., Richter, K., 2013. Functions of the Hsp90 chaperone system: lifting client proteins to new heights. Int. J. Biochem. Mol. Biol. 4,157-165.

Espiñeira, M., Herrero, B., Vieites, J.M., Santaclara, F.J., 2010. Detection and identification of anisakids in seafood by fragment length polymorphism analysis and PCR–RFLP of ITS-1 region. Food control. 21, 1051-1060.

Evans, C.G., Chang, L., Gestwicki, J.E., 2010. Heat Shock Protein 70 (Hsp70) as an Emerging Drug Target. J. Med. Chen. 53, 4585–4602.

Fast, N.M., Xue, L., Bingham, S. Keeling, P.J. 2002. Re-examining alveolate evolution using multiple protein molecular phylogenies. J. Eukaryot. Microbiol. 49, 30-37.

Fæste, C. K., Jonscher, K. R., Dooper, M. M., Egge-Jacobsen, W., Moen, A., Daschner, A., Egaas, E., Christians, U., 2014. Characterisation of potential novel allergens in the

fish parasite *Anisakis simplex*. EuPA Open Proteomics, 4, 140-155.

FDA/CFSAN, 2001. Fish and fisheries products hazards and controls guide, processing parameters needed to control pathogens in cold smoked fish. Potential hazards in cold-smoked fish: parasites. U.S. Food and Drug Administration. Center for Food Safety and Applied Nutrition.

Feder, M. E., Hofmann, G. E., 1999. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu. Rev. Physiol. 61, 243-282.

Feng, H.Z., Wang, L., Liu, Y.H., He, L., Li, M., Lu W.C., Xue, C.H., 2010. Molecular characterization and expression of a heat shock protein gene (HSP90) from the carmine spider mite, *Tetranychus cinnabarinus* (Boisduval). J. Insect Sci. 10, 112.

Fraga, J., Montalvo, A.M., De Doncker, S., Dujardin, J.C., Van der Auwera, G., 2010. Phylogeny of *Leishmania* species based on the heat-shock protein 70 gene. Infect. Genet. Evol. 10, 238-245.

Freeman B.C., Myers M.P., Schumacher R. Morimoto R.I., 1995. Identification of a regulatory motif in HSP70 that affects ATPase activity, substrate binding and interaction with HDJ-1. EMBO J. 14, 2281–2292.

Fu, N., Drinnenberg, I., Kelso, J., Wu, J.R., Pääbo, S., Zeng, R., Khaitovich, P., 2007. Comparison of protein and mRNA expression evolution in humans and chimpanzees.

PLoS One. 2, e216.

Gillan, V., Devaney, E., 2014. Nematode Hsp90: highly conserved but functionally diverse. Parasitol. 1-13.

Grabner, D.S., Schertzinger, G., Sures, B., 2014. Effect of multiple microsporidian infections and temperature stress on the heat shock protein 70 (hsp70) response of the amphipod *Gammarus pulex*. Parasite Vector. 7, 170.

Graefe, S.E., Wiesgigl, M., Gaworski, I., Macdonald, A., Clos, J., 2002. Inhibition of HSP90 in *Trypanosoma cruzi* induces a stress response but no stage differentiation. Eukaryotic Cell 1, 936-943.

Guiliano, D.B., Blaxter, M.L., 2006. Operon conservation and the evolution of trans-splicing in the phylum Nematoda. PLoS Genet. 2, 1871-1882.

Guo, Y., Xiao, P., Lei, S., Deng, F., Xiao, G.G., Liu, Y., Chen, X., Li L., Wu, S., Chen, Y., Jiang, H., Tan, L., Xie, J., Zhu, X., Liang, S., Deng, H., 2008. How is mRNA expression predictive for protein expression? A correlation study on human circulating monocytes. Acta Biochim. Biophys. Sin. (Shanghai). 40, 426-436.

