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TRANSMISSION DYNAMICS OF *SCHISTOSOMA JAPONICUM* WITHIN CHINA

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Thesis submitted for the degree of Doctor of Philosophy

Dedicated to *Yuanjun Wang*

DECLARATION

This PhD was carried out by Prof Joanne P. Webster's research group at Imperial College London. The research proposal was designed by Prof Webster and the candidate. Supervised by Prof Webster, the candidate selected the sampled villages. In Chapter 2, infection prevalence surveys in mammals and snails in Anhui Province of China were carried out under the assistance and support from Shitai County Centre for Disease Control, Tongling Schistosomiasis Control Station and Anhui Institute of Parasitic Diseases of China. The candidate participated in and was responsible for field surveys. James W. Rudge participated in infection prevalence survey in definitive hosts. Egg counting from sediments was done by two health workers from Anhui Institute of Parasitic Diseases. In Chapter 3, supervised by Prof Webster, the candidate performed the experiment of chronobiology of cercarial emergence from field-collected snails. In Chapter 4 to 6, the candidate participated in and was responsible for miracidia and cercariae collection during infection prevalence survey in field. James participated in miracidia collection in field, optimized the PCR reaction condition and ran PCR on five plates (96-well) of the miracidia samples. The rest of the miracidia samples (six plates) were run on PCR by the candidate and James. The candidate ran PCR on all cercariae samples and, supervised and instructed by Prof Webster, performed all the genetic analyses.

All written work presented in this thesis is the candidate's own:

…… ……

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ABSTRACT

Schistosoma japonicum, a multi-host parasite, remains highly endemic in China and has recently re-emerged in previously controlled regions. One reason may be, given the current human- and bovine-based control policy, due to a serious lack of knowledge of the potential role for other species of mammals, including dogs, cats and small rodents, in the transmission. This thesis aims to contribute to our understanding of transmission dynamics of *S. japonicum* by investigating the implications of different reservoirs in the transmission across two contrasting geographical regions/settings: the marshland with the disease persistence versus the hilly region where the disease was once controlled. Longitudinal characterization of *S. japonicum* infection at both definitive host and intermediate host levels was performed throughout 2006-2007, with the highest prevalence and infection intensity observed in rodents in the hilly region and in the agriculturally important domestic animals (cattle and goats) in the marshland. Three chronobiological trials of cercarial emergence were performed to identify any host (with nocturnal *vs* diurnal activity)-associated biological traits of the parasite. A late afternoon shedding pattern was observed in the hilly region, compatible with a nocturnal rodent reservoir, and a morning-afternoon dual shedding pattern within marshland areas, consistent with a diurnal bovine major reservoir. Characterization of the parasite population genetic diversity, using microsatellite markers, at both larval stages, also indicated cattle to be the main definitive host reservoir species in the marshland, which was further confirmed by sibling relationship analyses. In the hilly regions, however, epidemiological, biological and molecular data indicated that, in addition to the role of rodents as the main reservoirs to maintain the disease, dogs, with their higher mobility, may also play a significant role in *S. japonicum* transmission in these areas. The

implications of these results, in terms of parasite strain sub-structuring and targeted disease control, were discussed.

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Chapter 1: General introduction

1.1 INTRODUCTION

Schistosomiasis is one of the world's great neglected diseases, caused by blood flukes of the genus *Schistosoma*. Five species of schistosomes (Platyhelminth; Trematoda) are able to infect humans, and it is estimated that 200 million people are infected with schistosome parasites (Crompton, 1999; Chitsulo *et al.*, 2000). Based on prevalence, distribution and pathogenicity, the most important species in terms of human public health are *Schistosoma mansoni*, *S. japonicum* and *S. haematobium*, of which *S. japonicum*, also known as an Asian or Oriental blood fluke, is generally accepted as the most virulent due to its high egg output (Cheever *et al.*, 1994; Fan & Kang, 2003). *S. japonicum* remains endemic in China (Zhou *et al.*, 2005), the Philippines (Blas *et al.*, 2004) and parts of Indonesia (Garjito *et al.*, 2008), where 40million (Gray *et al.*, 2008), 6.7million (Riley *et al.*, 2008), and 0.7million (Dazo *et al.*, 1976) people are at risk of infection within the three countries, respectively.

1.1.1 The Life cycle of Schistosoma japonicum

Like all the other schistosomes, *S. japonicum* involves two obligatory host stages, a molluscan intermediate host and a mammalian definitive host, with transmission between the two via larval stages. Adult worms (males and females) typically reside in the mesenteric venules of the vertebrate host. They sexually reproduce and eggs are discharged in the excreta. One egg hatches upon contact with fresh water to release a free-living, motile larva, the miracidium. The miracidium in water locates an intermediate host *Oncomelania hupensis* snail (amphibious), penetrates the snail and then within the host forms a sporocyst. The sporocyst undergoes several rounds of asexual reproduction

and then produces thousands of cercariae, a second free living motile larval stage. The cercariae infect a definitive host via the host's skin, shed their bifurcated tails and transform into schistosomula, which then leave the skin via the blood vessels and draining lymphatics and reach the lungs. After several days, the male and female worms exit the lungs and arrive in the hepatic portal system, where they mature and pair up, and then migrate into and reside in mesenteric veins. The duration of time between penetration of the snail by the miracidium and the development of cercariae, the prepatent period, depending on temperature, lasts for 40 to 165 days (Yang *et al.*, 2007). Snails may remain infected for several months (Liang *et al.*, 2005). The prepatent of *S. japonicum* infection in definitive hosts, for example, in pigs, lasts for 27 to 33 days (Yason & Novilla, 1984) and most schistosome have a life span of over 5 years (Bush *et al.*, 2001).

1.1.2 Facing challenges in Schistosomiasis control in China

S. japonicum is believed to have been endemic in China for more than 2200 years, based on eggs discovered in two corpses exhumed in the Hunan and Hubei provinces in the 1970s (Mao & Shao, 1982) which were dated back to the Western Han dynasty. In 1950s, the disease was endemic in 400 counties or cities mainly along the Yangtze River and in the middle of the decade, out of the total population of approximately 600 million, an estimated 11.6 million were infected (Chen & Zheng, 1999). After over 50 years of integral control efforts, including health education, chemotherapy, molluscicide, environmental management, and sanitation improvement, by the year of 2000 the number of infected people had been reduced to about 0.7 million and the disease had been

eliminated in five of the 12 previously endemic provinces (Zhou *et al.*, 2005). However, epidemiological and surveillance data from 2000 to 2003 in the seven provinces, where *S. japonicum* remains endemic, suggest that schistosomiasis has re-emerged in 38 counties (Zhao *et al.*, 2005; Zhou *et al.*, 2005). Moreover, the results from a recent large-scale epidemiological survey of about 0.25million residents within 239 endemic villages across China, led to the estimation that the infection prevalence ranged from 0.02% to about 56% among villages (Wang *et al.*, 2006b).

Many factors, in terms of social, environmental and/or technical aspects (Utzinger *et al.*, 2005), may have contributed to such a worrying trend of the re-emergence and the presence of high infection prevalence in certain areas in China. However, one feature of the life cycle of this parasite, which distinguishes it from the other human schistosome species, is that a wide spectrum of potential definitive hosts, including over 40 species of domestic and wild mammals belonging to 28 genera, have been identified in China (Chen & Zheng, 1999), thereby leading to many gaps in our understanding of the transmission dynamics of the disease and thus hampering any control efforts.

1.1.3 Ignored host reservoirs for S. japonicum

A few species of definitive hosts such as bovines and pigs had been confirmed to be of potential threat to human infection. Cattle and water buffalo are generally accepted to be the most important reservoir hosts and in most endemic areas, a large proportion (70%) of the environmental contamination may be traced back to bovine defecation (Shen, 1992; Jiang *et al.*, 1997; Ross *et al.*, 2001; Wang *et al.*, 2005; Gray *et al.*, 2007). It has been

reported that, without the contribution of bovines, *S. japonicum* transmission would be interrupted and hence unsustainable (Gray *et al.*, 2008). Pigs were also once believed to play a very important role in the transmission and epidemiology of this disease in lake regions (Chen, 1989; Yuan, 1993), but as they are relatively short-lived and are often restricted to pens to fatten faster and provide faeces for biogas production if possible (Ross *et al.*, 2001), this may no longer be true. Goats and sheep are very susceptible to *S. japonicum* infection, but they are usually few in number and are thus unlikely to contribute much to overall transmission (He *et al.*, 2001). It is believed that, until recently, dogs were uncommon in rural China and were relatively unimportant to the human infection, and it was therefore assumed unnecessary to investigate these species during the three previous nation-wide surveys (Li *et al.*, 2005; Balen *et al.*, 2007). Wildlife such as small rodents and rabbits, were also previously assumed unimportant in the transmission and epidemiology of *S. japonicum* because of their low faecal production and/or small feral range (Ross *et al.*, 2001).

In the Philippines, in contrast, dogs, cats and rats, together with pigs, cows and water buffalo were all believed to play a potential role in the transmission of *S. japonicum* to humans (Pesigan *et al.*, 1958a; Fernandes *et al.*, 1982; Riley *et al.*, 2008; Rudge *et al.*, 2008). One recent research reported that human infections in 50 villages in Western Samar Province, Philippines may be due to the infection present in dogs and cats (McGarvey *et al.*, 2006). Furthermore, up to 95.5% (Fedorko, 1999) and up to 95.4% (Fernandez *et al.*, 2007) of infection prevalence in field rats were reported in some areas of the Philippines. Such may raise the question what roles such alternative reservoirs,

including dogs, cats and/or rodents, may have played in the transmission of the parasites in China.

1.1.4 Host-related adaptive behaviour for S. japonicum cercariae

The transmission of parasite *S. japonicum* between its obligatory host stages occurs via miracidia and cercariae and the probability of successful transmission of cercariae from snails to vertebrate hosts mainly depends on a temporal and spatial coincidence between the cercariae and the vertebrate, particularly due to the short lifespan of this free-living parasite stage (up to 25 hours on average, with rapidly reducing infectivity after the first hours), and their reliance on only non-renewable glycogen stores while locating their obligatory definitive hosts (Bruce *et al.*, 1969). However, vertebrates, in contrast to that of snails (aquatic or amphibious, depending on the schistosome species), are generally in contact with the water only for a short length of time, and the time point of which varies with species of mammal and/or their behaviour. Cercarial chances of encountering a suitable definitive host may therefore be enhanced, in addition to displaying host species-specific swimming behaviour, through peaks in emergence from their intermediate host in an appropriate chronobiological rhythm, which has been considered as an adaptive behavioural trait genetically controlled and shaped by definitive host behaviour, as has been well documented for other species of schistosome (Chasse & Theron, 1988; Theron, 1989; Pages & Theron, 1990b; Combes, 1991; Combes *et al.*, 1994). Therefore, any differences in such behaviour of *S. japonicum* cercariae, associated with diurnal versus nocturnal animals, would provide support for the existence of different main reservoir species.

