Chapter 1: Literature Review and Scope of the Thesis

1.1 Introduction

1.1.1 Schistosomiasis

Schistosomiasis is among the most severe parasitic diseases targeted in terms of morbidity and mortality [5-7] and has been highlighted for control by the World Health Organisation (WHO). It is estimated that 200 million people are infected, of which approximately 20 million are severely diseased, resulting in an estimated 200,000 deaths in 2003 [8]. Most infections are caused by the three major schistosome species; *Schistosoma haematobium*, *S. mansoni*, and *S. japonicum* [2, 8-10]. *S. haematobium* is endemic in Africa and the Eastern Mediterranean, *S. mansoni* is endemic in Africa and South America, while *S. japonicum* is endemic mainly in China and the Philippines [10] (Figure 1.1). Although the disease is ancient, with schistosome eggs being recovered from mummies from China and Egypt [10, 11], the first description of the disease caused by *S. japonicum* was made in the middle of the nineteenth century in Japan by Dr Yoshinao Fujii [12, 13]. Fujii named this new disease "Katayama Syndrome", but incorrectly attributed it to the liver fluke *Clonorchis sinensis* [11]. It was not until 1904 that the correct causative agent of Katayama Syndrome was discovered by Fijiro Katsurada who was investigating a disease that presented symptoms similar to Katayama disease (now known to be identical to schistosomiasis) [14]. Katsurada noted trematode eggs similar to those of *S. haematobium* in the faeces of some of his patients, and suspected that the disease was caused by a related parasite [11, 14]. He later went on to name this parasite *Schistosoma japonicum*, and identified the differences between the eggs of *S. haematobium* and *S. japonicum* [12, 14]. Although it has been eradicated from Japan, with the last case being identified in 1977-78 just before the availability of

an effective anti-schistosome drug, praziquantel, schistosomiasis continues to spread to new geographic areas [9, 15-21], including China [18], Senegal [20], Mali [21] and Brazil [19]. The main reasons for this spread are due to climate change and water resource development in China [18, 22], construction of dams effecting Mali and Senegal [20, 21] and the introduction of schistosomes in Brazil by African slaves in the mid 16th century [19]. The dual concerns that resistance to praziquantel is emerging, and the fact there are no anti-schistosome vaccines currently available, make the identification of vaccine and new chemotherapeutic targets a priority [9, 15].

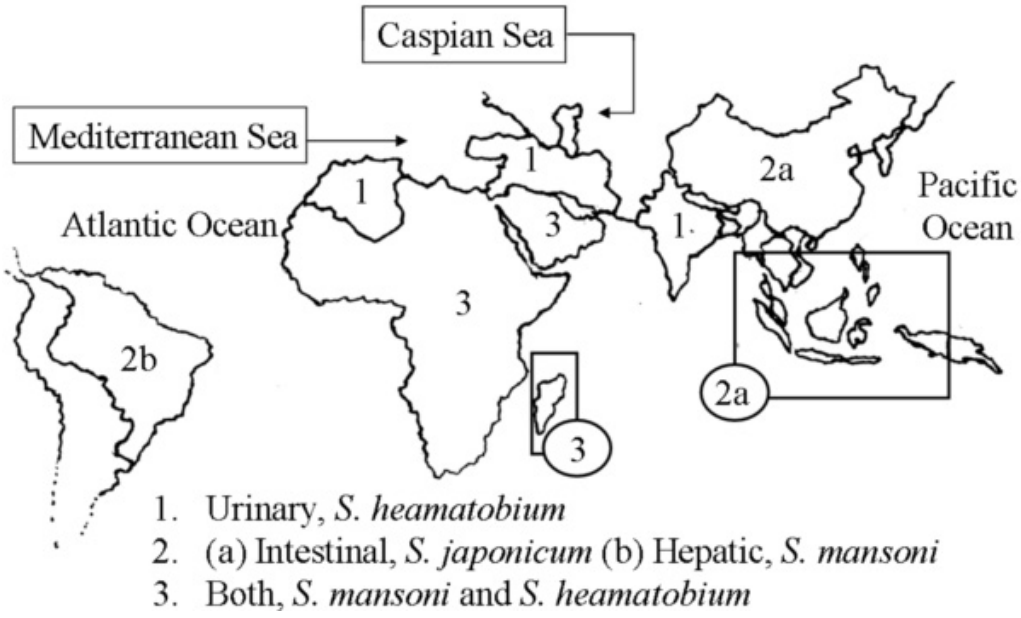

Figure 1.1 The distribution of schistosomiasis 'Adapted from [23]'. Intestinal/hepatic schistosomiasis includes *S. japonicum* (2a) and *S. mansoni* (2b) respectively; whereas urinary schistosomiasis is caused by *S. haematobium* (1). Schistosomiasis japonica has also been identified in South East Asia (Indonesia and the Philippines contained within the right hand square) [3, 23].