Gupta, R.S., 1995. Phylogenetic analysis of the 90 kD heat shock family of protein sequences and an examination of the relationship among animals, plants, and fungi species. Mol. Biol. Evol. 12, 1063-1073.

Gupta, R.S., Golding, G.B., 1993. Evolution of HSP70 gene and its implications

regarding relationships between Archaebacteria, Eubacteria, and Eukaryotes. J. Mol. Evol. 37, 573–582.

Gutierrez, Y., 2000. Diagnostic pathology of parasitic infections with clinical correlations. Oxford University Press.

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., and Vandesompele, J., 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol. 8, R19.

Him, N.A., Gillan, V., Emes, R.D., Maitland, K., Devaney, E., 2009. Hsp-90 and the biology of nematodes. BMC Evol. Biol. 9, 254.

Hochberg, N.S., Hamer, D.H., 2010. Anisakidosis: perils of the deep. Clin Infect Dis. 51, 806-812.

Hunt, C., Morimoto, R.I., 1985. Conserved features of eukaryotic hsp70 genes revealed by comparison with the nucleotide sequence of human hsp70. Proc. Natl. Acad. Sci. USA. 82, 6455-6459.

Iglesias, L., Valero, A., Adroher, F.J., 1997. Some factors which influence the in vitro maintenance of *Anisakis simplex* (Nematoda). Folia parasitol. 44, 297-301.

Iglesias, L., Valero, A., Benítez, R., Adroher, F. J., 2001. In vitro cultivation of *Anisakis simplex*: pepsin increases survival and moulting from fourth larval to adult stage. Parasitol. 123, 285-291.

Innes, E.A., Bartley, P.M., Rocchi, M., Benavidas-Silvan, J., Burrells, A., Hotchkiss, E., Chianini, F., Canton, G. Katzer, F., 2011. Developing vaccines to control protozoan parasites in ruminants: dead or alive? Vet. Parasitol. 180, 155-163.

Ishikura, H., Kikuchi, K., Nagasawa, K., Ooiwa, T., Takamiya, H., Sato, N., Sugane, K., 1993. Anisakidae and anisakidosis. Prog. Clin. Parasitol. 3, 43-102.

James, P., Pfund, C., Craig, E.A., 1997. Functional specificity among Hsp70 molecular chaperones. Science. 275, 387–389.

Johnson, J.L., 2012. Evolution and function of diverse Hsp90 homologs andcochaperone proteins. Biochim. Biophys. Acta. 1823, 607–613.

Kaiser, K., Camargo, N., Kappe, S.H.I., 2003. Transformation of sporozoites into early exoerythrocytic malaria parasites does not require host cells. J. Exp. Med. 197, 1045–1050.

Kim, K.H., Eom, K.S., Park, J.K., 2006. The complete mitochondrial genome of *Anisakis simplex* (Ascaridida: Nematoda) and phylogenetic implications. Int. J. Parasitol. 36, 319-328.

Kimmins, S., MacRae, T.H., 2000. Maturation of steroid receptors: an example of functional cooperation among molecular chaperones and their associated proteins. Cell stress chaperones, 5, 76.

Klimpel, S., Kellermanns, E., Palm, H. W., 2008. The role of pelagic swarm fish

(Myctophidae: Teleostei) in the oceanic life cycle of *Anisakis* sibling species at the Mid-Atlantic Ridge, Central Atlantic. Parasitol. Res. 104, 43-53.

Klimpel, S., Kellermanns, E., Palm, H.W., Moravec, F., 2007. Zoogeography of fish parasites of the pearlside (*Maurolicus muelleri*), with genetic evidence of *Anisakis simplex* (ss) from the Mid-Atlantic Ridge. Mar. Biol. 152, 725-732.

Klimpel, S., Kuhn, T., Busch, M.W., Karl, Horst., Palm, H.W., 2011. Deep-water life cycle of *Anisakis paggiae* (Nematoda: Anisakidae) in the Irminger Sea indicates *kogiid* whale distribution in north Atlantic waters. Polar Biol. 34, 899–906.