1.1.5 Host-related factors on population genetic structures of parasites

Different species (or even different groups within species) of definitive hosts may constitute different environments for *S. japonicum*, due to, in part, the difference in their immunocompetence (the ability to resist parasites). For humans, for example, adults have been reported to become less infected than they should given their exposure strength and frequency, and such insusceptibility (resistance) could reflect age-dependent acquired immunity (Ross *et al.*, 2001; Acosta *et al.*, 2002), which has been commonly demonstrated for *S. mansoni* (Butterworth *et al.*, 1985; Kabatereine *et al.*, 1999) and *S. haematobium* (Wilkins *et al.*, 1987). Water buffalo appear to have an innate level of resistance against *S. japonicum* greater than cattle in that only 6 to 11% of penetrated cercariae developed into adult worms within water buffalo (Hsu *et al.*, 1984; Shi *et al.*, 1990), far less than over 50% within cattle (Hsu *et al.*, 1983). In water buffalo, worm burdens, worm size, and worm fecundity all appeared to decrease 1 to 1.5 years after infection (Luo *et al.*, 1988), a reflection of self-cure, decreased egg viability, and/or fecundity-suppression of female worms, as has been documented for other species of schistosomes, such as *S. bovis* (Bushara *et al.*, 1983; Bushara *et al.*, 1994; De Bont & Vercruysse, 1997) and *S. mattheei* (Taylor, 1996). Pigs were also able to mount a partial immune response against re-infection with *S. japonicum* by 4 weeks after a primary infection (Sorensen *et al.*, 1999a). Although such resistance in pigs seemed to decrease 6 weeks after primary infection, it remained effective in mice, suggesting that the underlying mechanism in mice and pigs may not be the same (Sorensen *et al.*, 2000). The mixed pairs of the primary infection and challenge infection isolates indicates that worms in pigs are either polygamous or able to wait in solitude for up to 12 weeks for a partner

(Sorensen *et al.*, 1999a). No difference in susceptibility was seen between the different age-groups of pigs to a primary infection (Sorensen *et al.*, 1999b) and congenital transmission in pigs during mid-to-late pregnancy was once reported (Willingham *et al.*, 1999). In mice, congenital infection did not occur and infection of the mice during pregnancy did not affect their offspring (Bendixen *et al.*, 1999). Fifty to 60 days after infected with a Philippine isolate of *S. japonicum*, mice expressed strong resistance to re-infection (Garcia *et al.*, 1983), but little or no resistance was induced when infected with single sexual parasites (Moloney *et al.*, 1986). A variability in response to *S. japonicum* eggs was also observed among different strains of mice (Mitchell *et al.*, 1981), and moreover, natural resistance against *S. japonicum* infection was reported in wild *Microtus fortis* (common rodents) living in the Dongting Lake area of China (He *et al.*, 1999a) as well as in laboratory bred *Microtus fortis* (He *et al.*, 1999b). In rabbits infected with either male or female *S. japonicum* or both, the levels of specific antibodies were much higher in infection with male and female than in single sexual infection and closely related to the intensity and duration of infection (Zhu & Li, 1990). Chinese hamsters (*Cricetulus griseus*) seemed to be equally susceptible as mice to *S. japonicum* infection and more tolerant than mice in terms of mortality (Kutsumi *et al.*, 1988). For different laboratory animals infected with the same Indonesian strain of *S. japonicum*, adult worms harvested from the rabbit and Taiwan monkeys reached the largest size, but many worms collected from rats were immature; and, similarly, eggs from the Taiwan monkeys were larger than those from a dog and mice (Cross, 1976). Such various constraints derived from the immunity of definitive hosts may therefore be predicted to affect population dynamics of parasites during the establishment of infection, development and

reproduction of parasites within hosts (Anderson, 1998), although the mechanisms of the above immune response against *S. japonicum* remain unknown (Vercruysse & Gabriel, 2005).

In addition, the difference in biological traits of definitive hosts such as lifespan, patterns of foraging, mobility, home range and dispersal rates (Huyse *et al.*, 2005), as well as the immune response, among species of hosts may affect parasite populations during their recruitment of infective larvae (cercariae) and dispersal of propagules (eggs and then miracidia). A comparison of the population genetic structure of *S. japonicum* at two stages would, therefore, shed better insight into such host-associated transmission of the parasites.

1.1.6 The existence of potential strains of parasites

Any observed phenotypic variation for the parasites and/or, more importantly, the heterogeneity of transmission could be predicted to be associated with genetic diversity and/or potential multi-strains of *S. japonicum*. Whilst no or low level of intra-specific genetic variation of *S. japonicum* has been occasionally reported (Bowles *et al.*, 1993; Sorensen *et al.*, 1998), the majority of studies indicate that this parasite is highly genetically diverse, and a number of studies have described the existence of various strains among mainland China, the Philippines, Japan, Taiwan and Indonesia (Ruff *et al.*, 1973; Moloney *et al.*, 1985; Sobhon *et al.*, 1986; Kresina *et al.*, 1991; McManus & Hope, 1993; Hope *et al.*, 1996; Ohmae *et al.*, 2003). Indeed, some studies suggest that, even within mainland China, several strains of *S. japonicum* may exist (He *et al.*, 1991b; He *et*

al., 1991a; He *et al.*, 1994; Gasser *et al.*, 1996; Chilton *et al.*, 1999). Such potential strains of *S. japonicum* in mainland China could be accredited to geographical speciation of parasites due to isolation, or, more specifically, the speciation of parasites towards their each suitable definitive host under host-induced selection pressure (Theron & Combes, 1988). Indeed, a series of experimental studies show that significant variation occurs for mammals in their susceptibility to different *S. japonicum* isolates. For example, *Macaca mulatto* was susceptible to Chinese mainland and Japanese groups of *S. japonicum*, but not to the Taiwanese (Hsu & Hsu, 1960; Cheever *et al.*, 1974; He *et al.*, 1992). Such findings suggest that the genetic diversity or possible multiple strains of *S. japonicum* might influence the transmission of the disease.

1.1.7 Key methods

Molecular genetic markers have provided useful tools in investigating population genetic structure of parasites and then dissecting such infection process or patterns at the genetic, rather than microscopic, level and may offer the potential to elucidate natural transmission dynamics (Anderson *et al.*, 1995; Blouin *et al.*, 1995). Microsatellite markers, due to their codominant expression, allelism and being neutral (Curtis & Minchella, 2000), are one of the best tools for this kind of genetic analyses. Highly polymorphic microsatellite loci for *S. japonicum* have been recently identified (Shrivastava *et al.*, 2003) and have been successfully applied for such studies (Shrivastava *et al.*, 2005a; Shrivastava *et al.*, 2005b; Wang *et al.*, 2006a). Moreover, the simple, long-term and room temperature storage of field-collected larval samples on a Whatman FTA® card (Whatman International Ltd., Springfield Mill, James Whatman

Way, Maidstone, Kent, UK), a chemically-treated paper that extracts the DNA from a specimen, facilitates sample collection (Gower *et al.*, 2007), thus avoiding host-imposed selection by the laboratory mammals and/or molluscs used for required samples (LoVerde *et al.*, 1985; Bremond *et al.*, 1993; Shrivastava *et al.*, 2005a; Gower *et al.*, 2007). A multiplex PCR analysis further enables the multilocus genotyping of single larval samples, therefore allowing the tracing of given genotypes of parasites, as well as the alleles, between local spatial or temporary parasite populations in order to elucidate the transmission dynamics of the parasites between and/or across hosts.

1.1.8 Thesis aims

Besides host and parasite aspects, environmental heterogeneity may also be predicted to have an influence on the transmission process of *S. japonicum*. In Anhui Province of China, the focus region of my thesis, there are 41 counties (or cities) endemic for schistosomiasis in historical records and the disease is still highly endemic in fourteen counties or county-level cities, of which the majority belong to marshland/lake regions and a small part to hilly/ mountainous regions (Zhou *et al.*, 2005). Therefore, this thesis aims to contribute to our understanding of transmission dynamics of *S. japonicum* within China by investigating the implications of different reservoirs for *S. japonicum* in the transmission. It aims, by setting it in two contrasting geographical regions/settings, the marshland with the disease persistence versus the hilly region where the disease was once controlled, to assess the role of each definitive host species in the transmission and thus determine main reservoir species in either region. Assuming wildlife such as rodents with nocturnal activities as main definitive host reservoirs, in contrast to bovines with

diurnal activities, this thesis aims to identify any host-associated biological traits of the parasite through experiments of circadian rhythms of cercarial emergence. This thesis mainly aims, by describing the relative gene flow of parasites based on allele frequencies and the path of certain parasites based on multilocus genotypes, to elucidate the infection process or transmission pattern of *S. japonicum* from definitive hosts to intermediate hosts. Finally, this thesis aims to determine any potential multiple strains of *S. japonicum* due to geographical barriers and/or different main reservoirs.

1.1.9 Thesis components

This thesis consists of seven chapters with Chapter 2 to 6 being the main data contents. These data chapters are presented in the generalized format as a series of inter-related scientific papers. Each chapter, therefore, has its own abstract, introduction, and discussion with certain degrees of necessitated repetition generated by similar introduction or methodology. Chapter 2 shows the results from longitudinal parasitological surveys of *S. japonicum* prevalence and intensity at both the miracidial (definitive host) and cercarial (intermediate host) levels in the two geographical regions throughout 2006-2007. Based on the results from the first year of epidemiological surveys and under the assumption of rodents as potential reservoirs, the chronobiology of cercarial emergence from field-collected snails was examined and two distinct cercarial shedding patterns were identified, as fully displayed in Chapter 3. By applying microsatellite markers, Chapter 4 explores the possible transmission dynamics of the disease in each village from definitive hosts to intermediate hosts based on allele frequencies. Based on the asexual reproduction of *S. japonicum* within an intermediate

host and its associated characteristic, Chapter 5 investigates the above mentioned transmission dynamics of parasites between two larval stages using sibling relationship analysis. Chapter 6 investigates whether there was a population genetic substructure (or potential multiple 'strains') of parasites in relation to geographical barriers or/and definitive hosts. The general discussion of Chapter 7 puts key findings, their implications and applications together and also raises some further interests and challenges.

1.2 GLOSSARY

Infection intensity: The number of individuals of a particular parasite present in/on a single infected host. For *S. japonicum*, the measure of infection intensity is defined as eggs per gram of faeces with the Kato–Katz thick smear (Katz *et al.*, 1972), a standard method for the quantification of egg burdens in humans. The egg hatching is used for detection of infections in domestic animals with infection intensity calculated as either miracidia plus eggs (from sediments after hatched) per gram of faeces (Ministry of Health of P. R. China, 2000), or miracidia only per gram of faeces. Infection intensity here was also measured as the number of genetically unique adult worm pairs within a single host, as it is impossible to estimate the number of worm pairs due to the possibility of the repeated genetically identical pairs within the host.

