Diagnostic and microsatellite studies of *Schistosoma japonicum* in humans and animal reservoir hosts

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患者及び保虫宿主を対象とした日本住血吸虫症診断法の開発と寄生虫マイクロサテライト解析に関する研究

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Definition of Terms

Population- refers to the individual *S. japonicum* adult worms obtained in a single study site

Migrants- are individual parasites which are assigned into different population

Residents- are individual parasites which are assigned in their respective population

Abbreviations

ELISA	enzyme-linked immunosorbent assay
SjTPx-1	*Schistosoma japonicum* thioredoxin peroxidase-1
PCR	polymerase chain reaction
SEA	soluble egg antigen
SWAP	soluble adult worm antigen
OD	optical density
BSA	bovine serum albumin
TPBS	Tween 20-phosphate buffered saline
MLGs	multilocus genotypes
AMOVA	analysis of molecular variance
PCoA	principal coordinate analysis
WHO	World Health Organization
COPT	circumoval precipitin test
IHA	indirect hemagglutination assay
DDIA	dipstick dye immunoassay
MDA	mass drug administration

General Introduction

1. Schistosomiasis

Schistosomiasis is a waterborne parasitic disease caused by the blood fluke under the genus *Schistosoma.* The disease affects almost 240 million people with more than 700 million are considered to be at risk of infection in 78 countries throughout the world (WHO, 2016). Five species of schistosomes are known to cause infection in man, namely *S. haematobium*, *S. mansoni*, *S. mekongi*, *S. intercalatum*, and *S. japonicum*. *S. haematobium*, *S. mansoni* and *S. intercalatum* are mostly seen in the African continent with the first two species also endemic in the Middle East. On the other hand, Asian schistosomiasis is caused by *S. mekongi*, exclusively seen in Cambodia and Laos and *S. japonicum* found in China, Indonesia and the Philippines. Among these species, *S. japonicum* is considered as the most virulent because the parasite species can produce larger number of eggs as compared to other species causing severe disease pathology in affected organs.

2. *Schistosoma japonicum*

S. japonicum is the only schistosome species considered to be zoonotic. This species infects over 46 species of wild and domestic animals with dogs and water buffaloes act as major reservoir hosts contributing significantly in increased disease transmission (He *et al*., 2001). Humans and animal hosts acquire the infection when they come in contact with freshwater contaminated with the cercarial stage of the parasite (Figure 1). The cercariae penetrate the skin of the host and later develop into schistosomes. The adult schistosomes live in the mesenteric veins, and the female worm release eggs which are passed out in the feces. Some of the eggs are trapped in the tissues causing damage to the organs. The snail species identified as the intermediate host in the Philippines is *Oncomelania hupensis quadrasi*. When parasite eggs are excreted from the infected host and in contact with water, the miracidia emerge and infect the snail host. Inside the snail, the miracidia undergo asexual development and later develop into cercariae, keeping the parasite's cycle going (Figure 1).

3. Diagnosis

Detection of parasite eggs in stool samples remains to be the gold standard in diagnosing *S. japonicum* infection. The simple and relatively cheap Kato-Katz method is recommended by the World Health Organization (WHO) which require 40 to 50 mg feces. However, this technique is time consuming, require a skilled microscopist and has low sensitivity in areas with low intensity of infection. On the other hand, efforts have been made to increase its sensitivity by multiple stool examination and increased slide preparation. Sedimentation and concentration techniques such as the Formalin Ether Concentration Technique had also been used and may increase the diagnostic yield of patients with light infection. Furthermore, the highly specific and sensitive polymerase chain reaction (PCR) based assays have been developed for the detection of schistosome DNA in stool samples. However, PCR requires expensive equipment and reagents making this test not suitable in field studies which deals with a large number of samples.

Indirect immunodiagnostic assays used for the detection of schistosome specific antibodies have become important over the past years. These tests include the circumoval precipitin test (COPT), indirect hemagglutination assay (IHA), enzyme-linked immunosorbent assay (ELISA) and dipstick dye immunoassay (DDIA) (Olveda *et al*., 2014). The conventional ELISA for schistosomiasis uses crude soluble

egg antigen (SEA), or crude soluble adult worm antigen preparation (SWAP). However, large scale production of these antigens, its low sensitivity and specificity, and cross-reaction with antibodies against other helminths are the major pitfalls of using diagnostic crude antigens. Thus, the use of recombinant antigens in the development of a more accurate and reliable tests might improve the current diagnostic formats. So far, several recombinant antigens have been identified to have good diagnostic potentials for *S. japonicum* infection (Angeles *et al*., 2011; Jin *et al*., 2010; Qian *et al*., 2011; Zhou *et al*., 2010).

4. Disease Control

Vaccine has not yet been developed thus the disease control solely relies on chemotherapy using the cheap and well tolerated drug Praziquantel. Mass Drug Administration (MDA) with praziquantel is the cornerstone of the national control program and the central strategy in the disease-endemic countries to control morbidity. MDA requires 85% target coverage based on the WHO standards. The treatment kills the adult worms preventing the disease to progress into the severe chronic phase. However, the drug is not capable of killing juvenile worms (e.g. schistosomula) making re-infection possible in endemic areas (Olveda *et a*l., 2014). A follow-up program for assessment of the infection and retreatment of patients is indispensable for morbidity

control. There is a need therefore to develop a tool to monitor the efficacy of MDA, hence curtailing its improvement. In addition, detection and control of the infection in the animal hosts is also required to ensure disease control. Thus, diagnostic means with high sensitivity and specificity that can be applicable for both humans and important animal reservoirs would be helpful in an effective disease surveillance.

5. Population genetics

Microsatellite markers have recently been used in determining *S. japonicum* genetic diversity among different geographical areas and mammalian hosts. Furthermore, the results of such studies might estimate the levels of gene flow among the population. Microsatellites are repeating sequences of 2-6 base pairs of DNA and their polymorphism is based on their variation in the number of repeats (Curtis *et al*., 2001; Shrivastava *et al*., 2005). Previous studies have recommended the use of microsatellite markers to determine schistosome genetic diversity because of their codominant expression and as neutral markers. The allele frequencies are equivalent under natural selection in all parasite life cycle stages (Curtis *et al*., 2000; Shrivastava *et al*., 2005). Studying the genetic variation of *S. japonicum* populations provides an opportunity to link some genotypes associated with disease prevalence which can then be used in formulating effective control measures. Using these markers, previous

studies suggested that the prevalence of infection tends to be closely related with parasite genetic variation. High prevalence of infection was observed in those areas with high genetic diversity and low prevalence in those areas with low genetic diversity (Aemero *et al*., 2015; Ezeh *et al*., 2015). These information make DNA microsatellites useful in population genetics studies.

Moreover, microsatellites have been used in monitoring the effects of praziquantel on schistosome populations especially on changes in genetic diversity. Praziquantel has been the drug solely used for the chemotherapy in mass drug administration (MDA) strategy (Norton *et al*., 2010). Reduction in the parasite's diversity may indicate that the population is less likely to survive in the selective pressure imposed. These may provide information on the impacts of MDA on parasite population and disease epidemiology.

6. Objectives of the present study

The general objective of this study is to develop and perform tools that will lead to a better understanding of the transmission epidemiology of *S. japonicum*. These tools include a uniform diagnostic test that can be used for a variety of mammalian hosts and microsatellite markers that will give information on the genetic diversity of the parasite. These are important in assessing and monitoring the success of control programs done against schistosomiasis. Thus, the specific objectives of this study are the following: (1) to develop and optimize cocktail-ELISA for *S. japonicum* diagnosis in humans, water buffaloes and dogs, (2) to characterize the genetic variation of *S. japonicum* using microsatellite markers in different areas in the Philippines.