Klimpel, S., Palm, H.W., 2011. Anisakid nematode (Ascaridoidea) life cycles and distribution: increasing zoonotic potential in the time of climate change?, in: Mehlhorn, H. (ed.), Progress in parasitology. Springer, Berlin Heidelberg, pp. 201-222.

Klimpel, S., Palm, H.W., Rückert, S., Piatkowski, U., 2004. The life cycle of *Anisakis simplex* in the Norwegian Deep (northern North Sea). Parasitol. Res. 94, 1-9.

Koinari, M., Karl, S., Elliot, A., Ryan, U., Lymbery, A.J., 2013. Identification of *Anisakis* species (Nematoda: Anisakidae) in marine fish hosts from Papua New Guinea. Vet. Parasitol. 193, 126-133.

Krenek, S., Schlegel, M., Berendonk, T. U., 2013. Convergent evolution of heat-inducibility during subfunctionalization of the Hsp70 gene family. BMC Evol. Biol. 13, 49.

Kuhn, T., Garcı´a-Ma`rquez, J., Klimpel, S., 2011. Adaptive radiation within marine anisakid nematodes: a zoogeographical modeling of cosmopolitan, zoonotic parasites. PLoS ONE, 6, e28642.

Kumar, R., Pavithra, S.R., Tatu, U., 2007. Three-dimensional structure of heat shock protein 90 from *Plasmodium falciparum*: molecular modelling approach to rational drug design against malaria. J. Biosci. 32, 531-536.

Lamoureux, F., Thomas, C., Yin, M.J., Fazli, L., Zoubeidi, A., Gleave, M.E., 2013. Suppression of Heat Shock Protein 27 Using OGX-427 Induces Endoplasmic Reticulum Stress and Potentiates Heat Shock Protein 90 Inhibitors to Delay Castrate-resistant Prostate Cancer. Eur Urol. 66, 145-155.

Lanfranchi, A.L., Sardella, N.H., 2010. Anisakids Survival after Microwaving, Freezing and Salting Fish from Argentina. Food Sci. Technol. Res. 16, 499-504.

Levsen, A., Karl, H., 2014. *Anisakis simplex* (sl) in Grey gurnard (*Eutrigla gurnardus*) from the North Sea: Food safety considerations in relation to fishing ground and distribution in the flesh. Food Control, 36, 15-19.

Lindquist, S., 1986. The heat-shock response. Annu. Rev. Biochem. 55, 1151-1191.

Liu, Q., Zhang, Q., 2013. Analysis on long-term change of sea surface temperature in the China Seas. J Ocean Univ China. 12, 295-300.

Lindquist, S., Craig, E.A., 1988. The heat-shock proteins. Annu. Rev. Genet. 22, 631-677.

Mahroof, R., Yan Zhu, K., Neven, L., Subramanyam, B., Bai, J., 2005. Expression patterns of three heat shock protein 70 genes among developmental stages of the red flour beetle, *Tribolium castaneum* (Coleoptera: Tenebrionidae). Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 141, 247-256.

Lu, H.J., Lee, H.L., 2014. Changes in the fish species composition in the coastal zones of the Kuroshio Current and China Coastal Current during periods of climate change: Observations from the set-net fishery (1993–2011). Fish Res. 155, 103-113.

Mattick, J.S., 2003. Challenging the dogma: the hidden layer of non-protein-coding RNAs in complex organisms. Bioessays. 25, 930-939.

Marcogliese, D.J., 2008. The impact of climate change on the parasites and infectious diseases of aquatic animals. Rev Sci Tech. 27, 467-484.

Martinez, J., Perez-Serrano, J., Bernadina, W. E., Rodriguez-Caabeiro, F., 2001. Stress response to cold in *Trichinella* Species. Cryobiol. 43, 293-302.