1.1.2 Life cycle.

The schistosome life cycle includes a free swimming form called the cercaria (Figure 1.2). Cercariae secrete elastase located from special glands in their heads, which enable them to penetrate the skin of animals that come into contact with them in water [10]. At this time, the cercariae shed their tails and transform their trilaminate tegument, covered by a glycocalyx, replacing the cercarial lipid bilayer and glycocalyx with a double lipid bilayer into the heptalaminate form adapted to the definitive host environment [24, 25]. Now referred to as schistosomula, they migrate to the lungs of the host via blood vessels and draining lymphatics. After several days the schistosomula move to the hepatic portal system where they mature and pair up [10].

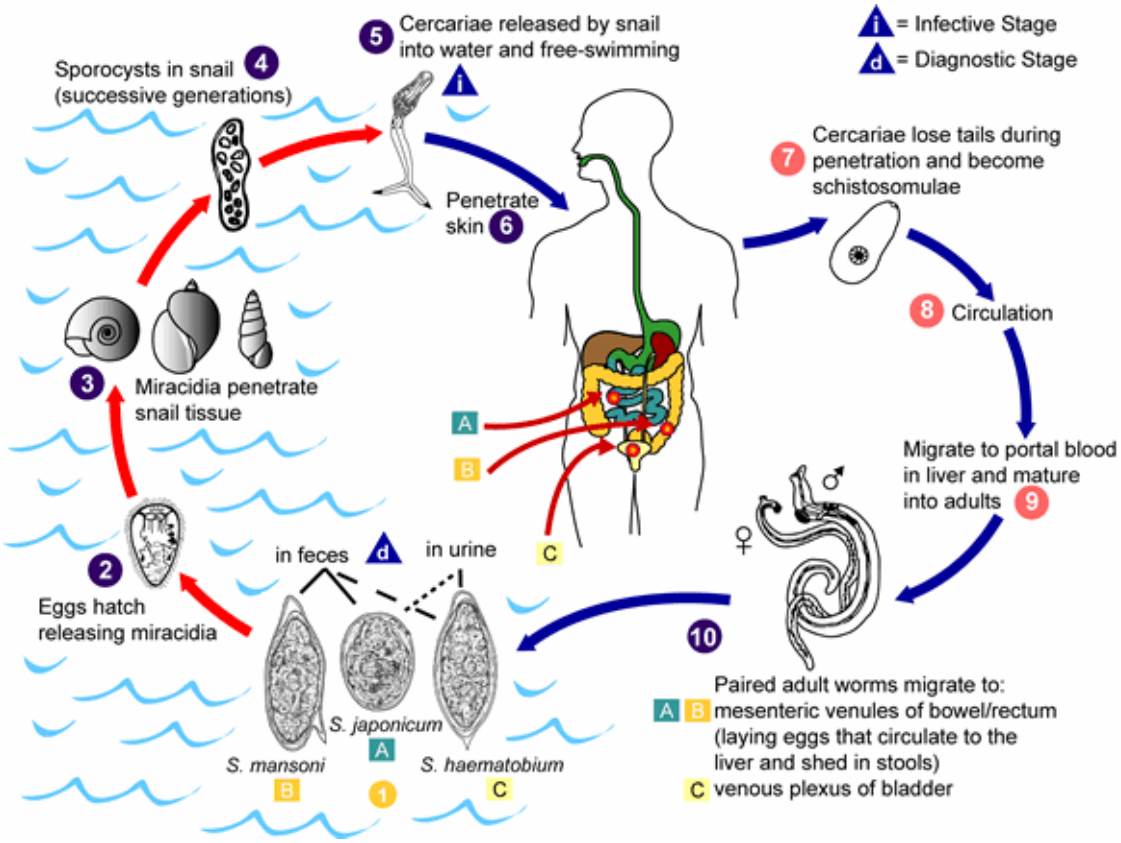

Figure 1.2 *Schistosoma* life cycle 'Taken from [3], with permission from DPDx: CDC's website for parasite identification; http://www.dpd.cdc.gov/dpdx, see appendix A'. Eggs are released into the water (1). The eggs hatch releasing miracidia (2) which infect fresh water snails (3). Sporocysts migrate to the snail's hepatopancreas (4) and asexually produce free swimming cercariae (5), which in turn penetrate the skin of animals (6). The cercariae now termed schistosomula (7) mature in the host (8) and lay eggs (9, 10) which are either trapped in the surrounding tissues or are released into the environment via faeces [10].