Circadian rhythms of cercarial emergence: This term is defined as a 24hrs cycle in the behavioural process of schistosome cercarial emergence from their intermediate host snails. This trait is generally considered to be adaptive for parasites to their relative hosts and genetically inherited, as has been demonstrated for other forms of schistosomes (Chasse & Theron, 1988; Theron, 1989; Pages & Theron, 1990b; Combes, 1991; Combes *et al.*, 1994).

Parasite infra-population: The group of parasites of the same species present within one individual host at a particular time (Bush *et al.*, 1997). Knowledge of the genetic structure of parasite infra-populations may provide useful information on the recruitment patterns of the cercariae into definitive hosts, thus improving our understanding of the transmission dynamics.

Parasite subpopulation: One term widely used in the description of population genetic structures. A parasite population may be divided into several clusters, namely subpopulations, of individuals using software such as STRUCTURE (Pritchard *et al.*, 2000) and BAPS (Corander & Marttinen, 2006). The former applies a model-based clustering method for using multilocus genotype data to infer population structure and assign individuals to subpopulations. The latter attempts either to identify a hidden population structure by clustering individuals into genetically divergent groups or to target at separating the ancestral sources of the alleles observed in different individuals.

Cross-infection: For *S. japonicum* in the current thesis, the term of cross-infection among species of definitive hosts is defined as a given genotype of parasite discovered in two or more sympatric species of mammals, due to the existence of many clonal cercariae released from snails.

Allopatric speciation: The formation of new species following either the geographical or the physical separation of populations of the ancestral species. For parasites, allopatric speciation includes speciation following host-switching and parasite speciation following host speciation (Huyse *et al.*, 2005).

Sympatric speciation: The formation of new species in the absence of either a geographical or a physical barrier that isolates populations of the ancestral species. For parasites, sympatric speciation can occur on the same host species (Combes & Theron, 2000; Huyse *et al.*, 2005).

Chapter 2: Contrasting reservoirs for *Schistosoma japonicum* between marshland and hilly regions in Anhui, China: inferred from a two-year longitudinal parasitological survey

A modified version of this chapter has been accepted by Parasitology *for publication with authors as Da-Bing Lu (corresponding author), Tian-Ping Wang, James W. Rudge, Christl A. Donnelly, Guo-Ren Fang, & Joanne P. Webster*

2.1 ABSTRACT

Schistosoma japonicum remains highly endemic in many counties in China and has recently re-emerged, to a large extent, in previously controlled areas. To test the hypothesis that small rodents and less agriculturally important domestic animals such as dogs (and cats) may play an important role in the transmission and potential re-emergence of this disease, an annual investigation of *S. japonicum* among humans, domestic animals and rodents, combined with detailed surveys of the snail intermediate host, was performed across three marshland villages and three hilly villages in Anhui province of China over two consecutive years. The highest infection prevalence and intensity observed across all mammals was in rodents in the hilly region; while in the marshland cattle were suspected as the main reservoirs. However, relatively high infection prevalence levels were also found in dogs and cats in both regions. Such results may have implications for the current human- and bovine-oriented control policy for this medically and veterinarily important disease, particularly in the hilly regions of mainland China.

2.2 INTRODUCTION

Schistosomiasis is one of the world's great neglected diseases, caused by blood flukes of the genus *Schistosoma*. Of the three major schistosome species infecting humans, *Schistosoma japonicum* presents one of the greatest challenges to control due, at least in part, to its zoonotic nature. Although great progress has been made in China over the past five decades where, for example, the estimated number of infected people was reduced from 11.6 million in 1950s to 0.7 million in 2000, the disease remains endemic in 110 counties within seven provinces in China (Zhou *et al.*, 2005), with an estimate of up to 56% infection in some villages (Wang *et al.*, 2006b). Moreover, particularly since the mid-1990s, *S. japonicum* has re-emerged in previously controlled areas and further snail infested areas have been found in formerly non-endemic provinces such as Shanghai, Zhejiang and Fujian (Zhou *et al.*, 2004). Such worrying trends in the re-emergence and/or the continued presence of high infection levels in certain areas may suggest some ongoing factors in the disease transmission which have been long neglected.

Based upon previous estimates of their relative transmission index (an index of the role each species may play in *S. japonicum* transmission based on the size of each species population, their infection prevalence, excreted faeces per day, and eggs per gram of faeces), bovines are generally considered as the most important species in terms of chemotherapy-based policy (Ross *et al.*, 2001; Wang *et al.*, 2005; Gray *et al.*, 2007). Thus, most effort has been invested in either the development of a potential vaccine against *S. japonicum* in bovines (McManus & Bartley, 2004; Wu *et al.*, 2005), the chemotherapeutic treatment of bovines (Gray *et al.*, 2007), and/or the elimination and

replacement of bovines with machines (Chen *et al.*, 2004), together with the fencing of pigs. Prevention or control measures for other agriculturally 'less important' domestic animals, such as dogs or cats, are not mentioned within the most recently passed 'The Legislation of Schistosomiasis Control'(The Central Government of P. R. China, 2006), nor indeed were even included during the three previous nation-wide surveys conducted in 1989, 1995 and 2004, respectively (Li *et al.*, 2005; Balen *et al.*, 2007) or annual monitoring surveillance across the whole endemic areas (Zhao *et al.*, 2005). Likewise, the potential importance of wild animals, in particular that of small rodents, in the transmission or re-emergence of *S. japonicum* has also long been neglected (Yao *et al.*, 1989; Zheng, 2006). Although previous studies have occasionally reported high infection prevalence levels in rodents of up to, for example, 64% in one area of Yunnan (Cheng & Gong, 1989) and 59.8% in two islets along the Yangtze river (Xu *et al.*, 1999), their subsequent role in the transmission of schistosomiasis has generally been dismissed. Reasons for this may relate to the relatively low amount of excreted faeces from rodents (a rodent per day produces only one twenty-fifth of that produced by a human (Mao, 1990)), a potentially low egg viability as reported by some studies (Ho, 1963; Mitchell *et al.*, 1990), and/or a potential 'natural resistance' in *Microtus fortis*, one common species of lake rodents in Hunan province of China, against this parasite (He *et al.*, 1999a; He *et al.*, 1999c). However, given contrasting reports that, for example, many viable eggs were excreted in the faeces from *Rattus norvegicus* infected with *S. japonicum* (Wu, 1957), and miracidia per gram of faeces from infected rodents can be 14.5 times higher than that obtained from the equivalent human samples (Mao, 1990), and in one plateau area of Yunnan the mean infection intensity (eggs per gram of faeces) in rodents was 5.8,

significantly higher than in humans, cattle, horses and pigs (Yang *et al.*, 2000), such a general omission and/or dismissal of the potential role of rodents in the transmission of *S. japonicum* may be surprising. Furthermore, recent molecular research in Anhui Province of China, using polymorphic *S. japonicum* microsatellite markers (Shrivastava *et al.*, 2003), revealed that a large proportion of alleles observed from cercariae samples from snails were not observed in any miracidia from the human or domestic animals sampled in the same areas (Wang *et al.*, 2006a), thereby indicating that additional potential definitive host reservoir species may play a role in the ongoing transmission of this important disease.

Such obvious contrasting views and gaps in our current understanding of this multi-host pathogen leads us to question what may be the actual infection profile of *S. japonicum* in dogs, cats and rodents within mainland China and, moreover, whether such species could maintain transmission of the disease even if infections within bovines, goats and/or pigs can be successfully controlled or eliminated through current targeted treatment programmes. Therefore, throughout 2006-2007, parasitological surveys of the infected snail distribution prior to transmission seasons and of the prevalence and infection intensity in all species of definitive hosts present near the end of annual transmission season were performed across two contrasting geographical regions within Anhui province of China: in the marshland where the disease persists, and in the hilly region where the disease was previously controlled, in order to evaluate the potential role of less agriculturally important domestic animals (dogs or cats) and wildlife (small rodents) in the transmission of the disease.

2.3 MATERIALS AND METHODS

2.3.1 Sites location

Research sites were set in Tongling County and in Shitai County, Anhui Province of China. Tongling County is located on the south bank of the Yangtze River, and is classified as marshland region with schistosomiasis persistence. According to the local historical recording (provided by Tongling Schistosomiasis Control Station, Anhui, China), at the end of 2004, a total of 126 villages within nine townships were endemic for the disease, with around 204,900 residents at risk of infection; the infected snail area was 7,144,300m^2, found on 34 habitats within six townships of 21 villages; the snail density on average was 0.37 per 0.11m^2 (frame), and infected snail density was 0.001 per 0.11m^2; the mean prevalence in humans was 3.3%, with the highest up to 7.1% in some villages; the infection prevalence level among cattle and water buffalo was 8.5% on average across the county, with the highest 30.8% in some villages; acute cases have annually occurred over recent years.

Shitai County, a hilly region in which the disease has re-emerged since 1995, lies in the southern Anhui. According to local historical recordings (provided by Shitai County Centre for Disease Control, Anhui, China), in 1985 disease control reached 'transmission control level' (Liang *et al.*, 2006) at the scale of county, with the prevalence in humans and in cattle both less than 1%, and no new infections in children under 12 years old nor any acute cases recorded. However, since 1995 the infected snail habitats have increased every year. During 2001-2005, the prevalence at the county-level in bovines fluctuated

between 0 and 1.7 % and in humans between 0.2 and 1.0%. In 2003, an outbreak of acute schistosomiasis occurred in one village (Cao & Wu, 2004).

Based on the accessibility of locations and the availability of accurate survey data recordings (annual reports to the Anhui Institute of Parasitic Diseases) from the previous three years, three villages in Tongling County, namely Guanghui, Heping, and Xingzhuang from Laozhou Township located on an island in the middle of the Yangtze River, were chosen for the current investigation. Meanwhile, in Shitai County three villages: Longquan from Dingxiang Township, Longshang from Chili Township and Yuantou from Ketian Township, were chosen. Table 2.7.1 shows general information on demography, schistosomiasis endemic status, and control measures in the six villages in 2005 and Figures 2.8.1-2 display their geographical locations.

2.3.2 Snail survey

Systematic surveys of snails were carried out in all suspected snail habitats across the six villages during March to April of 2006 and of 2007. According to the standard procedure (Ministry of Health of P. R. China, 2000), frames, each 0.11m^2, were set at regularly-spaced locations within snail habitats, and all snails found within each frame were collected in order to measure the density of snails and of infected snails. In the hilly region, where snail habitats are known to be concentrated within irrigation ditches, frames were placed at regular distances (5m or 10m) along these ditches. In the marshland region, where snails are more widely dispersed across marshy areas of land, frames were set in an equally-spaced lattice formation (20m×20m). The captured snails

from the field were checked in the laboratory the following day for death and then for infection by using a crushing method (Ministry of Health of P. R. China, 2000). The numbers of snails or infected snails were recorded by frames and habitats and infected habitats were subjected to a follow-up investigation in order to obtain additional infected snails for cercariae collection.