Mattiucci, S., Abaunza, P., Damiano, S., Garcia, A., Santos, M.N., Nascetti, G., 2007. Distribution of *Anisakis* larvae, identified by genetic markers, and their use for stock characterization of demersal and pelagic fish from European waters: an update. J. Helminthol. 81, 117-127.

Mattiucci, S., Cipriani, P., Webb, S.C., Paoletti, M., Marcer, F., Bellisario, B., Gibson, D.I., Nascetti, G., 2014. Genetic and morphological approaches distinguish the three sibling species of the *Anisakis simplex* species complex, with a species designation as *Anisakis berlandi* n. sp. for *A. simplex* sp. C (Nematoda: Anisakidae). J. Parasitol. 100, 199-214.

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., 2006. Molecular systematics, phylogeny and ecology of anisakid nematodes of the genus *Anisakis* Dujardin, 1845: an update. Parasite. 13, 99-113.

Mattiucci, S., Nascetti, G., Bullini, L., Orecchia, P., Paggi, L., 1986. Genetic structure of *Anisakis* physeteris, and its differentiation from the *Anisakis simplex* complex (Ascaridida: Anisakidae). Parasitol. 93, 383-387.

Mattiucci, S., Nascetti, G., Clanchi, R., Paggi, L., Arduino, P., Margolis, L., Brattey, J., Webb, S., D'Amelio, 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., 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). Syst. Parasitol. 61, 157-171.

Mattiucci, S., Paggi, L., Nascetti, G., Santos C.P., 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., 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.

Mercado, R., Torres, P., Muñoz, V., Apt, W., 2001. Human infection by *Pseudoterranova decipiens* (Nematoda, Anisakidae) in Chile: report of seven cases. Mem. Inst. Oswaldo. Cruz. 96, 653-655.

Mladineo, I., Simat, 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.

Moiseev, S.I., 1991. Observation of the vertical distribution and behaviour of nektonic squids using manned submersibles. Bull. Mar. Sci. 49,446–456.

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.

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. J. Parasitol. 91, 1413–1429.

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, 633-640.

Nemoto, T., Sato, N., Iwanari, H., Yamashita, H., Takagi, T., 1997. Domain structures and immunogenic regions of the 90-kDa heat-shock protein (HSP90) Probing with a library of anti-HSP90 monoclonal antibodies and limited proteolysis. J. Biol. Chem. 272, 26179-26187.

Newton-Howes, J., Heath, D.D., Shoemaker, C.B., Grant, W.N., 2006. Characterisation and expression of an Hsp-70 gene from *Parastrongyloides trichosuri*. Int. J. Parasitol. 36, 467– 474.

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. Syst. Parasitol. 40, 161-174.

Parsell, D.A., Lindquist, S., 1993. The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. Annu. Rev. Gene. 27, 437-496.

Pearl, L.H., Prodromou, C., 2006. Structure and mechanism of the Hsp90 molecular chaperone machinery. Annu. Rev. Biochem., 75, 271-294.

Peñalver, J., Dolores. E.M., Muñoz, P., 2010. Absence of anisakid larvae in farmed European sea bass (*Dicentrarchus labrax* L.) and gilthead sea bream (*Sparus aurata* L.) in Southeast Spain. J. Food Prot. 73, 1332-1334.

Péroval, M., Péry, P., Labbé, M., 2006. The heat shock protein 90 of *Eimeria tenella* is essential for invasion of host cell and schizont growth. Int. J. Parasitol. 36, 1205-1215.

Perry, R.N., Wharton, D.A. (Ed.), 2011. Molecular and physiological basis of nematode survival. CABI, 320 pp.

Petronijevic, T., Rogers, W.P., 1987. Undissociated bases as the stimulus for the development of early parasitic stages of nematodes. Int. J. Parasitol. 17, 911-915.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR, Nucleic Acids Research. 29, 2002-2007.

Picard, D., 2002. Heat-shock protein 90, a chaperone for folding and regulation. Cell. Mol. Life Sci. 59, 1640-1648.

Pirkkala, L., Nykanen, P., Sistonen, L., 2001. Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. FASEB. J. 15, 1118-1131.