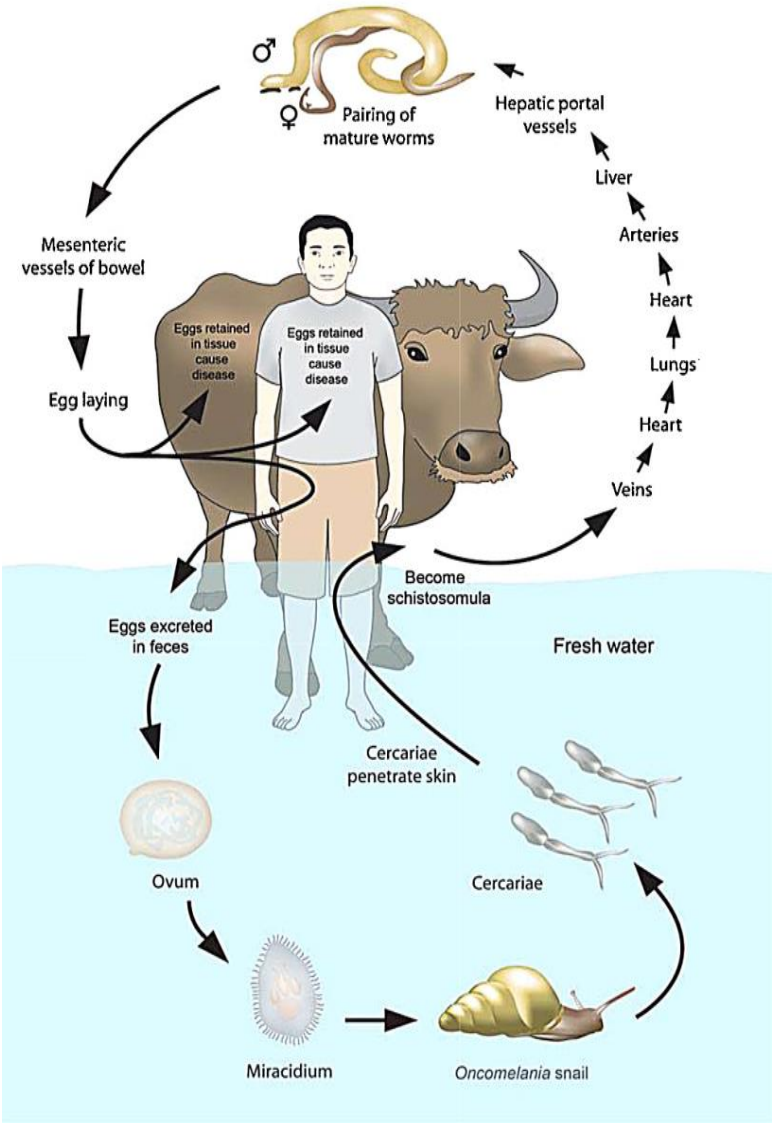

Figure 1. *S. japonicum* life cycle (Olveda *et al*., 2014)

Chapter I. Development and optimization of cocktail-ELISA for a unified surveillance of zoonotic schistosomiasis in multiple host species

1.1 Introduction

Generally, zoonotic transmission of schistosomiasis to humans depends largely on the presence of infected animal hosts which serve as carriers of infection. Such situation necessitates a unified surveillance to control zoonotic transmission across host species. Among the animal reservoir hosts for *Schistosoma japonicum*, water buffaloes and dogs were considered to be important in the zoonotic transmission of schistosomiasis. For instance in China, water buffaloes are known to account for a maximum of 99.1% of transmission of *S. japonicum* to humans as shown by mathematical modeling approaches (Gray *et al*., 2008). Water buffaloes have higher and constant exposure to schistosomiasis from their use as work animals in endemic areas. Furthermore, the huge volume of feces that these animals excrete everyday contributes substantially to contamination of the environment and subsequent transmission of the disease (Gray *et al*., 2007).

Meanwhile, previous studies show that human infection is strongly correlated with the intensity of *S. japonicum* infection in dogs in the Philippines (McGarvey *et al*., 2006). Such correlation is further supported by a population genetics study suggesting

strongly the high levels of transmission between humans and dogs (Rudge *et al*., 2008).

The zoonotic nature of *S. japonicum* therefore suggests the need for simultaneous testing of samples from different host species. This will be useful especially in large scale screening as it enhances the speed and cost effectiveness of the test in the effective control and management of zoonotic schistosomiasis. Enzyme-linked immunosorbent assay (ELISA) has been shown to be more sensitive than stool microscopy and cheaper than polymerase chain reaction (PCR) as the latter requires expensive equipment and reagents. However, conventional ELISA detection used for schistosomiasis is based on crude antigen like soluble egg antigen (SEA) and soluble adult worm antigen (SWAP). These crude antigens may detect the infection in many host species but possess very inferior specificity and sensitivity (Angeles *et al*., 2012; Doenhoff *et al*., 2004). Recombinant antigens are more promising with higher sensitivity compared to crude antigen-based ELISA. Several antigens have been identified to have good diagnostic potentials individually for *S. japonicum* infection (Cheng *et al*., 2007; Jin *et al*., 2010; Peng *et al*., 2008; Qian *et al*., 2011; Zhou *et al*., 2009; Zhou *et al*., 2010).

We have recently identified thioredoxin peroxidase-1 (SjTPx-1) and tandem repeat proteins (Sj1TR, Sj7TR) as promising diagnostic antigens in humans (Angeles *et*

al., 2011), water buffaloes (Angeles *et al*., 2012) and dogs (Angeles *et al*., unpublished data). In spite of the potential of the individual antigens, they had not been optimized for use in a multiple host species diagnostic test. With dogs and water buffaloes identified as significant reservoirs contributing to *S. japonicum* transmission, this study aims to develop an ELISA system simultaneously applicable for these animals as well as humans.

Multiple antigens, in contrast with single defined antigen have been proven to improve diagnostic capabilities in parasitic diseases such as malaria (Kim *et al*., 2003), leishmaniasis (Houghton *et al*., 2000) and clonorchiasis (Li *et al*., 2011) as well as bacterial infection (Hara *et al*., 2013; Houghton *et al*., 2002). Thus, the present study aimed to develop and optimize cocktail-ELISA by combining the recombinant *S. japonicum* antigens, SjTPx-1, Sj7TR and Sj1TR.

1.2 Materials and methods

Sera. Archived sera from *S. japonicum* infected humans (n=44), water buffaloes (n=25) and dogs (n=15) were used to evaluate the immunodiagnostic potential of the cocktail antigens. These samples were collected from schistosomiasis endemic areas in Negros Occidental, Mindoro Occidental, Leyte, and Northern Samar provinces in the Philippines and have been used in previous studies and were confirmed positive for

schistosome infection using either microscopy or stool PCR (Angeles *et al*., 2011; Angeles *et al*., 2012; Nara *et al*., 2007). *S. japonicum* negative sera from the three host species also obtained from the same schistosomiasis endemic areas were also tested with the cocktail-ELISA. Cross-reactions for other parasitic infections were already tested for each individual antigen in previous studies (Angeles *et al*., 2011; Angeles *et al*., 2012). Among the recombinant antigens, SjTPx-1 showed a very minimal cross reaction with *Opisthorchis viverrini* samples, while none for the tandem repeat proteins.

ELISA. Different combinations of recombinant proteins, i.e., SjTPx-1/Sj7TR/Sj1TR, SjTPx-1/Sj7TR, SjTPx-1/Sj1TR and Sj7TR/Sj1TR were used to coat the 96-well ELISA plates overnight at 4°C. These proteins are mixed in equivalent molar concentrations such that 200 ng SjTPx-1 and Sj1TR, and 400 ng of Sj7TR in 100 µl of carbonate/bicarbonate buffer at pH 9.0 were used per well for coating. Blocking was done using 1% bovine serum albumin in phosphate buffered saline with 0.05% Tween 20 (1%-BSA-TPBS) for 5 minutes. The human sera were diluted 400-fold while 200-fold dilutions were done for the animal sera in 1%-BSA-TPBS before loading into the coated plates. Although horseradish peroxidase-conjugated protein G was initially used as the conjugate for all the sera, good results were not obtained for the canine samples (data not shown). Thus, I resorted to using the species-specific secondary

antibody anti-canine IgG for the dogs. Protein G was diluted 1:10,000 whereas anti-canine IgG was used in 1:30,000 dilution. After the addition of the secondary antibody, the plates were incubated for 1 hour. The plates were then washed after incubation and 100 µl of substrate 3,3',5,5'-tetramethylbenzidine (KPL, Gaithersberg, MD, USA) was added and 50 µl of 1M phosphoric acid was used to stop the reaction. Optical density (OD) values were read at 450 nm using an ELISA plate reader (MTP-500, Corona Electric, Tokyo, Japan).

For each host species serum sample, the assay was done in triplicates and the mean OD values were calculated as presented in Figure 2. Cut-off values for each host species were computed as the mean absorbance values of the *S. japonicum* negative sera plus 3 standard deviations using a panel of sera collected from endemic areas in the Philippines (humans, n=44; water buffaloes, n=28; dogs, n=30). A sample was considered positive when the mean absorbance value was higher than the cut-off value.

Statistical analysis. Sensitivity, specificity, predictive values and kappa values were calculated using the statistics software GraphPad available online to evaluate the validity of the assay results (http://graphpad.com/quickcalcs/contingency1.cfm).