The adult schistosome survives by absorbing amino acids, and large amounts of glucose from the host, the equivalent of its dry weight every five hours [25]. The dorso-ventrally flattened male (5-10 mm) embraces the smaller but longer cylindrical female (10-15 mm) in its gynaecophoric canal (Figure 1.3) [11, 26]. Interlocked together they migrate downstream to the blood vessels of the bladder or intestine (Table 1.1) [27]. Once the worm pairs reach the portal or vesicular blood vessels of the definitive host, the mature female adult lays eggs which pass through the intestinal or bladder wall and/or are retained in the body of the host (Table 1.1 and Figure 1.4) [10]. The eggs that are discharged from the mammalian host release free swimming miracidia that infect freshwater snails, the genus being *Oncomelania* for *S. japonicum* (Figure 1.2) [3]. Harley (1864) speculated that there was an intermediate mollusc host, this was not accepted until Miyairi and Suzuki (1913, 1914) confirmed this in their studies on *S. japonicum* [11]. This observation was later confirmed by others, with the snail hosts of *S mansoni* and *S. haematobium* soon identified [11]. The parasite forms a sporocyst (mother sporocyst) at the site of penetration that produces daughter sporocysts that migrate to the snail's hepatopancreatic region [10]. There the parasite asexually produces cercariae, which in turn are released into water (Figure 1.2) [10]. The freshwater snails may remain infected for several months releasing cercariae daily [10].

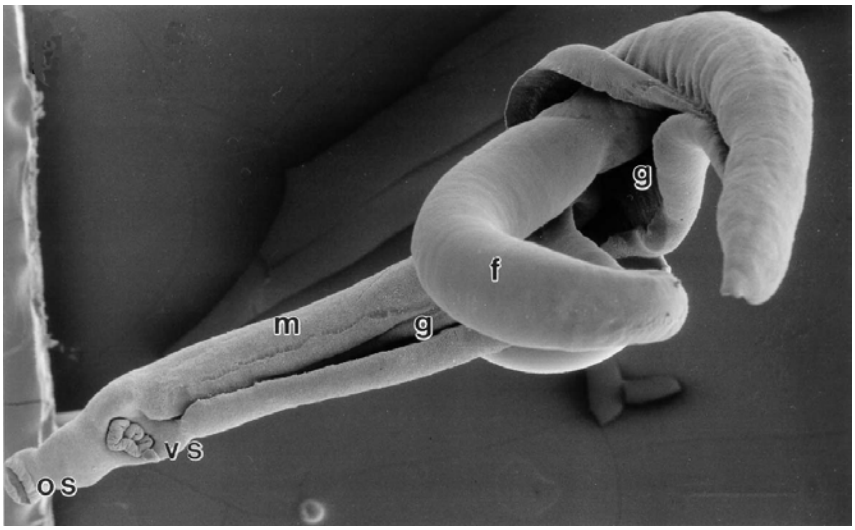

Figure 1.3 Adult male and female *S. japonicum* worms 'Taken from [26] with permission from Elsevier, see appendix A'. The male (m) is embracing the female (f) in the gynaecophoric canal (g), the oral sucker (os) and the ventral sucker (vs) are both used for movement.

Table 1.1 Comparison of *Schistosoma* species 'Adapted from [11]'.

Item	***S. japonicum***	***S. mansoni***	***S. haematobium***
Adult Worm			
Location in host	Mesenteric veins	Mesenteric veins	Vesical plexus
Female			
Length (mm)	20-30	10-20	16-26
Male			
Length (mm)	10-20	6-13	10-15
Mature Egg			
Shape	round	ovoid	ovoid
Size (μm)	60×100	61×140	62×150
Eggs/day/female	3,500	100-300	20-300

The Table shows a comparison of the adult and egg stages between the 3 major schistosome species that affect humans.