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.

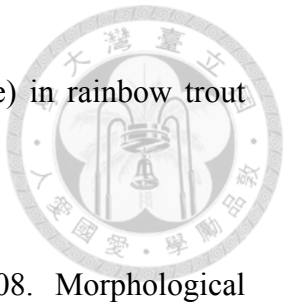
Prasad, B.D., Goel, S., Krishna, P., 2010. In silico identification of carboxylate clamp type tetratricopeptide repeat proteins in Arabidopsis and rice as putative co-chaperones of Hsp90/Hsp70. PLoS One. 5, e12761

Pratt, W.B., 1998. The Hsp90-based chaperone system: involvement in signal transduction from a variety of hormone and growth factor receptors. Proc Soc Exp Biol Med. 217, 420-434.

Prodromou, C., Panaretou, B., Chohan, S., Siligardi, G., O'Brien, R., Ladbury, J.E., Roe, S.M., Piper, P.W., Pearl, L.H., 2000. The ATPase cycle of Hsp90 drives a molecular 'clamp' via transient dimerization of the Nterminal domains. EMBO. J. 19, 4383-4392.

Qin, Y., Zhao, Y., Ren, Y., Zheng, L., Dai, X., Li, Y., Mao, W., Cui, Y., 2013. Anisakiasis in China: the first clinical case report. Foodborne Pathog Dis. 10, 472-474.

Quiazon, K.M., 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.

Quiazon, K.M., Zenke, K., Yoshinaga, T., 2013. Molecular characterization and comparison of four *Anisakis* allergens between *Anisakis simplex* sensu stricto and *Anisakis pegreffii* from Japan. Mol. Biochem. Parasitol. 190, 23-26.

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.

Rodríguez-Mahillo, A.I., González-Muñoz, M., de las Heras, C., Tejada, M., Moneo, I., 2010. Quantification of *Anisakis simplex* allergens in fresh, long-term frozen, and cooked fish muscle. Foodborne Pathog Dis. 7, 967-973.

Rogers, S.O., Bendich, A.J., 1985. Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. Plant Mol Biol. 5, 69-76.

Saunders, L.R., Verdin, E., 2009. Cell biology, stress response and aging. Science 323, 1021-1022.

Selkirk, M.E., Denham, D.A., Partono, F., Maizels, R.M., 1989. Heat shock cognate 70

is a prominent immunogen in Brugian filariasis. J. Immunol. 143, 299– 308.

Sharma, Y. D., 1992. Structure and possible function of heat-shock proteins in *Plasmodium falciparum* malaria. Comp. Biochem. Physiol. B. Comp. Biochem. 102, 437-444.

Sharp, M.F., Lopata, A.L., 2014. Fish allergy: in review. Clin Rev Allergy Immunol. 46, 258-271.

Shih, H.H., 2004. Myths and analysis of nematodes infections in marine fish. Fisheries extension NTU, 15, 15–34 (in Chinese).

Shih, H.H., 2004. Parasitic helminth fauna of the cutlass fish, *Trichiurus lepturus* L., and the differentiation of four anisakid nematode third-stage larvae by nuclear ribosomal DNA sequences. Parasitol. Res. 93, 188-195.

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.

Shiny, C., Krushna, N.S., Babu, S., Elango, S., Manokaran, G., Narayanan, R.B., 2011. Recombinant Wolbachia heat shock protein 60 (HSP60) mediated immune responses in patients with lymphatic filariasis. Microbes Infect. 13, 1221-1231.

Simonetta, M., Michela, P., Francesco, B., Massimo, P., Raffaele, P., Vincenzo, G., Alessandra C., Giuseppe, N., 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 Inf. Dise. 11, 82.

Simpson, A.G., Gill, E.E., Callahan, H.A., Litaker, R.W., & Roger, A.J., 2004. Early evolution within kinetoplastids (Euglenozoa), and the late emergence of trypanosomatids. Protist. 155, 407-422.