1.3 Results

Cocktail-ELISA results demonstrated that among the combinations tested, SjTPx-1/Sj7TR/Sj1TR detected the highest number of positive in the three host species (Figure 2). Statistical analysis as shown in Table 1 presented that this combination also has the highest sensitivity (84.1% in humans, 80% in water buffaloes and dogs) and specificity (100% in all host species). Interestingly, this was followed by different combinations in different host species namely Sj7TR/Sj1TR (63.6% sensitivity, 100% specificity) in humans, SjTPx-1/Sj1TR (72% sensitivity, 100% specificity) in water buffaloes and SjTPx-1/Sj1TR (53.3% sensitivity, 100% specificity) in dogs.

1.4 Discussion

Combination of Sj7TR/Sj1TR was shown to be better than that of SjTPx-1/Sj7TR contradicting the previous individual assessment that SjTPx-1 is better than Sj1TR in human diagnosis. Furthermore, the diagnostic potentials obtained by mixing two antigens do not perform as good as when they were used individually and when the three antigens were mixed together. Although the reason behind these contradicting results produced by using different number of antigens is unknown, it could possibly be due to the varying degree of antigen competition (Hara *et al*., 2013; Lyashchenko *et al*., 2000). Cocktail-ELISA containing the combination of

SjTPx-1/Sj7TR/Sj1TR resulted in improved specificities, positive predictive values and kappa values in each host species. Results of this study indicate the potential of the optimized cocktail-ELISA used in the development of a common diagnostic tool that will improve the surveillance for zoonotic schistosomiasis in multiple host species. This will also be an important step towards developing serodiagnostic test for zoonotic schistosomiasis in other significant host species of *S. japonicum* beside humans, water buffaloes and dogs. The necessity of using more number of samples in each host species possibly with a wider geographical scope including China is deemed important for further diagnostic evaluation of these cocktail antigens prior to their application in schistosomiasis surveillance programs.

1.5 Summary

The zoonotic characteristic of the human parasite *Schistosoma japonicum* infecting a significant number of wild and domestic animals highlights the need to develop a unified surveillance in multiple host species for a strengthened schistosomiasis control. Recombinant antigens like thioredoxin peroxidase-1 (SjTPx-1) and tandem repeat proteins (Sj1TR, Sj7TR) have been shown to be good diagnostic antigens individually in humans, water buffaloes and dogs in previous studies. Mixing these antigens together in a cocktail-ELISA might not only improve their diagnostic

potentials but rather produce a multi-host species detection means for zoonotic schistosomiasis. In this study, I aimed to develop and optimize cocktail-ELISA by testing different combinations of these recombinant antigens in humans, water buffaloes and dogs. Using samples collected from various endemic areas in the Philippines, results showed that the combination of SjTPx-1/Sj7TR/Sj1TR has the best results in identifying positive samples in the three host species. As compared with the diagnostic potential calculated for each of the three recombinant antigens used, their combination has presented improved specificities, positive predictive values and kappa values. This study therefore suggests the use of cocktail-ELISA in improving the zoonotic surveillance in schistosomiasis endemic areas.

Table 1

Diagnostic performance of cocktail-ELISA in humans, water buffaloes and dogs samples

Cocktail antigens	Sensitivity (%)	Specificity (%)	PPV (%)	NPV %	Kappa
Humans					
A.SjTPx-1/Sj7TR/Sj1TR	84.1	100	100	86.8	0.844
B.SjTPx-1/Sj7TR	59.1	100	100	71.9	0.596
C.SjTPx-1/Sj1TR	50.0	100	100	67.6	0.505
D.Sj7TR/Sj1TR	63.6	100	100	74.2	0.641
Water buffaloes					
A.SjTPx-1/Sj7TR/Sj1TR	80.0	100	100	84.8	0.809
B.SjTPx-1/Sj7TR	56.0	100	100	71.8	0.574
C.SjTPx-1/Sj1TR	72.0	100	100	80.0	0.731
D.Sj7TR/Sj1TR	56.0	100	100	71.8	0.574
Dogs					
A.SjTPx-1/Sj7TR/Sj1TR	80.0	100	100	90.9	0.842
B.SjTPx-1/Sj7TR	40.0	100	100	76.9	0.471
C.SjTPx-1/Sj1TR	53.3	100	100	81.1	0.604
D.Sj7TR/Sj1TR	40.0	100	100	76.9	0.471

PPV positive predictive values, *NPV* negative predictive values

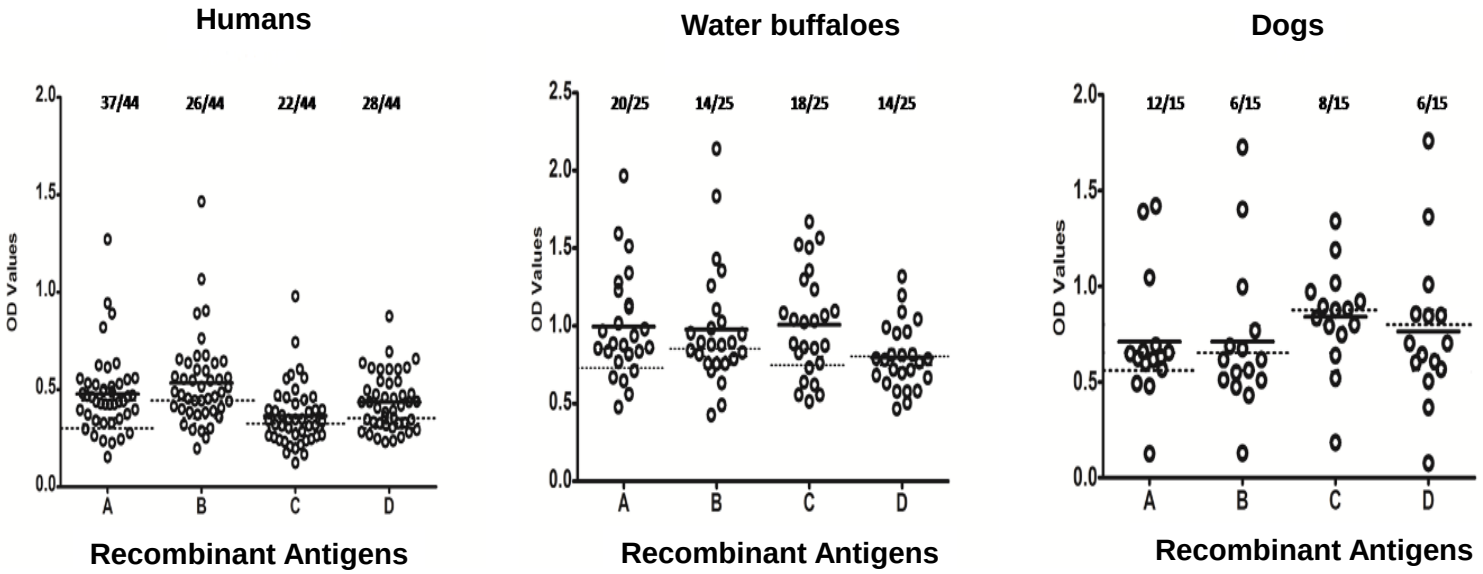

Figure 2. Cocktail-ELISA in humans, water buffaloes and dogs samples using the following cocktail-antigens (A) SjTPx-1/Sj7TR/Sj1TR (B) SjTPx-1/Sj7TR (C) SjTPx-1/Sj1TR (D) Sj7TR/Sj1TR**.** The graph shows that the combination of SjTPx-1/Sj7TR/Sj1TR has the highest number of positives in each host species. The dotted lines represent the cut-off values whereas the solid line for the mean optical density values.

Chapter II. Genetic diversity of *Schistosoma japonicum* in the Philippines using microsatellite markers

2.1 Introduction

Microsatellite markers have recently been used in determining *Schistosoma japonicum* genetic diversity in the population. Previous studies have recommended the use of microsatellite markers to determine schistosome genetic diversity because of their codominant expression and their ability to serve as neutral markers (Curtis *et al*., 2000; Shrivastava *et al*., 2005). The easy observation of heterozygosity and the reasonable number of alleles per polymorphic locus in the samples make microsatellite analysis a more powerful tool in genetic studies than the use of rapid amplified polymorphic DNA and mitochondrial DNA (Saeed *et al*., 2016).

Studying the genetic variation of *S. japonicum* populations provides an opportunity to link some genotypes associated with disease prevalence which can then be used in formulating effective control measures. Previous studies using microsatellite markers suggested that the prevalence of *S. haematobium* and *S. mansoni* infections could be closely related with the parasite's genetic variation (Aemero *et al*., 2015; Ezeh *et al*., 2015). High prevalence of infection was observed in those areas with high genetic diversity and low prevalence in those areas with low genetic diversity (Aemero *et al*.,

2015; Ezeh *et al*., 2015). This information makes DNA microsatellites useful in population genetics studies.