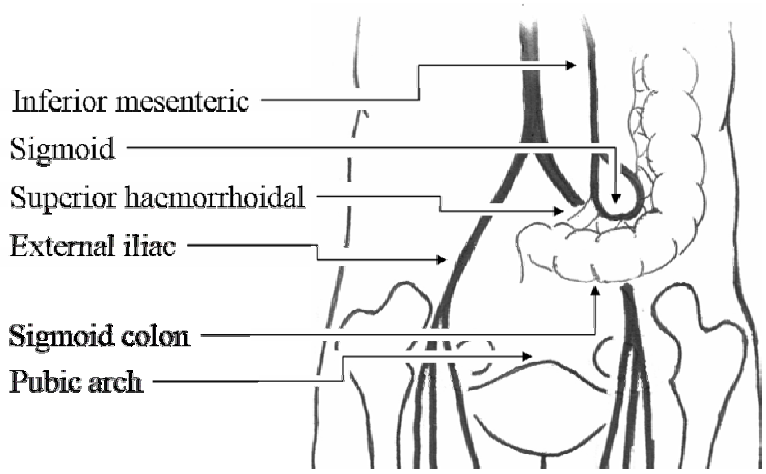

Figure 1.4 The principal veins of the pelvic region 'Adapted from [28]'. The superior haemorrhoidal vein, also known as the superior rectal, drains the upper-part of the rectal plexus into the inferior mesenteric vein connecting the portal system with the systemic circulation.

1.1.3 Pathology

Schistosomiasis, also known as bilharzia, is caused by the adult schistosome worm depositing eggs in the blood vessels of the bladder or intestine of vertebrates [10]. Schistosomes, similar to other helminths such as *Ascaris lumbricoides* and *Clonorchis sinensis*, induce in their mammalian hosts marked cytokine responses as part of an overall immune response [29]. There are two main conditions recognised in schistosome infected individuals; acute and chronic schistosomiasis. Acute schistosomiasis, also referred to as Katayama fever, can occur before the appearance of schistosome eggs in the stool [9, 10, 29]. Individuals may present nocturnal fever peaks, coughing, generalised muscle pain, headaches, and a tender enlarged liver [10]. This is accompanied by a rise in the level of immunoregulatory molecules in the blood, including tumour necrosis factor, interleukin-1 and interleukin-6, producing a cell mediated response induced by egg antigens [29]. Chronic schistosomiasis affects the circulatory system and severe infection that may be life threatening. This disease may

be accompanied by severe hepatic and periportal fibrosis, portal hypertension, and portosystemic shunting of venous blood [29, 30]. This immunological response from the host is due to the presence of schistosome eggs and the granulomatous reaction evoked by the secreted antigens [9]. The granulomas, developed where eggs accumulate, eventually destroy the egg, but also result in fibrotic deposition in host tissues, such as the liver (Figure 1.5) and intestine in the case of schistosomiasis japonica and mansoni or genitourinary tract in schistosomiasis haematobia infections [9]. Both the acute and chronic schistosomiasis improve with chemotherapy but may reoccur after reinfection with the parasite [10].

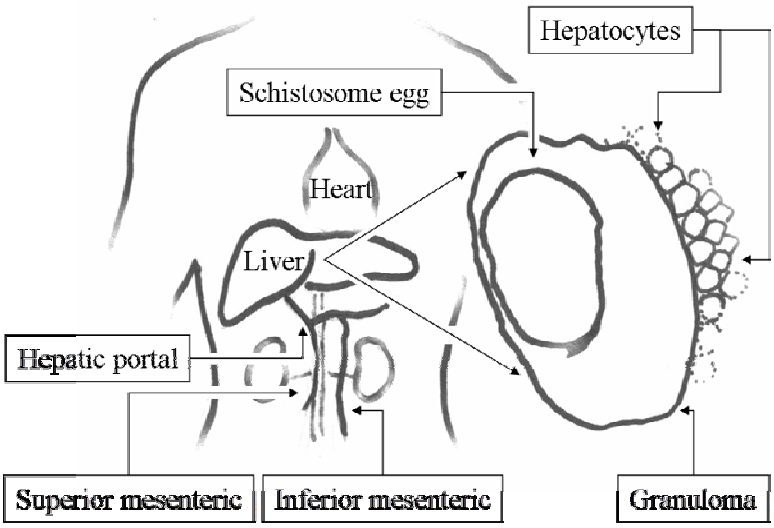

Figure 1.5 Granuloma in a human liver 'Adapted from [31]' The liver is the main site that is affected because many of the eggs are carried there by the blood. The granulomata that forms are composed of collagen fibres and cells including macrophages, eosinophils and $CD4^+$ T cells [29]. This schematic is not to scale, the stylised granuloma is magnified from the liver.