Skantar, A.M., Carta, L.K., 2004. Molecular characterization and phylogenetic evaluation of the Hsp90 gene from selected nematodes. J. Nematol. 36, 466-480.

Smith, J.W., 1983. *Anisakis simplex* (Rudolphi, 1809, det. Krabbe, 1878)(Nematoda: Ascaridoidea): morphology and morphometry of larvae from euphausiids and fish, and a review of the life-history and ecology. J Helminthol. 57, 205-224.

Song, H.M., Mu, X.D., Gu, D.E., Luo, D., Yang, Y.X., Xu, M., Luo, J.R., Zhang, J.E., Hu, Y. C., 2014. Molecular characteristics of the HSP70 gene and its differential expression in female and male golden apple snails (*Pomacea canaliculata*) under temperature stimulation. Cell Stress Chaperones. 19, 579-589.

Stechmann, A., Cavalier-Smith, T., 2003. Phylogenetic analysis of eukaryotes using heat-shock protein Hsp90. J. Mol. Evol. 57, 408-419.

Suzuki, J., Murata, R., Hosaka, M., Araki, J., 2010. 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.

Svanevik, C.S., Levsen, A., Lunestad, B.T., 2013. The role of muscle-invading anisakid larvae on bacterial contamination of the flesh of post-harvest blue whiting (*Micromesistius poutassou*). Food Control. 30, 526-530.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725-2729.

Tejada, M., Karl, H., De Las Heras, C., Vidacek, S., Solas, M.T., Garcia, M.L., 2013. Does the intensity of *Anisakis* infection affect the quality of hake muscle? J. Aquat. Food Prod. Technol. 23, 221-236.

Tejada, M., Olivares, F., de Las Heras, C., Careche, M., Solas, M.T., García, M.L., Fernandez, A., Mendizábal, A., Navas, A., Rodríguez-Mahillo, A.I., González-Muñoz, M., 2014. Antigenicity of *Anisakis simplex* s. s. L3 in parasitized fish after heating conditions used in the canning processing. J Sci Food Agric.

Tejada, M., Solas, M.T., Navas, A., Mendizábal, A., 2006. Scanning electron microscopy of *Anisakis* larvae following different treatments. J. Food Prot. 69, 1379-1387.

Tsan, M.F., Gao, B., 2004. Cytokine function of heat shock proteins. Am. J. Physiol. Cell Physiol. 286, 739-744.

Tseng, C.J., Shih, H.H., 2007. Study of the correlation between low temperature and the trehalose synthase activity of *Anisakis simplex*. National Taiwan university, college of

life science, master thesis. 65pp. (in Chinese, with English abstract).

Tsutsumi, S., Neckers, L., 2007. Extracellular heat shock protein 90: a role for a molecular chaperone in cell motility and cancer metastasis. Cancer Sci. 98, 1536-1539.

Tzeng, T.D., 2004. Morphological variation between populations of spotted mackerel (*Scomber australasicus*) off Taiwan. Fish Res. 68, 45–55.

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., 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, 267-271.

Valentini, A., Mattiucci, S., Bondanelli, P., Webb, S.C., Mignucci-Giannone, A. A., Colom-Llavina, M.M., & Nascetti, G., 2006. Genetic relationships among *Anisakis* species (Nematoda: Anisakidae) inferred from mitochondrial cox2 sequences, and comparison with allozyme data. J. Parasitol. 92, 156-166.

Van Oers, M.M., Vlak, J.M., Voorma, H.O., Thomas, A.A., 1999. Role of the 3' untranslated region of baculovirus p10 mRNA in high-level expression of foreign genes. J. Gen. Virol. 8, 2253-2262.

Van Thiel, P.H., Kuipers, F.C., Roskam, R.T., 1960. A nematode parasitic to herring, causing acute abdominal syndromes in man. Trop. Geogr. Med. 12, 97-113.