In addition, microsatellite markers had also been used in the identification of different geographical strains of *S. japonicum* in China (Shrivastava *et al*., 2005). These markers had also been utilized for assessing the efficacy of mass drug administration (MDA) with praziquantel in China by comparing the genetic diversity of *S. japonicum* population before and after the treatment program. The allelic richness was compared in the parasite populations between two time periods, however there was no obvious change in allelic richness, suggesting that the effect of drug treatment on *S. japonicum* genetic diversity may take more time to become apparent in the population (Yin *et al*., 2016). Hence, the information obtained on the parasite genetic diversity are useful in planning of disease control strategies.

In the Philippines, schistosomiasis has shown considerable variations in the intensity and prevalence of the disease from different endemic areas (Leonardo *et al*., 2012). However, no comparative study done so far on the population genetics of the schistosome parasites distributed in each endemic area in the Philippines. The parasite population in each endemic area was therefore characterized for their genetic backgrounds using the microsatellite markers. The information found in this study can

therefore provide basic information in the population genetics structure of *S. japonicum* in the Philippines that can be applicable to evaluate and modify the widely used current control strategies for human schistosomiasis.

2.2 Materials and methods

Ethics statement. The procedures involving animals were carried out in accordance with the guidelines from the University of the Philippines Manila Institutional Animal Care and Use Committee.

Parasite samples. The snail intermediate host, *Oncomelania hupensis quadrasi* were collected from seven municipalities (in seven provinces) in 2013 to 2015 where the disease is endemic, namely Catarman (in Northern Samar), Gonzaga (in Cagayan Province), New Corella (in Davao del Norte), Irosin (in Sorsogon), Talibon (in Bohol), Alang-Alang (in Leyte) and Socorro (in Oriental Mindoro) (Fig. 1). The snails were crushed to examine the presence of cercariae. The cercariae were then pooled separately for each endemic municipality for mice infection. Ten BALB/c mice were infected percutaneously with 50 cercariae from each municipality. The infected mice were sacrificed six weeks after the infection, and the adult worms were collected from their mesenteric veins and washed with saline for DNA extraction.

DNA extraction. Genomic DNA was extracted from individual male and female adult worms using the DNeasy Blood & Tissue Kit (QIAGEN, Japan) following the manufacturers' protocol. Table 2 showed the total number of DNA samples tested in each endemic municipality.

PCR amplification. PCR amplifications were performed using Veriti 96- Well Thermal Cycler (Applied Biosystems, Carlsbad, CA, USA). Amplifications were performed in 10 µl reactions containing 1 µl of PCR buffer, 0.4 µl of 1.5 mM $MgCl_2$, 0.2 µl of 2.5 mM dNTP, 0.2 µl of each 10 pmol/µl primer, 0.1 µl of 5 U/µl Taq DNA polymerase (Takara, Otsu, Japan), and 1 µl of template. The conditions for thermal cycling were as follows: 5 minutes at 94°C, followed by 30 cycles of 1 minute at 94°C, 1 minute at locus-specific temperature, 1 minute at 72°C, with a final extension at 72°C for 10 minutes (Shrivastava *et al*., 2003).

Microsatellite genotyping. The DNA of each individual *S. japonicum* worm was genotyped using the previously characterized microsatellite loci RRPS, M5A, TS2, MPA, 2AAA, J5, SJP1, SJP5, SJP6, and SJP9 (Shrivastava *et al*., 2003; Yin *et al*., 2008). The 5' end of the forward primer for each locus was fluorescently labeled with 6-FAM, VIC and Ned dyes. Different dyes were used for those loci with overlapping fragment size. Two µl of the PCR product with LIZ 600 labeled size standard (Applied

Biosystems) was subjected on the 3500 ABI Prism Genetic Analyzer for fragment analysis assay. The allele sizes were determined using the Gene Mapper software version 4.0 (Applied Biosystems). In each run, *S. japonicum* sample from Gonzaga, Cagayan which has good DNA volume and concentration served as the reference genotype for which the microsatellite sizes for the 10 loci had been determined by sequencing. *S. japonicum* Yamanashi strain (Japanese isolate) was also genotyped as a control group to confirm that the microsatellite markers could differentiate between samples from different origin. A total of 201 DNA samples were tested, however only 186 were successfully genotyped due to poor DNA quality.

Data analysis. For each population, the genetic diversity was examined by calculating the number of alleles using rarefaction analysis. Expected heterozygosity (gene diversity) (He) and observed heterozygosity (Ho) were determined using the GenAlEx 6.5 software (Peakall *et al*., 2012). Rarefaction analysis was performed to make the alleles comparable in the population. Genetic differentiation was determined using Wright's F-statistics (Fst) in Arlequin, and the significance of the Fst values was tested at p value <0.05 (Excoffier *et al*., 2015). The following qualitative guidelines were used for the interpretation of Fst genetic differentiation: 0-0.05 (little), 0.05-0.15 (moderate), 0.15-0.25 (great), and >0.25 indicate (very great genetic differentiation)

(Shrivastava *et al.,* 2005). The Analysis of Molecular Variance (AMOVA) was used to partition the genetic variation within and among populations using the software Arlequin version 3.5. The inbreeding coefficient (F_{IS}) which measures the extent of nonrandom mating was computed in the study. Nonrandom mating occurs when there is inbreeding. Inbreeding increases the homozygosity of the alleles.

2.3 Results

Genetic diversity. A total of 186 individual *S. japonicum* worms collected from seven endemic municipalities were analyzed. Highest gene diversity indices (He) was observed in Catarman (0.727) followed by Irosin (0.694), Socorro (0.677), and Gonzaga (0.605) while the lowest in Alang-Alang (0.495). Meanwhile, those from New Corella (0.587) and Talibon (0.566) were comparable. Similarly, allelic richness after sample size correction was highest in Irosin (4.630), Catarman (4.500) and Socorro (4.280), while the lowest in Talibon (2.920) and Alang-Alang (2.570) (Table 2).

Population-specific inbreeding coefficient was determined in this study to measure the extent of nonrandom mating. Highest inbreeding coefficient values (F_{IS}) was observed in Irosin (0.239) while the lowest in Catarman (0.012) (Table 2). The lowest inbreeding coefficient values in Catarman may be related to the increased heterozygosity in this area. Inbreeding increases the homozygosity of the alleles. The

pairwise F_{ST} values ranged from 0.019 to 0.0188, indicating varied levels of pairwise population genetic differentiation (Table 3). Great genetic differentiation was observed in the New Corella samples. The AMOVA showed that greater genetic variation in the samples occurred within the population (91.95%) rather than among populations (8.05%) (Table 4).

2.4 Discussion

Large number of different alleles have been observed in the samples examined, especially in Irosin, Catarman and Socorro were high prevalence of infections were reported (Leonardo *et al*., 2012) (Table 5). The presence of large number of different alleles in these areas indicate that there is a greater potential for these population to possess the alleles responsible for the parasites infectivity causing high infection (Aemero *et al*., 2015; Glenn et al., 2013). These findings were in agreement with that of previous studies where the prevalence of infection was directly proportional to the number of alleles (Aemero *et al*., 2015; Ezeh *et al*., 2015; Glenn *et al*., 2013). This situation somehow follows a general pattern in our current study where high prevalence of infection either in humans and snail hosts were observed in those areas with high allelic richness while low or zero prevalence in those areas with low allele numbers (Table 5). On the other hand, a low number of different alleles have also been observed

in the samples in Alang-Alang where high prevalence of the infection was reported. This could be due to the prolonged utilization of praziquantel from the annual MDA since Leyte has been one of the oldest endemic foci in the Philippines. It has been known in other parasitic infections such as malaria that selective drug pressure brought by extensive drug use can lead to a reduction in genetic diversity of the parasite (Mendes *et al*., 2013; Razak *et al*., 2016). Furthermore, alleles that might contribute to the high infection rate might be present in those area with high prevalence, however further studies should be done to confirm this. Currently, there are no microsatellite markers linked with parasites infectivity.

In this study, it has to be noted that the prevalence data presented in Table 5 was collected from 2013 to 2015, and our samples obtained from Talibon (Bohol) was collected prior to this period. Bohol is considered as a near elimination area based on the absence of human cases for many years now. However, the presence of infection in snails and water buffaloes continues to indicate an ongoing transmission even if there are no more human cases (Angeles *et al*., unpublished data). Hence the possibility of human infection is always present.