1.2 Vaccine development and current treatments

1.2.1 Overview

It has been known for the past 20 years that vaccination with ultraviolet radiated-attenuated cercariae (URAC) can induce almost complete protection to subsequent infection by promotion of antibodies against cercariae [32, 33]. This method however is impractical as a field application since there are significant problems with availability (large numbers of cercariae are required), sustainability (viability less than 24 hours), and practical deployment for field application [34-37]. Unfortunately to date, there have been no other vaccines developed that promote such protection as URAC, whilst the available drugs will only provide short-term relief [32].

1.2.2 Praziquantel and artemether

For over 25 years control of schistosomiasis has been primarily dependent on the use of praziquantel, a drug that causes the tegument of worms to disintegrate *in vivo* [8, 38-40]. It has been suggested that this is caused by numerous granular materials being deposited on worms resulting in damage to the schistosome tegument and inevitably host mediated attacks [40]. Unfortunately there are problems associated with regulation of worm burden dependent on praziquantel alone as praziquantel, when taken in the correct dosage, is only fully effective on mature worms [40]; the therapeutic effect of praziquantel is time limited, as the white crystalline powder has a half-life in serum of 50 – 90 minutes [41]; the use of praziquantel can induce an anaphylactic response, resulting in abdominal pain, nausea and vomiting, primarily due to the vesication and disintegration of the tegumental surface of the worm [42]; and there is some evidence of decreasing susceptibility of worms to praziquantel, which has been confirmed by laboratory studies [39, 41, 43-45]. Unfortunately, the limited knowledge of natural

variation in susceptibility of schistosomes to praziquantel causes a major problem when attempting to identify resistance [38].

The concerns over resistance to praziquantel make it important to investigate alternative drugs and combination therapies [46]. Another pharmaceutical product that is used to treat schistosomiasis is artemether. The medical properties of the plant *Artemisia annua* were investigated when China embarked on an examination of indigenous plants in 1967 [47]. Although its therapeutic properties had been known from early times, the plant antimalarial constituent artemisinin was not extracted until 1972 [47, 48]. Artemether, which is the β-methyl ether of artemisinin, has been only recently recognised for its anti-schistosomula properties [48, 49]. Detailed investigations have revealed that artemether is most effective against the juvenile stages of the major schistosome species [50]. Praziquantel has been combined with some success with artemether showing highest efficacies against the juvenile and mature worms [40, 51]. As a result of the broad-spectrum antischistomal activities displayed by these two drugs, it has been suggested that they may be beneficial for the treatment of all human schistosomiasis [51]. Unfortunately because of the short-term effectiveness of these drugs and common reinfection of patients, continual chemotherapeutic treatment is not an ideal option. Future combinations could include existing drugs with vaccines, but in order to facilitate the development of such vaccines and new chemotherapies, more information on the schistosome genome, transcriptome and proteome is needed.

1.3 Genome

Schistosomes have eight chromosomes with ZW female and ZZ male sex chromosomes in adults and fertilised eggs stages [5, 52]. The schistosome haploid genome size is 270

megabases, with 15,000 to 20,000 genes estimated to be present in *S. japonicum* [1, 5, 25]. The generation of whole genome maps for the schistosome species has not been achieved but there is some progress in producing low-resolution maps for chromosomes of *S. mansoni* [53]. The physical mapping of the *S. mansoni* chromosomes have been made possible using centromere-banding (C-banding) to identify chromosomes and *in situ* fluorescence hybridization (FISH) to map genes [54-56]. More recently a second strategy of using HAPPY mapping (haploid cells and the polymerase chain reaction) [57] has been used to generate maps for *S. mansoni* [56]. HAPPY mapping provides an *in vitro* equivalent of a genetic map that can generate a physical map [56]. This has been made possible by using existing database sequences such as the expressed sequence tags (ESTs) for *S. mansoni* and *S. japonicum.* Hu *et al*. [25] assigned 43,707 ESTs from adults and eggs of *S. japonicum*, using Chinese strain (SJC) miracidium, cercaria, and adult worm complementary DNA (cDNA) isolated from polyadenylic acid (poly(A)$^+$) messenger RNA (mRNA). They discovered that there was greater sequence similarity of SJC ESTs to humans (Table 1.2) than to other eukaryote genomes, such as the fruit fly *Drosophila melanogaster* or the nematode *Caenorhabditis elegans* [25]. Similarly, Verjovski-Almeida *et al*. [58] generated 163,000 ESTs from six developmental stages of *S. mansoni*. As a result of the work undertaken by Hu *et al*. [25] and Verjovski-Almeida *et al*. [58], two major schistosome databases have been constructed; the *Schistosoma japonicum* EST database (http://schistosoma.chgc.sh.cn/) [25] and the *Schistosoma mansoni* Gene Index (http://www.tigr.org/tdb/e2k1/sma1/) [58]. Together this work and other recent publications of ESTs have added another 168,347 new schistosome sequences to gene information banks [1]. Despite this, compared to the 1980s, the last fifteen years have seen few new putative vaccine candidates identified for SJC [32]. New technologies may promote future discoveries