2.3.3 Schistosomiasis prevalence and intensity epidemiological survey

All the humans and domestic animals from each village were regarded as subjects of this survey, and stool was collected from them during Sept. to Oct. of 2006 and of 2007. Stool examinations were conducted using the miracidia hatching test (Ministry of Health of P. R. China, 2000; Yu *et al.*, 2007) to diagnose *S. japonicum* infection for all species of definitive hosts. Stool samples were first placed into a mesh-made container with holes on the bottom and wall (150 holes per square inch), filled with water and stirred with bamboo sticks. Filtered faeces went into a 260-mesh nylon bag and washed with water until the sediment in the bag cleared. Sediment was collected from the bag and placed into a flask containing nonchlorinated or natural water. The flask was then left in a well-lit room with the temperature set at 25–30°C. The neck of the flask was strongly illuminated from one side, and examined with a magnifying glass to detect the presence of free-swimming miracidia after 2, 4, 8 and 12h. If no miracidium could be detected after 12h, a host was considered negative for infection. For humans, only those antibody-positive during an annual Indirect Haemagglutination Assay (Gui *et al.*, 1991) were asked to provide faecal material, which, if positive in egg hatching, were subjected to a further

Kato-Katz test with three slides thick-smeared per sample to quantify the intensity of infection with the index of eggs per gram of faeces (Feldmeier & Poggensee, 1993).

A veterinarian was hired to obtain faeces from cattle, water buffalo, goats and dogs by direct rectal sampling wherever possible. Faecal samples from cats were obtained through scrutinizing the neighbouring ground of the house to which a cat belonged. Only a sub-sample of pigs were given stool examinations, as pigs tend to be always fenced due to their value in meat and/or behaviour in terms of destroying crops when free-roaming, and therefore have few opportunities for water contact. Small rodents were captured by setting live traps in the late afternoon and checking at dawn for three consecutive days and captured rodents were kept inside overnight for obtaining their faecal samples. For domestic animals and small rodents, infection intensity data were calculated as miracidia per gram of faeces, or of miracidia plus eggs (from sediments after hatched) per gram (EPG) (Ministry of Health of P. R. China, 2000).

Throughout the two years of investigation in the sampled villages, control measures were implemented as usual by local health staff. These involved selective chemotherapy of humans, with praziquantel at a dose of 40 mg/kg body weight (Chen, 2005) based on the Indirect Hemagglutination Assay (Zhu, 2005), health education, and snail control with niclosamide 50%WP (Nanjing Essence Fine-Chemical Co., Ltd., China), a chemical with solubility in water 1.6 (pH 6.4) and 110 (pH 9.1) (both in mg/l, 20ºC) and molluscicide through respiratory and stomach action, widely used in China (Yuan *et al.*, 2005). Due to the increasing concern, from both officials and health workers, over the high prevalence

of schistosomiasis in bovines and goats in the marshland revealed in 2006, with special funding from the local government, all bovines and goats raised in the marshy villages have been eliminated since Jan. 2007.

2.3.4 Statistical methods

Snail density and infected snail density were measured as the number of snails per 0.11 m^2 (Ministry of Health of P. R. China, 2000) at levels of villages. A significant difference in infection prevalence in snails between years was tested with Chi-square test. Due to low examination rates and small sample sizes of definitive hosts in each village (See Table 2.7.2 and Table 2.7.4), infection prevalence in definitive hosts was measured at the level of region (county) and the significant difference among species was shown by their 95% confidence intervals. Infection intensity in definitive hosts was calculated as the geometric mean of miracidia per gram of faeces and the geometric mean of miracidia plus eggs per gram of faeces. The significant difference in infection intensity among species was tested with the non-parametric Kruskal-Wallis test using SPSS 11.0 software (**SPSS Inc.**, 2002).

2.3.5 Ethical considerations

Ethical clearance for the study was obtained from the Scientific Committee, Anhui Institute of Parasitic Diseases. A blood test for antibodies to *S. japonicum* in all residents at home, conducted by local health workers, is an annual routine task required from the local health department, for selecting targeted individuals for treatment. Oral informed consent was obtained from all adults and from parents or guardians of minors who were

involved in the project, and from owners whose domestic animals were sampled. Owners and local village leaders were informed of the infected dogs or cats and control advice was given. Captured rodents were humanely euthanized with ether in black bags if positive for *S. japonicum* in stool examination; otherwise, they were released at their original point of capture the following day.

2.4 RESULTS

2.4.1 Infection prevalence in intermediate host snails

Infected snails were identified in all the sampled villages across both years (Table 2.7.3). In the marshland, both the infected snail density and the proportion of infected snails was lower in 2007 relative to the numbers in 2006, with a significant difference in prevalence seen in Heping (χ^2=7.97, d.f.=1, p<0.01). In contrast, in the hilly villages, with the exception of Yuantou, both indices were higher by 2007, with a noteworthy difference in prevalence found in Longshang (χ^2=11.32, d.f.=1, p<0.01). This apparent increase in prevalence in Longshang was accompanied by an increase in the number of infected snail habitats, with ten out of 38 snail habitats found with infected snails in 2007, whereas only six infected snail habitats were observed in 2005 and three in 2006 (Table 2.7.1 and Table 2.7.3).

2.4.2 Infection prevalence and intensity in definitive hosts

The infected species, not including humans, in the marshland, in order of infection prevalence, were goats, cattle, water buffalo and dogs, whereas in the hilly region were rodents, dogs and cats in 2006 (Table 2.7.4). In 2007 after bovines and goats had been removed in the marshland, the prevalence profile in definitive hosts changed with cats and dogs on the top of the list, while in the hilly region the dogs became the species with the highest infection prevalence among all species, with rodents second (Table 2.7.4). However, the prevalence in dogs and cats was not significantly higher in 2007, compared to in 2006, in either region (in marshland, χ^2=0.03, d.f.=1, p=0.86; in hilly region, d.f.=1, χ^2=0.0001, p=1.0).

Infected rodents were found in each hilly village in both years, often at high prevalence levels of between 11.8% and 30.0% by village, but no infected rodents were found in the marshland. In contrast, within the marshland villages, very high infection prevalence levels were observed within cattle in 2006, ranging from 21.05% to 71.05% by village, but no infected cattle were identified within the hilly region. It was also noted that infected dogs were identified in each hilly village across both years. Infections in human populations, who were positive in the previous Indirect Haemagglutination Assay test here, were identified in all six villages during 2006-2007, with a non-significant increase between the two years in the marshland (χ^2=1.87, d.f.=1, p=0.17) and in the hilly region (χ^2=0.47, d.f.=1, p=0.49) (See Table 2.7.4).

Using the Kato-Katz test, no eggs were found among the majority of the infected humans previously identified through egg hatching, with the exception in the hilly region in 2007, and, as a result, the intensity in humans should be very low (Zheng *et al.*, 1995). Highest infection intensities in the hilly region were observed in rodents, across both years, while in the marshland there appeared to be no distinct difference in such indices among infected host species (cattle, water buffalo, dogs, cats and pigs; for the index of miracidia per gram of faeces, χ^2=1.15, d.f. = 4, p=0.88; for EPG, χ^2=0.79, d.f. = 4, p=0.94) (See Table 2.7.4).

2.5 DISCUSSION

The present survey demonstrated that, based on the infection prevalence and intensity in each species, small rodents, with an ability of rapid reproduction and potentially more opportunities to come into contact with water sources and defecate on snail inhabited ditches, may play an important role in the transmission of *S. japonicum* in the hilly region, in contrast to the marshland region where cattle, and to some extent goats, are primarily responsible for ongoing transmission. Furthermore, a relatively high infection prevalence was discovered in dogs (and cats) in both geographical regions, raising potentially important implications for current control criteria of *S. japonicum* in China (Liang *et al.*, 2006), which is essentially based on infection in humans and bovines, rather than any current concern over other less agriculturally important domestic animals.

Small rodents have long been considered of little or no importance in *S. japonicum* transmission in China (Chen & Zheng, 1999) due mainly to the much lower amount of excreta from rodents compared to that from humans or bovines. Another important reason is that recent research has either generally documented a very low prevalence (0.3-3.0%) of *S. japonicum* in small rodents (Yang *et al.*, 2000; Zheng, 2006), or involved no attempts to collect faecal samples from such animals (Li *et al.*, 2005; Wang *et al.*, 2005; Zhao *et al.*, 2005; Balen *et al.*, 2007), which is potentially due to the logistical difficulty inherent in the sampling of wild rodents (Webster & Macdonald, 1995). It may not be surprising that no quantitative estimation of the role of such species in the transmission of *S. japonicum* in China has (to my knowledge) been made, as the necessary parasitological survey data have generally not been available.

In contrast, bovines (water buffalo and domestic cattle) have been considered as the most important contributors to *S. japonicum* transmission within the lake/marshland areas of China due to their large herds, free-roaming behaviour, long life span and potentially high number of eggs passed with their faeces (Ross *et al.*, 2001; Guo *et al.*, 2006; Gray *et al.*, 2007). Schistosomiasis control in bovines has therefore long been regarded as a major part of the Chinese national schistosomiasis control programme (Ministry of Health of P. R. China, 2000). Indeed, in this study, I also found that the highest infection in the marshland areas was in bovines in 2006, and also goats, which is consistent with previous reports from within one marshy village within Anhui (Wang *et al.*, 2005; Zhao *et al.*, 2005). However, within the hilly region of the current survey, no infected cattle were found. This difference may be explicable by two factors: one is that very few cattle were raised in local hilly villages and the other may be attributed to a specific pattern of rearing cattle there. In the hilly region, cattle graze on the slopes of the hills, and therefore have little or no contact with snail habitats (which are largely concentrated in irrigation ditches), unlike bovines in the marshland region which graze on snail-inhabited pastures. Generally bovines are suspected to be less important within the hilly region relative to that of the marshland or lowland areas, as has been discussed by Shrivastava and colleagues (Shrivastava *et al.*, 2005b) and Seto and colleagues (Seto *et al.*, 2002), although the latter supposed that transmission may still be maintained in these areas indirectly through agricultural fertilization practices which involve usage of untreated human and animal excrement. However this was not the case in the hilly region of the current study, as bio-gas toilets, using faeces from humans or agriculturally important domestic animals as material to produce bio-gas, during which most pathogens are killed

under anaerobic conditions inside the digester (Jha, 2003), are relative common. The coverage of the toilets, for example, is 39% in Longquan village and 48% in Yuantou village (see Table 2.7.1), thus reducing the opportunities for faecal contamination of the water sources.