Among the seven endemic municipalities analyzed, the Catarman samples showed the highest gene diversity indices (Table 2). The results of the study are reasonable to conclude that the genetic diversity could be one possible criterion to assess the infection status in Catarman, and that there is a greater potential for such population to possess the alleles that may be responsible for the parasites infectivity and pathogenicity causing high prevalence of infection (Agola *et al*., 2009). The high genetic diversity in Catarman may also be explained by the lowest inbreeding coefficient values. Inbreeding increases the similarity of the alleles in the parasite population. Lowest inbreeding coefficient values increases the heterozygosity of the alleles. Previous studies suggested that co-infection by multiple genotypes decreases the possibility of inbreeding (Karvonen *et al*., 2012). Furthermore, Catarman has been reported with high prevalence of infection both in humans and snail intermediate hosts (Leonardo *et al*., unpublished data, Table 5). In addition, this study also revealed that water buffaloes and dogs in this municipality had high prevalence of infection (Angeles *et al*., unpublished data). High infection rate in humans and animal hosts will then increase the probability of snail infection. Therefore, the role of animal reservoir hosts should therefore be seriously considered in the increased disease transmission (Angeles *et al*., 2012; Gray *et al*., 2007). Genetic surveillance using microsatellite markers with

the parasite samples collected from reservoir animals should also be conducted in future studies.

The level of genetic differentiation differ between endemic areas. Great genetic differentiations were observed in the New Corella samples than those from other endemic sites (Table 3). The large geographical distance separating New Corella from other endemic sites could possibly limit the contact between the hosts eventually resulting to high genetic differentiation in this municipality (Figure 3). New Corella is located in Southern Mindanao and is expected to be more genetically differentiated because of its geographic location. Previous studies showed that the high genetic differentiation observed among peripheral populations such as those of New Corella can be explained by their strong spatial isolation (Wagner *et al*., 2011).

Furthermore, the possibility of the snail hosts influencing the genetic variation of the parasite population should also be taken into consideration. Currently, there is no study using microsatellites on the genetic variation of the snail population in the sampling areas. Previous studies suggested that co-evolutionary relationships and close genetic interactions between the snails and their parasites occur, thus a snail population may reflect the population genetic parameters of their parasites (Saijuntha *et al*., 2014; Webster *et al*., 2007). Therefore, future studies on the snail host population should

therefore be done to obtain results which can be analyzed together with those of *S. japonicum* population.

The genetic variation observed using AMOVA was greater within each *S. japonicum* population (91.95%) than the variation among the populations (8.05%) (Table 4). This might be due to the snails being infected by genetically different cercariae having multiple genotypes within the endemic areas (Aemero *et al*., 2015; Agola *et al*., 2009). Mixing of infected snails and of their parasites brought about by flooding, may explain the higher genetic variation within the population (Shrivastava *et al*., 2005; Yin *et al*., 2016). Also, the continuous rainfall and subsequent floods in these endemic areas might facilitate host-parasite contact exposing people and animals to contaminated waters resulting to high infection. Thus, people and animals moving from one village to another because of flooding could facilitate parasite transmission contributing to high genetic variations within each endemic area. Another reason could be due to the snail sampling being done in three villages for each endemic municipality. There might also be a high village-level variation in each municipality. Genetic variance within population was accounted for most of the genetic diversity of *S. japonicum* population in endemic provinces in China (Shrivastava *et al*., 2005). A more in-depth characterization of the genetic variation within the population of *S. japonicum* from

different endemic areas in the Philippines should be done.

Genetic diversity found in this study among the parasite population in each endemic site in the Philippines is vital in the parasites' ability to survive the effect of selective pressures such as those brought by drug treatment. On the other hand, selection pressures increase the frequency of favorable alleles across all populations (Curtis *et al*., 2000). In this sense, the present finding of high diversity among the parasite populations imply that the MDA with praziquantel has varying degree of impact in interrupting the parasite's life cycle. Although treatment failures have just been reported for *S. haematobium* in Africa, this should also be anticipated for *S. japonicum* (Alonzo *et al*., 2006; Silva *et al*., 2005). The patients did not respond to two single oral doses (40 mg/kg) of PZQ for *S. haematobium* (Alonzo *et al*., 2006; Silva *et al*., 2005). The problem arises when the drug is administered in two separate doses without assurance that the second dose will be taken by the MDA participants when they reach home. Aside from looking at several factors that can contribute to possible treatment failures including low compliance and the quality of the drugs used, it is also important that the effects of MDA be monitored on schistosome populations for its genetic background. This can be done by using microsatellite markers to measure the genetic diversity parameters which include the allelic richness and the heterozygosity of

the alleles in the parasite population before and after MDA implementation. Alleles contributing to the severity of the disease such as the ones responsible for the fecundity and survival of *S. japonicum* inside the host should be identified and need to be further studied. It is also recommended that the genetic diversity in the snail population in the Philippines should be studied to evaluate its effects on the genetic diversity of the parasite.

2.5 Summary

Microsatellites have been found to be useful in determining genetic diversities of various medically-important parasites leading to an effective disease management and control programs. In China, the presence of different geographical strains of *Schistosoma japonicum* as determined through microsatellites could pave the way for a better understanding of the transmission epidemiology of the parasite. Thus, the present study aims to apply the microsatellite markers in analyzing the populations of *S. japonicum* from different endemic areas in the Philippines for possible strain differentiation. Experimental mice were infected using the cercariae of *S. japonicum* collected from infected *Oncomelania* snails in seven endemic municipalities. Adult worms were harvested from infected mice after 45 days of infection and their DNA analyzed against ten previously characterized microsatellite loci. The degree of genetic

differentiation of the parasite population between endemic areas vary. Geographical separation was considered as one of the factors accounting for the observed difference between populations. Large number of different alleles and high genetic diversity have been observed in the samples examined, especially in the highly endemic Irosin, Catarman and Socorro. The results of the study are reasonable to conclude that genetic diversity could be one possible criterion to assess the infection status in highly endemic areas. There could be a greater potential for such population to harbor the alleles which confer parasites infectivity causing high prevalence of infection. The responsible alleles for the infectivity of the parasite should be studied further. Genetic surveillance using microsatellites is therefore important to predict the ongoing gene flow and degree of genetic diversity which indirectly reflects the success of the control program in schistosomiasis-endemic areas.

Table 2

Population genetic analysis of *S. japonicum* from seven endemic municipalities in the Philippines

Population	Sample size	A[a]	(A)[b]	Gene diversity (He) [c]	Ho [d]	F_{IS} [e]
Catarman (Northern Samar)	32	8.300	4.500	0.727	0.842	0.012
Gonzaga (Cagayan)	21	6.700	3.800	0.605	0.646	0.161
New Corella (Davao del Norte)	25	5.000	3.460	0.587	0.750	-0.176
Irosin (Sorsogon)	45	11.500	4.630	0.694	0.725	0.239
Talibon (Bohol)	25	3.800	2.920	0.566	0.681	0.197
Alang-Alang (Leyte)	16	2.900	2.570	0.495	0.811	0.082
Socorro (Mindoro)	22	6.800	4.280	0.677	0.844	0.059

[a] mean number of alleles, [b] mean number of alleles corrected for sample size,

[c] gene diversity indices (expected heterozygosity), [d] observed heterozygosity,

[e] population-specific inbreeding coefficient values

Table 3

Pairwise genetic differentiation (F_{ST}) among populations based on 10 microsatellite loci [a]

Population	Catarman	Gonzaga	New Corella	Irosin	Talibon	Alang-Alang	Socorro
Catarman							
Gonzaga	0.093						
New Corella	0.141	0.168					
Irosin	0.060	0.068	0.145				
Talibon	0.081	0.062	0.162	0.042			
Alang-Alang	0.084	0.113	0.188	0.028	0.057		
Socorro	0.071	0.081	0.156	0.019	0.058	0.049	

[a] All p values are significant below the level of 0.05 based on 100 permutations.