of the functional biology of schistosomes as well as identification of novel putative vaccines and drug candidates. One way this may be done is through the use of cDNA microarrays, an approach that has been explored in this thesis.

Table 1.2 Summary of human genes with sequence similarity to *S. japonicum* 'Adapted from [25]'.

Unigene Number	Gene Name	Sequence Similarity
Hs.211539	EIF2S3 Eukaryotic translation initiation factor	83%
Hs.1023	PDHA1 Pyruvate dehydrogenase	81%
Hs.173965	RPS6KA3 Ribosomal protein	86%
Hs.31314	RBBP7 Retinoblastoma-binding protein	81%
Hs.287867	SNX12 Sorting nexin	81%
Hs.125856	ABCB7 ATP-binding cassette	80%
Hs.100293	OGT Transferase	91%
Hs.78771	PGK1 Phosphoglycerate kinase	87%
Hs.1904	PRKC1 Protein kinase	86%
Hs.56145	TMSNB Thymosin	86%
Hs.56	PRPS1 Synthetase	81%
Hs.178391	RPL44 Ribosomal protein	85%
Hs.272497	GLUD2 Glutamate dehydrogenase	86%
Hs.82794	CETN2 Centrin (Acidic phosphoprotein)	82%
Hs.155103	EIF1AY Eukaryotic translation initiation factor	81%

Data obtained by Basic Local Alignment Search Tool X, (BLAST) X program by studies undertaken by Hu *et al*. [25]. The data represents human genes that have a greater than 75% nucleotide sequence similarity with known *S. japonicum* ESTs.

1.4 Scope and aims

The major thrust of this thesis was to aid in the design of a microarray resource and use it to explore the transcriptome of *Schistosoma japonicum*. The Chinese (SJC) and Philippine (SJP) forms of *S. japonicum* exhibit a number of morphological and other phenotypic differences, including pre-patent period [26 and 28 days, SJC and SJP respectively], tegument topography, adult worm size [SJC longer than the SJP],

virulence [SJC is more pathogenic] and sub-species of snail intermediate host infected [59-61]. Despite this, major genotypic differences have yet to be identified between the two strains [61]. Designing a microarray on this parasite will help in the exploration of transcriptional expression differences between and within the Chinese (SJC) and Philippine (SJP) strains of *S. japonicum*. Additionally, information from this study will further the understanding of the complex interplay between male and female schistosomes of the Chinese and Philippine strains. Therefore the aims of this study were to:

(1) Design a custom made microarray based on ESTs of *S. mansoni* and *S. japonicum*.

(2) Identify differential gene expression between the Chinese and Philippine strains of *S. japonicum*.

(3) Show differential gene expression between the sexes of the Chinese and Philippine strains of *S. japonicum*.

(4) Use real time polymerase chain reaction (PCR) to further explore the expression of selected genes through different life stages of the Chinese *S. japonicum*.

Although previously no major genetic differences had been found [61], significant DNA microsatellite genetic variation has been shown between strains and isolates in *S. japonicum* [62]. In this work, it is hypothesized that there is major expressional differences between strains. Additionally these expressional differences can highlight important genes of interest that will provide a greater understanding of the schistosome transcriptome. This thesis comprises seven chapters, including a literature review (Chapter-1) and a review on Microarrays in Parasitology (Chapter 2). Chapter 3 provides a description and characterization of the microarray including its design and construction. Chapter 4 explores the expression differences of probes between the

Chinese and Philippine strains of *S. japonicum*. Chapter 5 compares the differential expression of probes between the male and female worms of the Chinese and Philippine strains of *S. japonicum*. Chapter 6 describes an investigation of selected genes of interest in different life stages of the Chinese *S. japonicum* using real time PCR. The final chapter (Chapter 7) summarises the results of the work described in the thesis and provides some insights for future studies based on the findings arising from this project.