The role of infected humans in the maintenance or re-emergence of *S. japonicum* transmission, through migration into new regions, application of fresh faeces as fertilizer or occasionally through 'night soil' due to personal behaviours, has been demonstrated (Zheng *et al.*, 1991; Cheng *et al.*, 1994). However, in both Shitai County and Tongling County, there seems to be no strong evidence to support the hypothesis that humans might currently be a main factor in transmission. The infection prevalence in humans, despite being overestimated here (since it was estimated among sero-positive individuals rather than among the total population), at 0.4-1.8% in the marshland and 4.0-6.3% in the hilly villages, was still very low compared to that in cattle or rodents, and the infection intensity was nearly negligible. Furthermore, great improvement in sanitation was noted to have occurred in the sampled villages as mentioned above, which, along with annual control measures such as health education and selective treatment, would further reduce the chances of humans contaminating snail habitats (Guo *et al.*, 2005).

Infection in dogs (and cats) has infrequently or rarely been reported in China for over 10 years due primarily to logistical difficulties in sampling faeces from these species, as indeed was encountered in the current survey (See Table 2.7.2 and Table 2.7.4). In this study, the considerable prevalence levels observed among dogs in both regions, coupled

with their high mobility, might indicate a significant role of these animals in the transmission and/or dispersal of *S. japonicum* in endemic communities in China. Indeed, there is increasing evidence to suggest that dogs may play an important role in *S japonicum* transmission in the Philippines (McGarvey *et al.*, 2006; Rudge *et al.*, 2008). However, from parasitological data alone it is unclear whether transmission within my study regions could be maintained within dogs, or whether they are mainly just 'spill-over' hosts. Pigs are also potentially important hosts (Johansen *et al.*, 2000), although they are relatively short-lived and often restricted to pens to fatten faster and/or provide faeces for biogas production, both in the marshland and in the hilly region. After examination of 133 pigs, however, two infected pigs which were not fenced were observed here in one marshland village, despite the fact that the local government has forbidden bovines and goats to live in the marshland since Jan. 2007, thereby highlighting the need for continued vigilance across all potential definitive host species for successful disease control.

The different trend in changes of infection prevalence in snails and infected snail density between the two regions could partly reflect the effects of contrasting reservoirs or different control measures (and/or frequencies or coverage) between regions. Indeed, although intensive molluscicide had been conducted annually in the hilly region, two hilly villages showed an increase in snail indices, indicating the possible existence of other important reservoirs apart from humans. The decrease in snail measures in Apr. 2007 in the marshland may not be easily accredited to the replacement of bovines (and goats) implemented in Jan. 2007, as most contamination of snail habitats by these animals

would likely have already occurred during the previous year (Liang *et al.*, 2005). Since snail measures are variable across seasons (Davis *et al.*, 2006), more caution should be given when interpreting the implication of such snail indices.

It should, however, be mentioned that there are several inherent limitations within the current study. First, the dilemma in the detection of infection with stool examination in humans and animals still remains. The Kato–Katz test, one standard method for the detection and quantification of egg burdens in humans (Katz *et al.*, 1972), has poor sensitivity when one stool sample per host is used, especially when infection intensity is very low (Yu *et al.*, 2007), and also cannot be conducted on faeces from animals with a high content of fibrous material. Here I could not detect any eggs with the Kato-Katz test in some infected humans, who here were identified through the previous hatching test. The hatching test indeed improves test sensitivity (Guo *et al.*, 2001), but the required time, natural water, suitable temperature and the disposal of large amount of waste limit its application in field surveys. Therefore, prevalence surveys using the hatching test were limited to the examination of samples from a single time point without considering day-to-day variation of egg excretion in stool (Carabin *et al.*, 2005) and, also due to time limitations of field work, infection intensity was not measured for each infected animal. Second, differences in the amount of faeces examined or in the sampling procedures, for example from cats, among species might be slightly problematic when directly comparing infection prevalence and intensity between species. Due to the logistic difficulty in the sampling of dogs, cats and small rodents, it was impossible to get the required amount of faeces each time as proposed in (Wang *et al.*, 2005), and thus each

sample as a whole, after being weighed, was examined with the miracidia hatching and eggs from the miracidia-positive sediments after hatching were counted. Finally, I could not calculate the relative transmission index when comparing the roles of species, one reason for which was due to the difficulties in accurately determining actual population sizes of wild rodents. On the other hand, although such an index is very useful to assess the relative contribution by different species of hosts and indeed has found quite a number of successful applications (Ross *et al.*, 2001; Wang *et al.*, 2005; Gray *et al.*, 2007), it does not take into account the hatchability of faeces and, more importantly, faecal deposition habits, which may also be essential factors in the assessment of epidemiological importance of each host species.

The contribution of different species of hosts to the transmission of *S. japonicum* varies with regions, particularly between mountainous/hilly regions (Zheng *et al.*, 1997) and marshland/lake regions (Gray *et al.*, 2007). Indeed, the present study clearly demonstrates contrasting infection profiles of *S. japonicum* in mammals between the two piloted regions, and strongly suggests that in the previously controlled hilly areas, rodents may be the most important reservoir for *S. japonicum*, whereas in the marshland, cattle may have been playing a major part in the transmission of the disease. The role of dogs (and cats) should not be ignored in both regions, and human infection seems inevitable in such areas. The practical implications from such results are that, in the hilly areas, human- or bovine-based control strategies alone may not be sufficient for controlling transmission and therefore more efforts should perhaps focus on intermediate host snail control and environmental management, in order to minimize the snail density and infected snail

habitats. Moreover, chemotherapy of dogs (and cats) might also help reduce the degree to which the disease is transmitted if dogs (and cats) play a role in the transmission. This would merit further investigation.

2.6 ACKNOWLEDGEMENTS

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2.7 TABLES

Table 2.7.1 General information in the sampled villages in 2005

Variables	Guanghui(M)	Heping(M)	Xingzhuang(M)	Longquan(H)	Longshang(H)	Yuantou(H)
Coordinates						
Latitude	30°56.96'	30°59.15'	30°58.54'	30°18.66'	30°21.83'	30°04.95'
Longitude	117°44.87'	117°44.96'	117°45.25'	117°31.64'	117°50.40'	117°30.24'
Registered population	2686	1758	2039	1137	866	759
Houses	713	450	560	282	245	209
With piped water or water from well	100%	100%	100%	40.43%	76.33%	52.15%
With a bio-gas toilet (in hilly region) or parasite-free toilet (in marshland)	44.88%	37.78%	35.71%	39.01%	11.84%	47.85%
Schistosomiasis endemic status						
Snail habitats	2	2	1	29	35	34
Infected snail habitats	1	2	1	4	6	5
Infected snail density (/0.11m^2)	0.004(7/1741)	0.0018(2/1115)	0.0423(13/307)	0.0030(14/4658)	0.012(11/920)	0.0154(32/2072)
Infected snail prevalence (%)	1.23(7/569)	0.17(2/1180)	5.31(13/245)	0.06(14/23802)	0.51(11/2172)	0.14(32/22116)
Number of acute schistosomiases	2	1	0	0	0	0
Expected prevalence in humans	≥10%	5-10%	5-10%	5-10%	5-10%	5-10%
Control measures						
Molluscicide						
Infected snail habitats	2 times	1 time	1 time	4 times	3 times	3-4 times
non-infected snail habitats	0	0	0	1-3 times	1-3 times	2-3 times
Selective treatment in humans	yes	yes	no, but in 2004	yes	no, but in 2004	no, but in 2004
Health education in humans	yes	yes	yes	yes	yes	yes

Note: M, for marshland; and H, for hilly region

Table 2.7.2 Censored population sizes of each species of definitive hosts in two regions in 2006 and 2007

Hosts	Year	Tongling, marshland				Shitai, hilly region			
		Guanghui(M)	Heping(M)	Xingzhuang(M)	Total	Longquan(H)	Longshang(H)	Yuantou(H)	Total
Humans*	2006	325(1213)	107(866)	91(984)	523(3063)	107(553)	76(493)	62(343)	245(1389)
	2007	125(1244)	39(910)	24(895)	188(3049)	52(601)	61(507)	37(311)	150(1419)
Cattle	2006	281	22	45	348	1	7	-	8
	2007	-	-	-		1	6	-	7
Buffalo	2006	36	11	13	60	-	-	-	
	2007	-	-	-		-	-	-	
Pigs	2006	37	34	48	119	303	257	212	772
	2007	31	27	35	93	284	329	241	854
Goats	2006	-	-	21	21	-	-	-	
	2007								
Dogs	2006	20	18	36	74	43	29	17	89
	2007	32	35	34	101	36	48	26	110
Cats	2006	30	3	1	34	25	45	43	113
	2007	10	8	4	22	18	49	28	95
Rodents†	2006	(146)	(100)	(88)	(334)	(141)	(142)	(141)	(424)
	2007	(113)	(112)	(111)	(336)	(106)	(108)	(110)	(324)

Note: * Numbers in parentheses refer to the people who underwent an antibody test for detection of *S. japonicum* and numbers out parentheses to the people positive with the test. † Numbers in parentheses refer to trap×days used for small rodents.

M, for marshland; and H, for hilly region

Table 2.7.3 Snail distribution in the sampled villages in 2006 and 2007

Villages	Years	Snail habitats	Infected snail habitats*	Snail areas (m^2)	Snail density (/0.11m^2)	Infected snail density (/0.11m^2)	Infection prevalence (%)
Guanghui(M)	2006	2	1(1)	600200	0.73	0.0036(6/1667)	0.49(6/1222)
	2007	2	1(1)	600200	0.32	0.0018(3/1704)	0.55(3/544)
Heping(M)	2006	2	2(2)	275800	0.75	0.0166(14/843)	2.22(14/632)
	2007	2	2(2)	399650	0.67	0.0026(3/1135)	0.40(3/756)
Xingzhuang(M)	2006	1	1(1)	100000	1.10	0.0062(2/322)	0.56(2/355)
	2007	1	1(1)	123100	0.92	0.0027(1/366)	0.30(1/336)
Longquan(H)	2006	30	5(0)	90754	2.82	0.0063(17/2722)	0.22(17/7683)
	2007	30	5(1)	90754	2.28	0.0073(16/2204)	0.32(16/5018)
Longshang(H)	2006	35	3(1)	62612	1.79	0.0098(10/1020)	0.55(10/1823)
	2007	38	10(1)	53676	2.67	0.0454(56/1233)	1.70(56/3296)
Yuantou(H)	2006	34	5(1)	83977	2.48	0.0178(24/1352)	0.72(24/3349)
	2007	34	2(1)	83977	2.23	0.0034(4/1174)	0.15(4/2613)

Note: *Numbers in parentheses indicate the same infected habitats also found in the previous year

M, for marshland; and H, for hilly region

Table 2.7.4 Prevalence and infection intensity in populations of different definitive hosts in 2006 and 2007