Table 4

Analysis of molecular variance (AMOVA) for *S. japonicum* population from different endemic municipalities

Source of variation	Degree of freedom	Sum of squares	Variance components	Percentage of variation % [a]
Among population	6	118.129	0.33814	8.05
Within populations	331	1277.708	3.86014	91.95

[a] Significance test (1023 permutations), p value <0.001

Table 5

Prevalence of *S. japonicum* infection in humans and snail hosts from different endemic areas in the Philippines (2013-2015) [a]

Endemic areas	Snail infection rate % (N)	Human prevalence rate % (N) [b]
Socorro (Oriental Mindoro)	1.2 (500)	7.92 (442)
Alang- Alang (Leyte)	0.57 (1588)	7.10 (183)
Irosin (Sorsogon)	1.25 (1123)	6.73 (202)
Talibon (Bohol)	0 (300)	0 (143)
Gonzaga (Cagayan)	1.1 (1500)	1.66 (421)
New Corella (Davao del Norte)	0.5 (1810)	0 (187)
Catarman (Northern Samar)	12.77 (329)	10.06 (358)

[a] Data obtained from unpublished work by Leonardo *et al*., [b] determined by Kato-Katz technique

Figure 3**.** Study areas. The map shows the seven endemic municipalities in the Philippines included in this study. They are clustered into three major island groups Luzon, Visayas, and Mindanao.

Chapter III. Clustering and migrant detection analysis of *Schistosoma japonicum* using microsatellite markers

3.1 Introduction

Microsatellites have been used in estimating the levels of gene flow in schistosome population. Gene flow plays an important role in the dispersal of alleles from one population to another. These may increase the genetic distribution of alleles responsible for the parasites infectivity, and often leads to the spread of virulence in the parasite population (Gandon *et al*., 1996). Migration and movement of infected hosts may be responsible for gene flow between *Schistosoma japonicum* population. Therefore, knowing the parasite gene flow gives insights into the movement and persistence of alleles which might confer severe schistosome infection. These information may possibly help in the design of effective control strategies for schistosomiasis. Moreover, information on parasite gene flow is important in evaluating the efficacy of mass drug administration (MDA) as well as in monitoring of the parasite genotypes circulating in endemic areas.

In this chapter, clustering and migrant detection analysis using the data in chapter 2 were carried out to assess the gene flow of *S. japonicum* from different endemic areas. These were performed to determine the population structure of *S. japonicum* population.

3.2 Materials and methods

Microsatellite data. The data from chapter 2 was used in this study to perform clustering and migrant detection analysis of *S. japonicum* samples.

Data analysis. Principal Coordinate Analysis (PCoA) was done to determine the clustering pattern of *S. japonicum* population based on their genetic distance using GenAlEx 6.5. Cercariae derived from a snail infected with only a single miracidium is assumed to be genetically identical. Hence, duplicate multi locus genotypes in a population is a consequence of clonal replication within snails (Yin *et al*., 2008). Duplicate multilocus genotype (MLG) was therefore removed leaving a single representative of each in the dataset. The GENECLASS software 2.0 was used to identify migrant individuals (Piry *et al*., 2004).

To visualize relationships among populations, a Neighbor-joining tree was constructed based on Nei's genetic distance using 1000 bootstrap replications in POPTREE2 (Takezaki *et al*., 2014). The *S. japonicum* Yamanashi strain was used as an

outgroup.

3.3 Results

Population structure. The PCoA showed no particular geographical structuring among the *S. japonicum* populations (Fig. 4). The neighbor-joining tree method showed clustering of the samples into two groups. Populations from Catarman, New Corella, Gonzaga, Talibon and Irosin grouped together indicating high genetic similarity among them whereas populations from Alang-Alang and Socorro belong to a separate cluster. However, there was no correlation between the clustering of populations and their geographic distribution as shown in the neighbor joining tree (Fig. 5). These findings further support the results of the PCoA (Fig. 4) suggesting the existing gene flow in the population. The presence of two subgroups in Catarman (Northern Samar) may account for the high genetic variation within population (Table 1). Six samples namely 3 from Gonzaga, 2 from Irosin and 1 from New Corella were identified by GENECLASS as migrants (Table 6). These individuals showed a probability below 0.05.

3.4 Discussion

S. japonicum samples obtained from different endemic areas did not form a particular spatial structuring. The lack of geographical structuring suggests that there is still an ongoing gene flow among the *S. japonicum* populations in all the study areas despite execution of control measures (Agola *et al*., 2009). These findings might imply that there is a continuing transmission of *S. japonicum* across geographic areas, and therefore reflect the inadequate effect of MDA implementation. The current national control strategy for schistosomiasis in the Philippines is annual MDA using 40 mg/kg of praziquantel in all schistosomiasis-endemic villages including the sampling areas. However, the compliance was <50% (Ross *et al*., 2014; Tallo *et al*., 2008).

The ongoing gene flow in the population might be attributed to migration and movement of infected hosts as also suggested otherwise by previous studies done on *S. mansoni* (Agola *et al*., 2009). The infected hosts could therefore serve as means of allele dispersal in endemic sites. Therefore, the existence of gene flow among the schistosome populations might increase the opportunity for the spread of alleles conferring parasite traits such as infectivity, virulence and drug resistance (Agola *et al*., 2009; Ezeh *et al*., 2015).

Two subgroups were observed in the Catarman samples using the PCoA analysis (Fig. 4) indicating that co-infection with several genotypes of the parasite might be infecting the hosts in this endemic site. The high genetic variation within population (Table 4) may also be attributed to the presence of subgroups in the Catarman samples. Catarman is surrounded by other endemic areas so the possibility of intermixing of the parasite is very high leading to high genetic variation within the area. High prevalence of infection in humans and snail hosts was reported in Catarman (Table 5). A higher transmission and infection success is expected to occur more in mixed parasite genotypes than single-genotype infection as reported in previous studies (Karvonen *et al*., 2012). There might be a decrease in the effectiveness of the host immune system to cope up with the infection due to the simultaneous attack of the parasite with different genotypes leading to a higher infection success (Karvonen *et al*., 2012).

Some parasite populations in Gonzaga, New Corella and Irosin were identified as migrants using GeneClass 2.0 software (Table 6). Gonzaga has just been identified as a new endemic foci at the start of the 21st century (Leonardo *et al*., 2015) and it is presumed that some parasite populations are introduced in this area from Talibon and Catarman. Infected people or animals might have moved from these areas and started the disease transmission in Gonzaga. In the past, the theory proposed that there was a

big geothermal project by PNOC (Philippine National Oil Company) in Gonzaga that recruited workers from the Visayas and Mindanao (Figure 3). The movement of people from endemic areas into Gonzaga brought in cases, and the presence of snail hosts led to the emergence of disease (Leonardo *et al*., 2015). Interestingly, migrants detected in Irosin were rooted from Gonzaga which solidify the continuous transmission flow of the parasite among the endemic municipalities in the Philippines (Table 6). Migrant detection in the study supports the clustering analysis using the neighbor joining method wherein the population including those individuals that were considered to be migrants clustered together with their source population (Figure 5). However, there was no correlation between the clustering of populations and their geographic distribution as shown in the neighbor joining tree (Figure 5). These findings further support the results of the PCoA (Figure 4) suggesting the existing gene flow in the population.

3.5 Summary

The use of microsatellites in this study has shown that there is an ongoing gene flow among the *S. japonicum* population from different endemic areas indicating the active movement of infected humans and animals from one endemic area to another. An effective surveillance to monitoring these movements in humans and animals will involve both the medical and veterinary sectors. Thus, a better cooperation between these two sectors would be highly recommended to ensure a strengthened control program for schistosomiasis. In addition, the diversity will indirectly explain the varying degree of the effects of the ongoing control programs done in these endemic areas. A regular MDA should be implemented and monitored regularly for its efficacy in endemic areas. Genetic surveillance using microsatellites is therefore important to predict the ongoing gene flow and degree of genetic diversity which indirectly reflects the success of the control program in schistosomiasis-endemic areas.

Table 6

Results of migrant detection analysis performed in GENECLASS [a]

Sample (individuals)	GENECLASS migrant likelihood ratio (L_h/L_{max}) $p<0.05$	Catarman	Gonzaga	New Corella	Irosin	Talibon	Alang- Alang	Socorro
Gonzaga	2.378	**25.119**	27.497	28.232	26.467	25.486	30.796	28.012
Gonzaga	0.692	19.157	7.410	22.428	21.506	**6.718**	25.575	24.229
Gonzaga	1.052	15.990	5.134	18.335	16.199	**4.082**	21.891	18.183
New Corella	5.706	**15.072**	21.517	20.779	21.082	22.944	21.800	20.741
Irosin	1.850	18.679	**16.293**	23.914	18.143	18.931	28.000	26.432
Irosin	0.371	**15.691**	30.700	24.214	16.062	25.013	22.569	20.470

[a] The most likely source population for each individual is shown in bold.