Vidaček, S., De Las Heras, C., Solas, M.T., Mendizábal, A., Rodriguez-Mahillo, A.I., Tejada, M., 2010. Antigenicity and viability of *Anisakis* larvae infesting hake heated at different time-temperature conditions. J. Food Prot. 73, 62-68.

Vidaček, S., De Las Heras, C., Solas, M.T., García, M.L., Mendizábal, A., Tejada, M., 2011. Viability and antigenicity of *Anisakis simplex* after conventional and microwave heating at fixed temperatures. J. Food. Prot. 74, 2119-2126.

Wang, J.Y., Zhang, J.H., Lin, Q., Zhang, Q.T., He, W.X., Li, K.F., Xu, X., 2010. Infection and physico-chemical characteristics of *Anisakis* among marine fish caught in Zhoushan Fishery. Zhonghua Liu Xing Bing Xue Za Zhi. 31, 1001-1004. [Article in Chinese]

Wang, S., Zhu, X., Yang, Y., Yang, J., Gu, Y., Wei, J., Haoa, R., Boireau, P., Cui, S., 2009. Molecular cloning and characterization of heat shock protein 70 from *Trichinella spiralis*. Acta Trop. 110, 46-51.

Wang, Z., Lindquist, S., 1998. Developmentally regulated nuclear transport of transcription factors in *Drosophila* embryos enable the heat shock response. Development. 125, 4841-4850.

Yang, Y., Qin, W., Zarlenga, D., Cao, L., Tian, G., 2013. TsDAF-21/Hsp90 is expressed in all examined stages of *Trichinella spiralis*. Vet. Parasitol. 194, 171-174.

Yang, J., Yang, L., Lv, Z., Wang, J., Zhang, Q., Zheng, H., Wu, Z., 2012. Molecular

cloning and characterization of a HSP70 gene from *Schistosoma japonicum*. Parasitol. Res. 110, 1785-1793.

Yeh, F. L., Hsu, T., 2002. Differential regulation of spontaneous and heat-induced HSP 70 expression in developing zebrafish (*Danio rerio*). J. Exp. Zool. 293, 349-359.

Young, P.C., 1972. The relationship between the presence of larval *Anisakis* nematodes in cod and marine mammals in British home waters. J. Appl. Ecol. 459-485.

Yu, H.S., Park, S.K., Lee, K.H., Lee, S.J., Choi, S.H., Ock, M.S., Jeong, H.J., 2007. *Anisakis simplex*: analysis of expressed sequence tags (ESTs) of third-stage larva. Exp. Parasitol. 117, 51-56.

Zhang, Z., 2013. Animal biodiversity: An update of classification and diversity in 2013. In: Zhang, Z.-Q. (Ed.) Animal biodiversity: An outline of higher-level classification and survey of taxonomic richness. Zootaxa. 3703, 5–11.

Zhang, L., Du, X., An, R., Li, L., Gasser, R.B., 2013. Identification and genetic characterization of *Anisakis* larvae from marine fishes in the South China Sea using an electrophoretic-guided approach. Electrophoresis. 34, 888-894.

Zhu, F., Wang, F., Wang, D., Zuo, Q., Zhu, X.F., Wang, Y.Y., Duan, Y.X., Chen, L.J., 2012. Gene Expression Profiles of Hsp90 Associated with Root-Knot Nematode *Meloidogyne incognita* and Tomato. Journal of Shenyang Agricultural University. 43, 435-443. (in Chinese, with English abstract).

Zhu, X., Zhao, X., Burkholder, W. F., Gragerov, A., Ogata, C.M., Gottesman, M. E., Hendrickson, W.A., 1996. Structural analysis of substrate binding by the molecular chaperone DnaK. Sci. 272, 1606-1614.

Zullo, A., Hassan, C., Scaccianoce, G., Lorenzetti, R., Campo, S. M., Morini, S., 2010. Gastric anisakiasis: Do not forget the clinical history. J. Gastrointestin. Liver Dis. 19, 359-359.