Hosts	Years	Tongling, marshland							Shitai, hilly region						
		No. infected/No. examined				Prevalence % (95% CI)	Infection intensity *		No. infected/No. examined				Prevalence % (95% CI)	Infection intensity *	
		GH (M)	HP (M)	XZ (M)	Total		Mira/g faeces (Range)	EPG (Range)	LQ (H)	LS (H)	YT (H)	Total		Mira/g faeces (Range)	EPG (Range)
Humans †	2006	1/325	0/107	1/88	2/520	0.4 (0.0-1.4)		No eggs	0/96	5/51	3/52	8/199	4.0 (1.8-7.8)		No eggs
	2007	2/107	1/33	0/24	3/164	1.8 (0.4-5.3)		No eggs	2/51	6/59	1/34	9/144	6.3 (2.9-11.5)		4.8 (0-88)
Cattle	2006	42/100	4/19	27/38	73/157	46.5 (38.5-54.6)	1.4 (0.1-9.6)	1.9 (0.3-9.8)	0/1	0/7	-	0/8	0 (0-31.2)		
	2007	-	-	-					0/1	0/6	-	0/7	0(0-34.8)		
Buffalo	2006	3/25	0/6	1/7	4/38	10.5 (3.0-24.8)	3.4	3.4							
Pigs	2006	0/7	-	0/12	0/19	0 (0-14.6)			-	0/23	0/19	0/42	0 (0-6.9)		
	2007	2/25	0/1	0/26	2/52	3.9 (0.4-13.2)	1.9 (0.3-11.4)	2.6 (0.5-13.5)	-	0/20	-	0/20	0(0-13.9)		
Goats	2006	-	-	11/20	11/20	55 (31.5-77.0)	-	-	-	-	-				
Dogs	2006	2/8	0/10	0/24	2/42	4.8 (0.5-16.2)	-	-	9/34	1/23	4/17	14/74	18.9 (10.7-29.7)	1.1 (0.4-2.2)	1.3 (0.4-2.8)
	2007	4/25	0/29	3/29	7/83	8.4 (3.5-16.6)	1.7 (0.1-210.6)	3.1 (0.6-214.0)	7/27	5/21	3/23	15/71	21.1 (12.3-32.4)	0.5 (0.0-15.4)	0.7 (0.1-16.6)
Cats	2006	0/1		0/1	0/2	0 (0-77.6)			1/5	0/17	0/17	1/39	2.6 (0.1-13.5)	-	-
	2007	3/6	0/2	-	3/8	37.50 (8.4-75.6)	3.0 (0.2-27.2)	3.9 (0.4-27.2)	0/1	1/3	0/15	1/19	5.3(0.1-26.0)	0.1	0.2
Rodents	2006	0/7	0/1	0/1	0/9	0 (0-28.3)			6/18	5/22	2/9	13/49	26.5 (14.9-41.1)	249.5 (40.3-3307.5)	230.7 (40.3-3311.7)
	2007	0/3	-	-	0/3	0 (0-63.2)			4/12	3/22	2/17	9/51	17.7 (6.4-35.1)	48.5 (4.8-3246.7)	51.9 (4.8-3268.3)

Note: * Infection intensity is calculated as geomean of miracidia per gram of faeces or EPG. † Only the individuals positive with an antibody test received stool examination, therefore prevalence in humans is estimated among seropositive individuals. Mira, for miracidia. GH, HP and XZ refer to Guanghui, Heping and Xingzhuang respectively; LQ, LS and YT for Longquan, Longshang and Yuantou, respectively. M, for marshland; and H, for hilly region

2.8 FIGURES

Figure 2.8.1 Schematic map of geographical locations of sampled villages from Tongling County and Shitai County in Anhui of China.

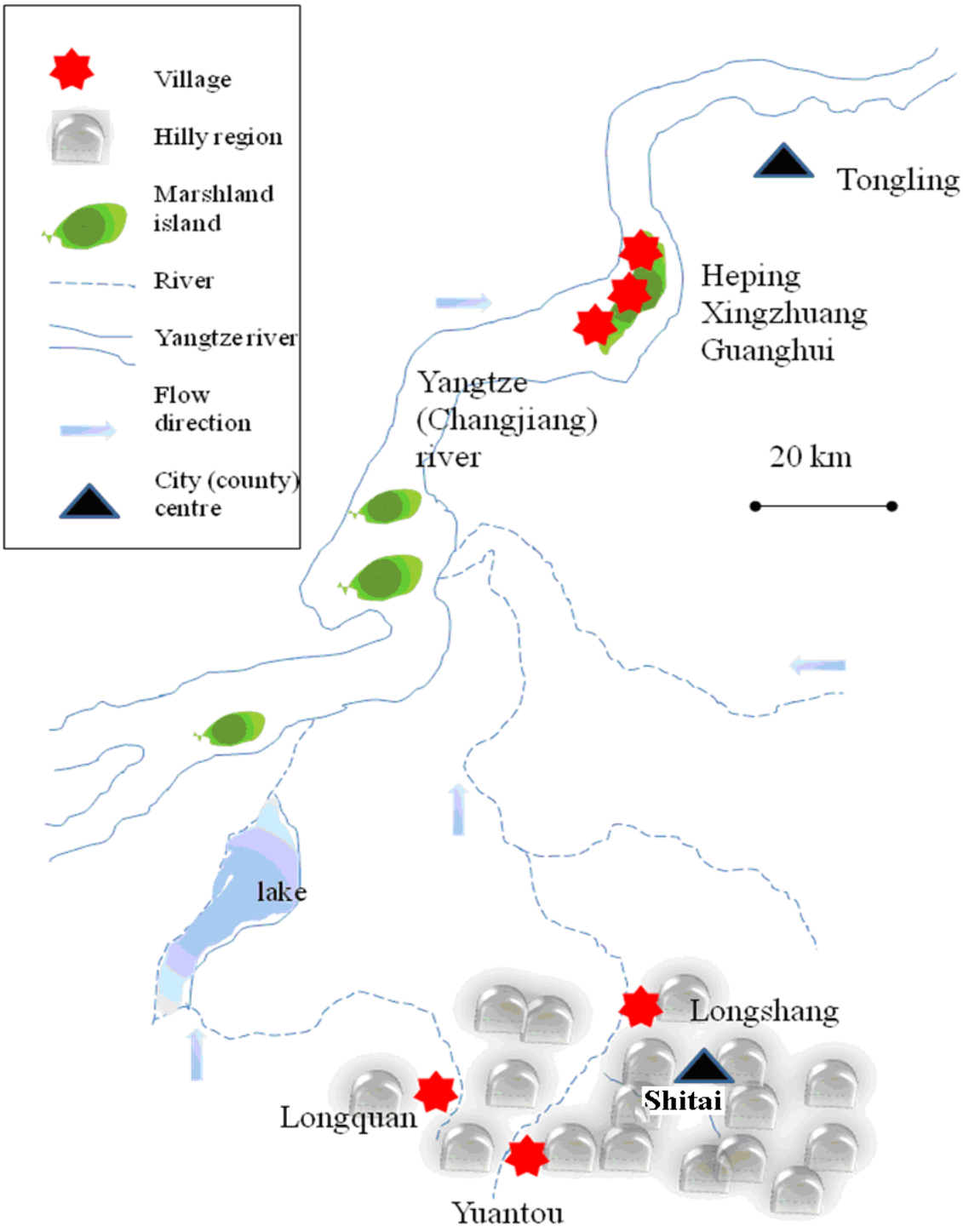
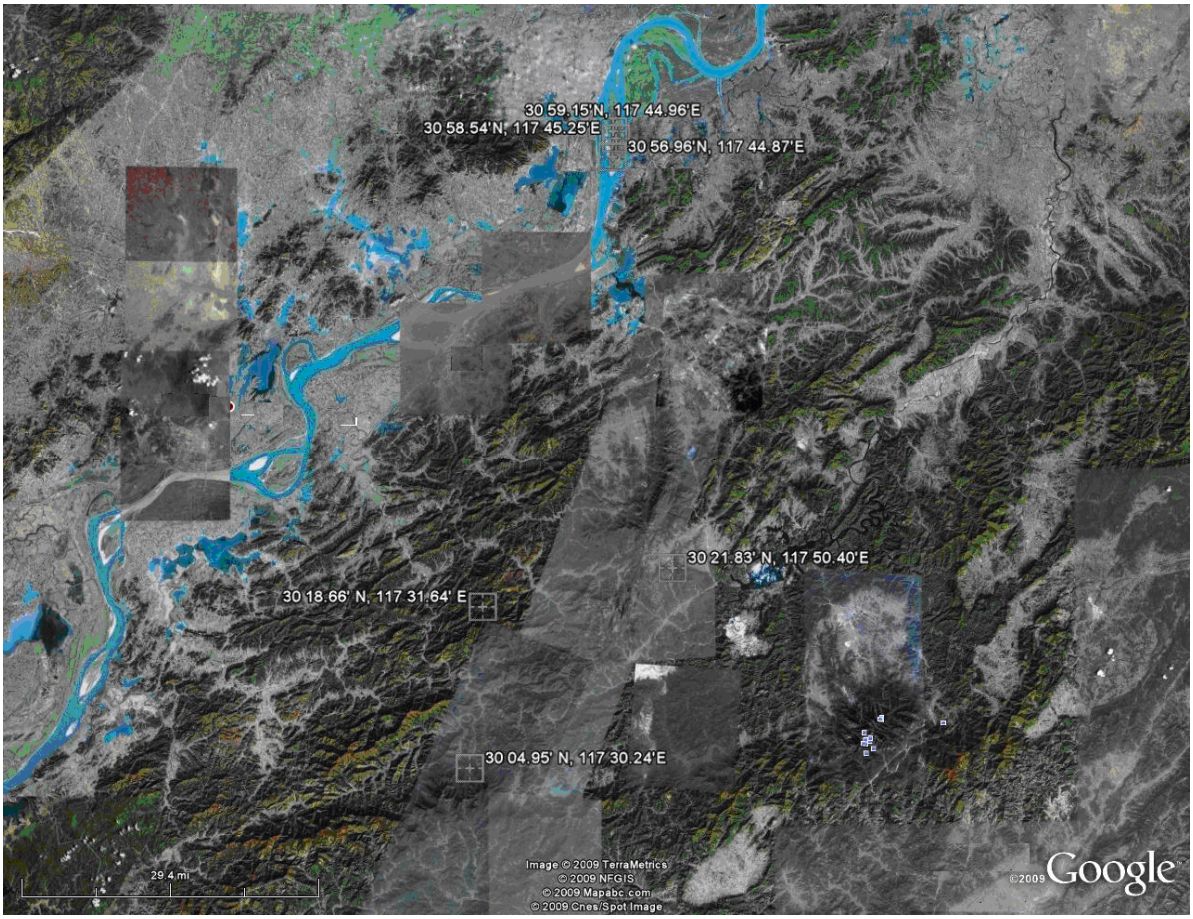


Figure 2.8.2 Map of geographical locations of sampled villages from Tongling County and Shitai County in Anhui Province of China. The villages labelled with coordinates are, from top to bottom, Heping, Xingzhuang, Guanghui in Tongling County (marshland) and Longshang, Longqun and Yuantou in Shitai County (hilly region). Image courtesy of Google Earth (http://earth.google.co.uk/)



Chapter 3: Contrasting reservoirs for *Schistosoma japonicum* between marshland and hilly regions in Anhui, China: inferred from circadian rhythm of cercarial emergence

Results were presented at BSP Spring Meeting 2008 and were integrated into the manuscript entitled 'Evolution in a multi-host parasite?: Chronobiological circadian rhythm and population genetics of *Schistosoma japonicum* cercariae indicates contrasting definitive host reservoirs by habitat' with authors as 'Da-Bing Lu, Tian-Ping Wang, James W. Rudge, Christl A. Donnelly, Guo-Ren Fang, & Joanne P. Webster (Corresponding author)', which has been accepted by *International Journal for Parasitology* for publication.