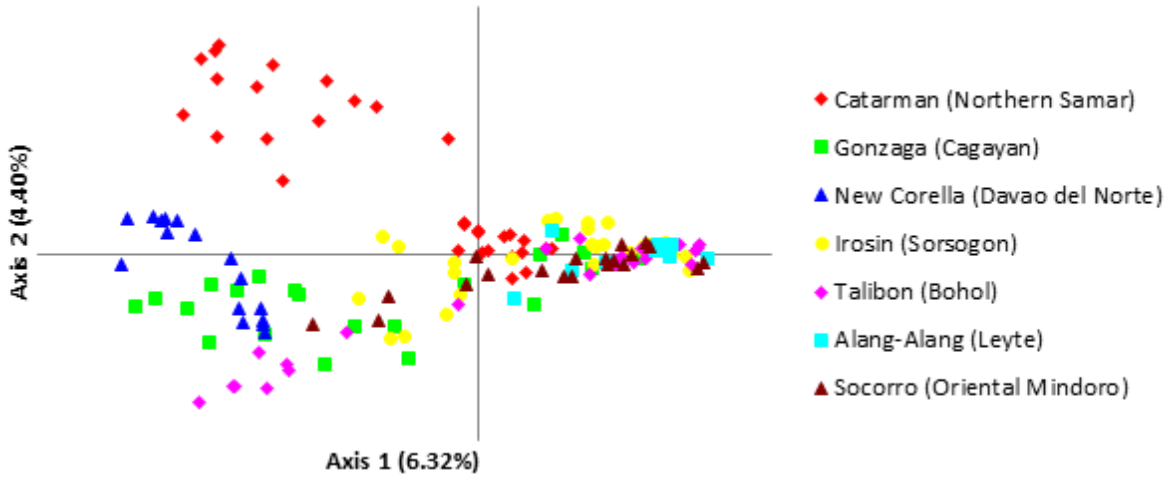

Figure 4. A two-dimensional plot of the Principal Coordinate Analysis (PCoA) of microsatellite data showing the clustering of *S. japonicum* populations after removal of duplicate genotypes. The proportion of variation is explained by each axis in parenthesis.

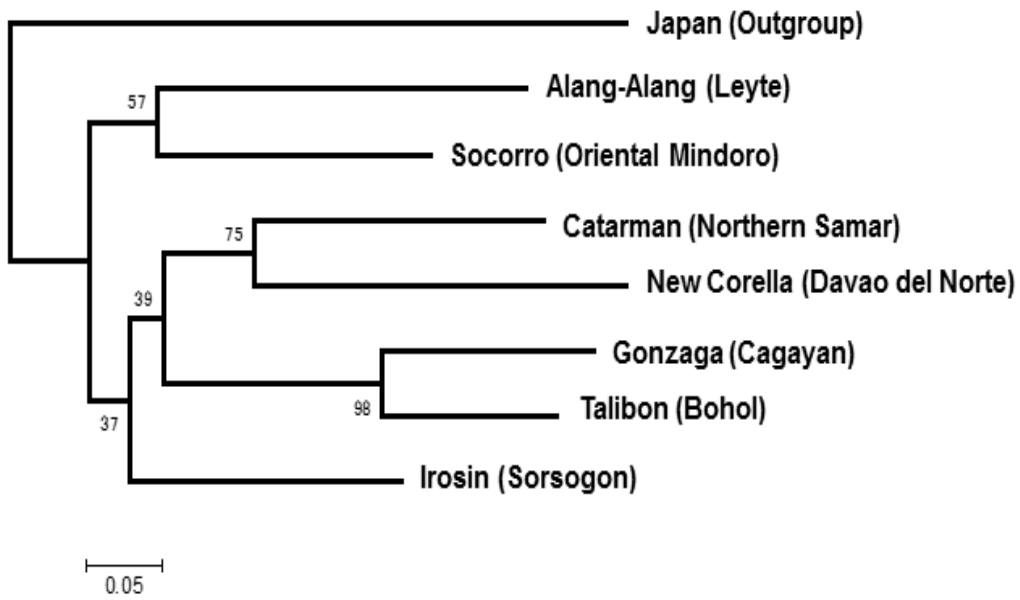

Figure 5. Neighbor joining tree showing the phylogenetic relationships of *S. japonicum* originating from each endemic site.

General Discussion

This study aims to develop and perform tools that will provide a better understanding of the transmission epidemiology of *S. japonicum*. The first chapter of this study focused on the development of a cocktail-ELISA for the diagnosis of *S. japonicum* infection in different host species. The zoonotic nature of *S. japonicum* has made the control of the disease difficult. Water buffaloes and dogs have been identified as important reservoirs in disease transmission. A uniform diagnostic test for the detection of zoonotic schistosomiasis in humans and important reservoir hosts will be helpful in the unified surveillance of the disease.

Taking advantage of the recombinant antigens thioredoxin peroxidase-1 (SjTPx-1) and tandem repeat proteins (Sj1TR, Sj7TR) previously tested and identified as good diagnostic antigens in humans (Angeles *et al*., 2011), water buffaloes (Angeles *et al*., 2012) and dogs (Angeles *et al*., unpublished data), these antigens were evaluated in different combinations to determine the diagnostic potentials of cocktail-ELISA. The use of multiple antigens have been proven to improve diagnostic capabilities in other parasitic diseases (Hara *et al*., 2013; Houghton *et al*., 2000; Houghton *et al*., 2002; Kim *et al*., 2003; Li *et al*., 2011). Results of this study has showed that combinations of SjTPx-1/ Sj7TR/ Sj1TR has the highest sensitivity in humans (84.1%), water buffaloes

(80%), and dogs (80%) and specificity (100%) in all host species. The use of these cocktail-antigens in serological tests will not just help improve the surveillance of zoonotic schistosomiasis but also stressed the importance of the neglected animal reservoirs.

The following two chapters of this study aimed to perform a microsatellite analysis to determine the presence of genetic diversity in the parasite in different areas in the Philippines. Characterization of *S. japonicum* strains in different endemic sites with varying prevalence will provide information on the genetic diversity of the parasite. The diversity will indirectly explain the varying degree of the effects of the ongoing control programs done in these endemic areas. Among the seven endemic municipalities in the Philippines analyzed, the Catarman samples has showed the highest genetic diversity. Two subgroups were observed suggesting that a mixed genotype of the parasite might be causing the infection in the area. It was reported that Catarman has high prevalence of infection both in humans and snail hosts (Leonardo *et al*., unpublished data, Table 5). A higher transmission and infection success is expected to occur more in mixed parasite strain than single-strain infection as reported in previous studies (Karvonen *et al*., 2012; Taylor *et al*., 1997). There might be a decrease in the effectiveness of the host immune system to cope up with the infection due to the

simultaneous attack of the parasite with different genotypes leading to a higher infection success (Karvonen *et al*., 2012).

In addition, a large number of different alleles have been observed in the samples examined, especially in Irosin, Catarman and Socorro (Table 2). These findings were in agreement with that of previous studies where the prevalence of infection was directly proportional to the number of alleles (Aemero *et al*., 2015; Ezeh *et al.*, 2015; Glenn *et al*., 2015). Thus, the prevalence of infection may be closely related with the genetic diversity of parasite populations (Aemero *et al*., 2015; Ezeh *et al*., 2015; Glenn *et al*., 2013). On the other hand, low number of different alleles have been observed in the samples in Alang-Alang where high prevalence of the infection was reported. This could be due to the prolonged use of praziquantel from the annual MDA since Leyte has been one of the oldest endemic foci in the Philippines. Alleles responsible for causing high infection rates might be present in those area with high prevalence, however further studies should be done to confirm this.

The analysis of molecular variance showed that the genetic variation was generally attributed to the variance within the population (91.95%). This might be due to the snails being infected by genetically different cercariae having multiple genotypes (Aemero *et al*., 2015; Yin *et al*., 2016). These results were also observed from previous

studies done on *S. japonicum* in China (Shrivastava *et al*., 2005; Yin *et al*., 2016). Thus, further characterization of the genetic variation within the population of the samples from different endemic areas should be done, as well as the genetic variability in its snail host species.

Furthermore, the absence of spatial structuring among the analyzed samples suggested that there is still an ongoing gene flow of the parasite throughout the endemic areas examined. The migration and movement of infected host occurring between endemic areas might be attributed for the ongoing gene flow in the population. Previous studies suggested that human movements can lead to significant gene flow in *S. mansoni* populations. Therefore, the higher gene flow among the schistosome populations might increase the opportunity for the spread of alleles conferring parasite traits such as infectivity, virulence and drug resistance (Agola *et al*., 2009; Ezeh *et al*., 2015). An effective surveillance to monitoring these movements in humans and animals will involve both the medical and veterinary sectors. Thus, a better cooperation between these two sectors would be highly recommended to ensure a strengthened control program for schistosomiasis. This will prevent emergence of the disease in new foci and re-emergence in areas already declared as schistosomiasis-free in the Philippines.