3.1 ABSTRACT

Schistosomiasis japonica is a disease of profound medical and veterinary importance which has remained endemic in many regions and re-emerged where previously controlled. One potential explanation for this persistence is that *Schistosoma japonicum* is unique in being a zoonotic human schistosome, with over 40 species of mammals suspected as definitive host reservoirs, although their relative roles, particularly concerning wildlife, remains to be ascertained. Based on the results from the first year of epidemiological surveys in the marshland and hilly regions, as shown in Chapter 2, and under the assumption of rodents as potential reservoirs, infected snails were collected from two contrasting ecological regions in Apr. 2007 and three chronobiological trials of *S. japonicum* cercarial emergence, an adaptive behavioural trait genetically controlled and shaped by definitive host behaviour, were performed. Two distinct modes of cercarial emergence were identified, with a late afternoon shedding pattern found in the hilly region, compatible with a nocturnal rodent reservoir, and a morning-afternoon dual shedding pattern within the marshland region consistent with a diurnal bovine major reservoir. The results obtained raise applied, in terms of targeted control, and theoretical, in terms of parasite evolution and speciation, implications.

3.2 INTRODUCTION

Schistosoma japonicum is the causative agent of one of the most important human parasitic diseases in the Far East, including the Philippines, China and Indonesia, with 40million at risk within China alone (Gray *et al.*, 2008). Of the human schistosomes, *S. japonicum* is unique in being the only schistosome for which zoonotic transmission is considered important, with over forty species of wild and domesticated animals suspected of serving as reservoir hosts for this parasite (He *et al.*, 2001). The role of both humans and agriculturally important domestic animals, in particular bovines, in the transmission of the disease has been acknowledged and control measures have focused upon them (see 2.2 in Chapter 2). In contrast, potential wildlife reservoirs, for example, small rodents, have received scant, if any, attention in China. Considering their nature of biomass and behavioural patterns, such wildlife may well play an important role in the transmission, and perhaps particularly in the maintenance of *S. japonicum* at low levels, reflected by low prevalence in humans, or indeed in the reported re-emergence in previously eliminated areas, mostly belonging to the mountainous/hilly regions (Zhou *et al.*, 2005; Liang *et al.*, 2006). However, the inherent logistical difficulties in sampling from such wildlife in a routine parasitological survey may have hampered, to a larger extent, any efforts in elucidating the potential role of wildlife in the transmission or re-emergence of *S. japonicum*.

Transmission of *S. japonicum* between hosts occurs through two larval stages, miracidia, infective to the mollusc, and cercariae, infective to the mammal. The probability of successful transmission of cercariae from snails to vertebrate hosts may be increased through peaks of *S. japonicum* cercariae in emergence from their intermediate host

Oncomelania hupensis snail in an appropriate chronobiological rhythm. Such circadian rhythm of cercarial emergence has been considered as an adaptive behaviour and has been well documented for other species of schistosome (Theron, 1989; Pages & Theron, 1990b; Combes, 1991; Combes *et al.*, 1994). Intra-specific cercarial chronobiology diversity within *Schistosoma*, associated with different definitive host species (e.g. rodents versus humans), has been demonstrated for *S. mansoni* on certain Caribbean islands (Chasse & Theron, 1988). Within certain Caribbean island populations, *S. mansoni* has been demonstrated to display different circadian rhythms of cercarial emergence between populations of schistosome originating from human hosts and those originating from murine hosts, where cercariae for rodent transmission peaks in the evening, according to the nocturnal activities of these host species, whilst those transmitted through humans show peak cercariae release only at midday, coinciding with peak exposure activities of this host species. Polymorphisms in cercarial emergence have also been observed in *S. japonicum*. Within the laboratory, using snails infected with *S. japonicum* miracidia from mainland China, Theron and Xia (Theron & Xia, 1986) demonstrated diurnal periodicity of emergence during the light period, with a peak emergence occurring between 2pm and 5pm. In contrast, in the Philippines it has been reported that the maximum emergence of *S. japonicum* cercariae was around 6-8pm (Pesigan *et al.*, 1958b), where a different snail (*Oncomelania hupensis quadrasi*) serves as the intermediate host. Such timing differences in cercarial shedding have also been shown to be genetically controlled either from within species (*S. mansoni*) (Theron & Combes, 1988) or from different species (*S. haematobium, S. intercalatum, S. bovis*) (Pages & Theron, 1990b). To date no data are yet available relating to potential

associations of definitive host species, for example, diurnal versus nocturnal animals, with the rhythmic behaviour of *S. japonicum* cercariae. However, such differences in *S. japonicum* cercarial circadian shedding patterns may well be predicted in relation to definitive host species.

The epidemiological investigations of *S. japonicum* among definitive hosts in two different geographical regions in 2006 revealed the highest infection prevalence in cattle (and goats in one village) within the marshland areas, whilst infection prevalence was highest within rodents within the hilly areas (Chapter 2). The current study thus tested, for the first time, if there is any intra-specific variation by potential definitive host species in the circadian rhythms of cercarial emergence of *S. japonicum* from infected snails collected in April of 2007 from two contrasting endemic settings, marshlands with schistosomiasis persistence versus hilly habitats with the re-emergence of the disease. In particular I predicted an early diurnal shedding in the marshland where cattle were suspected as key reservoirs, and a late for the nocturnal wild rodent species which was suspected from the prevalence survey to be key reservoirs within the hilly region.

3.3 MATERIALS AND METHODS

3.3.1 Infected snails

Systematic surveys of snails implemented in 2007 were detailed in Chapter 2. Habitats with infected snails were subjected to a follow-up investigation in order to obtain additional infected snails where necessary. During a second snail survey, out of 2784 snails sampled from the three marshland villages and 7017 snails from the three hilly villages, a total of 66 and 91 infected individuals respectively were identified using the cercariae shedding method (Ministry of Health of P. R. China, 2000). Infected snails from the same habitat were kept in a labelled pan (30cm ×18cm×6cm) and raised in the laboratory at the Anhui Institute of Parasitic Diseases, China, without air conditioning or artificial light, for two weeks prior to experiments.

3.3.2 Circadian rhythm of cercarial emergence

Three chronobiological trials, starting at different times: either 7am, 1pm or 7pm, each of a consecutively 24 hrs, were carried out in a laboratory at the Anhui Institute of Parasitic Diseases, China. A routine chronobiological test of cercariae emergence, as described by (Mouahid & Theron, 1986) for *S. mansoni*, usually lasts over two or more consecutive days. However, it was deemed unreasonable for *Oncomelania hupensis hupensis* snails to be restricted in water over such a long period, because this species of amphibious snails prefer living on moist earth at maturity (Mao, 1990). Furthermore, previous field surveys of cercariae in waters within the marshland/lake regions using sentinel mice have demonstrated a different peak of infection during daytime between provinces, one cause of which may be due to an occasional report of the ability for *S. japonicum* cercariae to

exit rapidly within a short time of contacting water (Mao, 1990). Therefore, in addition to the trial started at 7am based on natural dawn-dusk, two further time-trials, from 1pm or 7pm, were also performed here to test the influence of starting time on cercarial emergence. Table 3.7.1 lists the grouping of living snails used for each test. All time-trials followed the same experimental procedures. Tissue culture plates (24-well), with a cover to prevent snails from climbing out, were used for cercarial shedding in order to directly count cercariae under a binocular microscope. Each well contained one snail with 2.8 ml of dechlorinated water. Snail transfer was performed at 2h interval throughout a 24h period. Considerable attention was made during the transfer of each snail into a new plate to ensure no cercariae adhered to the snail. Cercariae quantification was carried out after staining of the individual cercariae with Lugol's iodine solution (Pages & Theron, 1990b), which stains glycogen in non-keratinized squamous epithelia to brown. Cercariae on the cover were also counted and classified into the corresponding well during analyses.

Air temperatures and light intensity, inside and outside the laboratory, with a Digital Lux Meter (TES1332A, No 040903033, Taiwan), were measured at the beginning of each 2hr interval. The total numbers of cercariae from each infected snail over 24hrs were compared between the two regions for each trial. Analyses of circadian rhythms were conducted with calculation of the mean (standard errors of the mean, SEM) number of emerging cercariae within each 2hr interval. As the number of cercariae produced per snail from each region in the three trials did not always fit a normal distribution, even after log-transformation, the Mann-Whitney U test, a nonparametric method, was employed to explore any differences between the two regions. Spearman's rank

correlation coefficient, between the quantity of cercariae per snail at each 2hr interval and the room temperature (˚C) and light intensity (lux), without making any assumptions about the frequency distribution of cercariae production, was calculated to investigate the effect of environmental factors on cercarial emergence. All calculations were conducted with SPSS11.0 software (**SPSS Inc.**, 2002).

3.4 RESULTS

3.4.1 Chronobiology of cercarial emergence

The number of infected snails from each village used in the following experiments can be seen in Table 3.7.1. From the three time-trials, as seen in Figure 3.8.1(a-c), the cercariae from the hilly region consistently revealed a distinctive emergence rhythm with maximum emergence around 3pm to 9pm, referred to here as a late afternoon shedding pattern; however, in the marshland region, a morning shedding pattern around 7am to 11am was apparent. When comparing trial 1 and trial 2 (Figure 3.8.1(a-b)), a high peak of cercariae emergence appeared at the beginning of the trial period, referred to here as an initial shedding pattern. This shedding pattern may be masked or overlapped by a temporal morning shedding pattern as shown in Figure 3.8.1(a). On trial 2, all the snails from the marshland each showed a high peak of initial cercariae shedding in the afternoon and a small shedding peak on the next morning.

Complex phenotypes of cercariae emergence were found in the marshland in trial 3, which started from 7pm (Figure 3.8.1(c)). After a subsequent analysis of the results by village, it was clear that the circadian rhythm of cercariae emergence in one marshland village, Guanghui, was nearly the same as that found in the hilly region (Figure 3.8.1(c-d)). Further analyses by individual snails showed that three snails in Guanghui shed cercariae in a similar pattern to the snails from the hilly villages, while one snail displayed the same shedding pattern as those in the other marshland villages. Meanwhile, it was also apparent that among snails from another marshland village, Xingzhuang, an initial shedding pattern may not happen when shedding time started at night (Figure 3.8.1(d)).