General Summary

Schistosomiasis is a waterborne parasitic disease caused by the blood fluke under the genus *Schistosoma.* The disease affects almost 240 million people with more than 700 million are considered to be at risk of infection in 78 countries throughout the world. Among the five species that can infect humans, *Schistosoma japonicum* is considered as the most virulent because of the larger number of eggs it can produce as compared to other schistosomes that cause severe disease pathology in affected organs. In addition, the zoonotic nature of *S. japonicum* contributes in increased disease transmission making the schistosomiasis control difficult. The general objective of this study is to develop and perform tools that will lead to a better understanding of the transmission epidemiology of *S. japonicum*. These tools include a uniform diagnostic test that can be used for a variety of mammalian hosts and microsatellite markers that will give information on the genetic diversity of the parasite. These are important in assessing and monitoring the success of control programs done against schistosomiasis.

The first chapter of this study focused on the development of a cocktail-ELISA for the diagnosis of *S. japonicum* infection in different host species. The zoonotic nature of *S. japonicum* has made the control of the disease difficult. Water buffaloes and dogs have been identified as important reservoirs in disease transmission. A uniform

diagnostic test for the detection of zoonotic schistosomiasis in humans and important reservoir hosts will be helpful in the unified surveillance of the disease. Therefore suitable combination of recombinant proteins that had already been evaluated as potential antigen for each host species was examined for its common use in humans and other animal species. Result of this study has showed that combination of three recombinant antigens, SjTPx-1/ Sj7TR/ Sj1TR has the highest sensitivity in humans (84.1%), water buffaloes (80%), and dogs (80%) and specificity (100%) in all host species. The use of these cocktail-antigens in serological tests will not just help improve the surveillance of zoonotic schistosomiasis but also stressed the importance of the neglected animal reservoirs.

The second chapter of this study aimed to perform a microsatellite analysis to determine the presence of genetic diversity in the parasite in different endemic areas to utilize the results for monitoring and modifying the disease control program. Characterization of *S. japonicum* population in different endemic sites in the Philippines with 10 microsatellite markers will provide information on the genetic diversity of the parasite. A high genetic diversity has been observed in the samples examined, especially those in the highly endemic municipalities of Irosin, Catarman and Socorro. There was a positive correlation between the genetic diversity in the parasite population and the

prevalence of the disease as previously reported in other countries where schistosomiasis is endemic.

The third chapter of this study aimed to determine the population structure of *S. japonicum* using the microsatellite information obtained in the previous chapter. Results showed that the parasite samples obtained from different endemic areas in the Philippines did not form a particular spatial structuring. The lack of geographical structuring suggests that there is still an ongoing gene flow among the *S. japonicum* populations in spite of extensive control measures. These findings might imply that there is a continuing transmission of *S. japonicum* across geographic areas, which might be attributed to the migration and movement of infected hosts. This information provides an idea that a safeguarding precaution should be implemented in these areas with high genetic diversity in the parasite population to ensure localized elimination of the disease.

In conclusion, this study has presented two important tools useful for a more effective disease surveillance for schistosomiasis. The cocktail-ELISA can be useful in formulating a unified disease surveillance among various mammalian hosts leading to a more wholistic approach in eliminating schistosomiasis. On the other hand, genetic surveillance using microsatellites is important to predict the disease outcome and the

success of control programs in schistosomiasis-endemic areas.

要　約

住血吸虫症は *Schistosoma* 属吸虫の寄生を原因とする水系感染症である。ヒトをはじめとする哺乳類宿主（終宿主）への感染源は，中間宿主となる水棲巻き貝から放出されるセルカリアで，この寄生虫ステージが農作業や水浴などで水中に暴露された手足から経皮感染する。虫卵を含む終宿主の糞便あるいは尿が，中間宿主貝が棲息する水系に排泄されると，虫卵からふ化したミラシジウムが貝に感染して，寄生虫の生活環が一周する。住血吸虫症は世界 78 ヶ国で流行が認められ，3 億 4000 万人がこの寄生虫病に感染し，7 億人がその感染のリスクの基に日々の生活を送っている。住血吸虫症の患者は，血管内に寄生する成虫が産卵する虫卵が，肝臓をはじめとする様々な臓器に栓塞して虫卵結節を形成することで，肝硬変をはじめとする様々な症状を呈する。ヒトに感染する住血吸虫は 5 種知られているが，このうち *Schistosoma japonicum*（日本住血吸虫）は成虫の産卵数が最も多く，従って病原性も最も強い。また，日本住血吸虫はヒト以外の哺乳類宿主（保虫宿主）にも感染する人獣共通感染症で，これがこの寄生虫病の感染拡大と対策の難しさの一因になっている。治療には，第一選択薬の Praziquantel が用いられる。一般に，この薬による流行地住民への集団投薬（mass drug administration: MDA）が，住血吸虫症の流行が認められる国と地域での主要な寄生虫病対策になっている。

この研究では，日本住血吸虫症の疫学調査や，現行の対策評価に有用な手法を開発あるいは試行することを目的とした。具体的には，保虫宿主も対象とした包括的な疫学調査のツールとして，患者と保虫種宿主に共用可能な血清診断法を開発し，また，MDA による寄生虫病対策の評価や考察に有用な情報を提供するツールとして，マイクロサテライトマーカーを応用した寄生虫の集団遺伝解析を試行した。

第一章では, 患者と保虫種宿主に共用可能な酵素抗体法（ELISA）を開発した。日本住血吸虫症の伝搬においては，スイギュウとイヌが保虫宿主として重要な役割を果たしていることが知られており，患者とこれら保虫宿主での日本住血吸虫症の感染状況を正確に把握することが，流行地での感染症対策の要になる。しかしながら，これら全ての哺乳類宿主での感染を包括的にモニターするためのツールは未だ開発されていない。そこで，既に各宿主での抗体検出に有用と評価された数種の組換え体抗原を組み合わせて使用することで，患者とこれら保虫宿主の感染を検出することができる一つの ELISA 法の開発を試みた。組換え体抗原 SjTPx-1，Sj7TR および Sj1TR について組み合わせを変えて評価した結果, これら 3 種の抗原を同じモル数で組み合わせた“カクテル抗原”で最も良い成績が得られた。ヒト血清，スイギュウ血清およびイヌ血清に対する“カクテル抗

原”の陽性検出の感度は，それぞれ，84.1%，80.0%，および 80.0%であった。また，各血清に対する陰性検出の特異性は，等しく 100.0%であった。“カクテル抗原”を用いた ELISA 法を，日本住血吸虫症の対策に導入することで，流行地での包括的な疫学調査が可能になると同時に，これまで見過ごされてきた，保虫宿主を対象とした対策の重要性を再提起できると考える。

第二章と三章では，マイクロサテライトマーカーを応用して，各流行地に分布する寄生虫集団の遺伝的背景を解析し，そこから得られた情報をもとに，現行の感染症対策を評価し，更に有効な対策手法を考察することを試みた。

第二章では，フィリピン国内 8 ヶ所の日本住血吸虫症流行地に分布する寄生虫集団について，10 のマイクロサテライトマーカーを用いて，その遺伝的多様性を解析した。その結果，これら流行地に分布する寄生虫集団には，大きな遺伝的多様性が観察された。今回の成績を，各流行地の疫学情報とあわせて考察したところ，他国での先行研究で示唆された知見に同じく，感染症の有病率と遺伝的多様性の大きさの間に正の相関が認められた。

第三章では，第二章での解析を基に，フィリピンに分布する日本住血吸虫集団の遺伝的構造を推定した。その結果，フィリピンに分布する日本住血吸虫集団は，流行地毎に個別の遺伝的背景を有した固有の集団に収斂していないことが示唆された。これは，現行の MDA を中心とする寄生虫症制御対策が巧く機能していないことを示唆している。また同時に，各流行地に分布する寄生虫集団間に遺伝子流動があることも意味している。この遺伝子流動の背後には、国内の寄生虫病流行地間で患者と保虫宿主が頻繁に移動している実態が推測できる。

今回の研究から，このように，寄生虫の集団遺伝学解析が，感染症対策に有用な情報を提供する有用なツールになることが確認できた。更に有効な対策を実践し，またその効果を正確に評価するため，特に遺伝的多様性が大きな寄生虫集団が分布する地域では，MDA の更なる効率化を図る，他流行地域からの患者や感染動物の移入をモニターするなどの対策を新たに講ずるとともに，寄生虫を対象とした集団遺伝学解析を継続しておこなってゆく必要があると考える。

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