3.4.2 Relationship between cercarial emergence and light and temperature

The mean total count of cercariae per snail was significantly higher for the parasites with late emergence in the hilly than for those with morning emergence in the marshland (Table 3.7.2). Figure 3.8.2 shows daily change in air temperature (˚C) and light intensity (Lux) inside and outside laboratory in three trials. The maximum temperature variation inside the room between day and night was 2.5 ˚C in trial 1, 1 ˚C in trial 2, and 1.5 ˚C in trial 3. The maximum light intensity in the room over 24 h in the three tests was 790, 934 and 1064 Lux, respectively, always at around 11am. The mean number of cercariae (with morning emergence) shed by snails from the marshland areas during each 2 h interval was significantly and positively correlated with light intensity in all trials, whereas a significant and positive correlation between room temperature and quantity of cercariae per snail from the hilly areas was found in trial 1 and trial 3 (Table 3.7.3).

3.5 DISCUSSION

Within the current study, analyses of the cercarial shedding patterns of *S. japonicum* from two different geographical regions (marshland versus hilly region) within Anhui Province in China demonstrated chronobiological polymorphism of cercarial emergence with an obvious difference between the two regions. Two shedding modes, at the level of the snail intermediate host were identified, with a late afternoon shedding pattern found in the hilly villages, and a morning plus initial shedding (a shedding peak within the first 2h of the shedding process) pattern generally found in snails from the marshland region (although a small number of snails from the marshland region also displayed a late afternoon shedding pattern); in no individual infected snails did both modes showed simultaneously. These results lend further support to my hypothesis that the main reservoirs for the parasite in the hilly areas may be different from those in the marshland, and that *S. japonicum* may be speciating towards different definitive hosts in these areas.

Research on *S. mansoni* populations within certain island populations have found that this parasite may show an early or a late shedding pattern of their cercarial emergence depending on the predominance of either human or murine definitive hosts in each transmission zone (Theron & Mone, 1984; Chasse & Theron, 1988). Correspondingly, for *S. japonicum* in this study, a late afternoon shedding pattern may be suitable to infect predominantly rodents, while a morning shedding pattern may be better to infect predominantly bovines. Such chronobiological results, provided by consideration of the differential infection profiles by host species observed in Chapter 2 (Table 2.7.4), would have a substantial practical implication for schistosomiasis control in both regions. For

example, in the hilly region, where frequent infections were also found in dogs and humans, no *S. japonicum* with a character of early shedding from the local snails was observed, potentially suggesting a transmission dynamics of rodents to intermediate host snails, and then to rodents, humans and dogs, given that human infections were once eliminated in these areas. Whereas, in the marshland, although no infected rodents were identified, *S. japonicum* cercariae from a few snails showed a late shedding pattern. One explanation may relate to the smaller number of rodents sampled in these areas. This raises a future concern of the possibility of the transmission persistence or re-emergence in the marshland even if the parasite in humans and domestic animals is eliminated.

As mentioned above, two modes (late afternoon shedding, and morning with initial shedding) were observed in the same marshland village, but each was on different individual snails. Previous studies have shown that mixed infection of the freshwater snail *Biomphalaria glabrata* by *S. mansoni* and *S. rodhaini* influenced cercarial production and chronobiology, but such inter-specific competition between species did not result in a large change in chronobiological emergence of cercariae for either species (Norton *et al.*, 2008). When *B. glabrata* was infected with two trematodes of a single species, *S. mansoni*, one with an early and the other with a late cercarial chronobiology, each strain of schistosome kept its own cercarial emergence rhythm (Theron *et al.*, 1997). Furthermore, field investigations have shown that a single *Bulinus globosus* snail can be infected naturally by up to six different genotypes of *S. haematobium* miracidia (Davies *et al.*, 1999), thus the possibility of multiple *S. japonicum* infections in snails should not be ruled out in this experiment here. Such may lead to interesting avenues for future

research regarding potential intra-intermediate host interactions and intra-definitive host interactions in mixed infections of both *S. japonicum* with early and late shedding cercarial phenotypes.

S. japonicum displaying only a morning shedding pattern appears to always be accompanied by an initial shedding pattern, the latter occurring only during daytime. The phenomenon of an additional initial shedding of *S. japonicum,* which appeared from the snails collected from the marshland, is a feature not reported for *S. mansoni*. One could speculate that the mechanism underlying this might therefore relate to the biological property of *O. hupensis* snails. It is evident that, although the intermediate host snails *O. hupensis* are amphibious, they almost immediately climb out of water (if possible) when submerged, preferring to live on moist earth rather than in water (Mao, 1990), in contrast to the more aquatic nature of snail hosts of the other human schistosome species (*S. mansoni, S. haematobium,* and *S. intercalutum*). Consequently, it seems likely that there will be very strong selective pressures placed upon *S. japonicum* cercariae for rapid shedding within a short period before their intermediate hosts leave the water in order to maintain maximum transmission in nature. However, it may be surprising that *S. japonicum* with a late afternoon shedding pattern, which were found both in the hilly region and in the marshland, did not show a character of initial shedding, one highly possible explanation for which may be accredited to the different 'strains' of *S. japonicum*.

Indeed, the phenotypic chronobiological divergence reported here was complemented by a range of other phenotypic traits contrasting between the two habitat types. For instance,

S. japonicum cercariae production per snail differed significantly between snails from the two contrasting habitats or between snails with different cercarial shedding patterns (Table 3.7.2). One could perhaps speculate that this may reflect trade-offs due to the different definitive host reservoirs, with higher cercarial shedding in the hilly region reflecting a compensatory mechanism for the parasite to counteract the shorter-lived rodent host in comparison to the longer-lived bovine main hosts of the marshlands. Cercarial emergence from marshland snails also appeared to be more sensitive to light intensity than snails from hilly areas, whereas cercarial shedding in the hilly region appeared to be more sensitive to temperature change (Table 3.7.3). A possible mechanism underlying this difference may relate to a difference in microhabitats within each region. For example, the irrigation ditches of the hilly areas are likely to have cooler water and be more shaded compared to the more open marshy habitats along the river flood plains in the warmer marshland areas, where temperature fluctuations may be relatively frequent and rapid.

However, alternative, not necessarily mutually exclusive explanations, rather than strain differences between the two sites in relation to main definitive host reservoirs, should also be considered for the traits observed. For example, as regards the observed infection intensity profiles of snails collected from the contrasting habitat sites, one could propose that this reflected a difference in terms of how long the snails had been infected at the time of these particular experiments, due to a temporal difference in the transmission 'seasons'/periodicity between regions. Indeed, *S. japonicum*-infected snails from both China and the Philippines have been observed to go through high- and low-shedding phases according to time post-infection (Ni *et al.*, 1992; Ishikawa *et al.*, 2006). However,

while this might account for a difference in overall shedding intensities, it is unlikely to explain the different chronobiological shedding patterns, nor the difference in sensitivity to light and temperature.

One must also consider that parasite diversity may reflect intermediate host diversity as well as, or potentially instead of, definitive host diversity, due to close co-evolved host-parasite relationships (Blair *et al.*, 2001; Zavodna *et al.*, 2008). Although a significant difference in daily shedding peaks between *S. intercalatum* cercariae emerging from *B. crystallinus* and *B. globosus* was once reported (Pages & Theron, 1990a), no difference was found in the emergence of *S. bovis* cercariae from two intermediate host species, *B. truncatus* and *Planorbarius metidjensis* (Mouahid & Theron, 1986), and of *S. mansoni* cercariae from *B. tenagophila* and *B. glabrata* (Favre *et al.*, 1997). Given that the snail-intermediate hosts from all locations in this study are of the same subspecies and morphology (ribbed-shelled *O. h. hupensis*), one may therefore suspect that the observed phenotypic differentiation of *S. japonicum* cercariae are more likely to reflect the proposed differences in main definitive host species by habitat type on parasite diversity rather than intermediate host pressure in this region.

To conclude, novel chronobiological trials of parasite larval emergence from field-collected snail intermediate hosts from two contrasting habitats within the same province of China, combined with complementary phenotypic analyses, strongly suggests here that the main reservoirs for the parasite in the hilly region may be nocturnal animals, different from cattle in the marshland. Furthermore, the phenotypic differentiation of the parasites

identified here between and within the two contrasting ecological regions might reflect the evolution of a strain complex.

3.6 ACKNOWLEDGEMENTS

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3.7 TABLES

Table 3.7.1 Infected snails by village used in chronobiological time-trials

Villages	Sampled snails	Infected snails	Infected snails alive until tests	Snails used in Time-trial 1 (From 7am)	Snails used in Time-trial 2 (From 1pm)	Snails used in Time-trial 3 (From 7pm)
Guanghui(M)	481	39	24	11	9	4
Heping(M)	1243	0				
Xingzhuang(M)	1060	27	23	11	6	6
Longquan(H)	2272	30	27	0	14	13
Longshang(H)	2382	27	26	22	4	0
Yuantou(H)	2363	34	22	0	6	16

Note: M, for marshland; and H, for hilly region

Table 3.7.2 Statistical comparisons in quantity of emerged cercariae per snail between two regions

Trials	Regions	Snails	Mean cercariae per snail	Std. Deviation
Trial 1	Tongling, marshland	22	751.36	395.24
	Shitai, hilly region	22	1530.55	866.13
	Mann-Whitney U, p=0.001, 2-tailed			
Trial 2	Tongling, marshland	15	1142.60	996.86
	Shitai, hilly region	24	2121.96	1008.11
	Mann-Whitney U, p=0.002, 2-tailed			
Trial 3	Tongling, marshland*	10(7)	1254.70(810.00)	998.85(488.57)
	Shitai, hilly region	29	1654.17	965.90
	Mann-Whitney U, p=0.157(0.016), 2-tailed			

Note: * the numbers in the brackets exclude the snails or cercariae with late emergence

Table 3.7.3 Rank (Spearman) correlation coefficient for the correlation between the mean cercariae per snail at intervals and the light and temperature inside room

	Time-trial 1		Time-trial 2		Time-trial 3	
Snail origins	Light intensity (Lux)	Temperature (˚C)	Light intensity (Lux)	Temperature (˚C)	Light intensity (Lux)	Temperature (˚C)
Tongling, marshland *	0.77	0.19	0.69	-0.56	0.50(0.78)	0.23(-0.06)
p (2-tailed)	0.003	0.561	0.012	0.060	0.099(0.003)	0.476(0.86)
Shitai, hilly region	-0.25	0.82	-0.10	0.35	-0.15	0.65
p (2-tailed)	0.438	0.001	0.750	0.265	0.651	0.023

Note: * the numbers in the brackets in trial 3 exclude the cercariae with late emergence

3.8 FIGURES

Figure 3.8.1 Chronobiology of S. japonicum cercarial emergence from snails. Figure shows shedding patterns of cercariae from snails in Tongling (marshland) and in Shitai (hilly region) over a 24hrs, expressed as mean (±SEM) cercariae production per snail within each 2h interval (a, starting at 7 am; b, starting at 1pm; c, starting at 7pm; and d, shedding patterns between two marshland villages starting at 7pm. Arrows refer to the time point when snails were put into water).

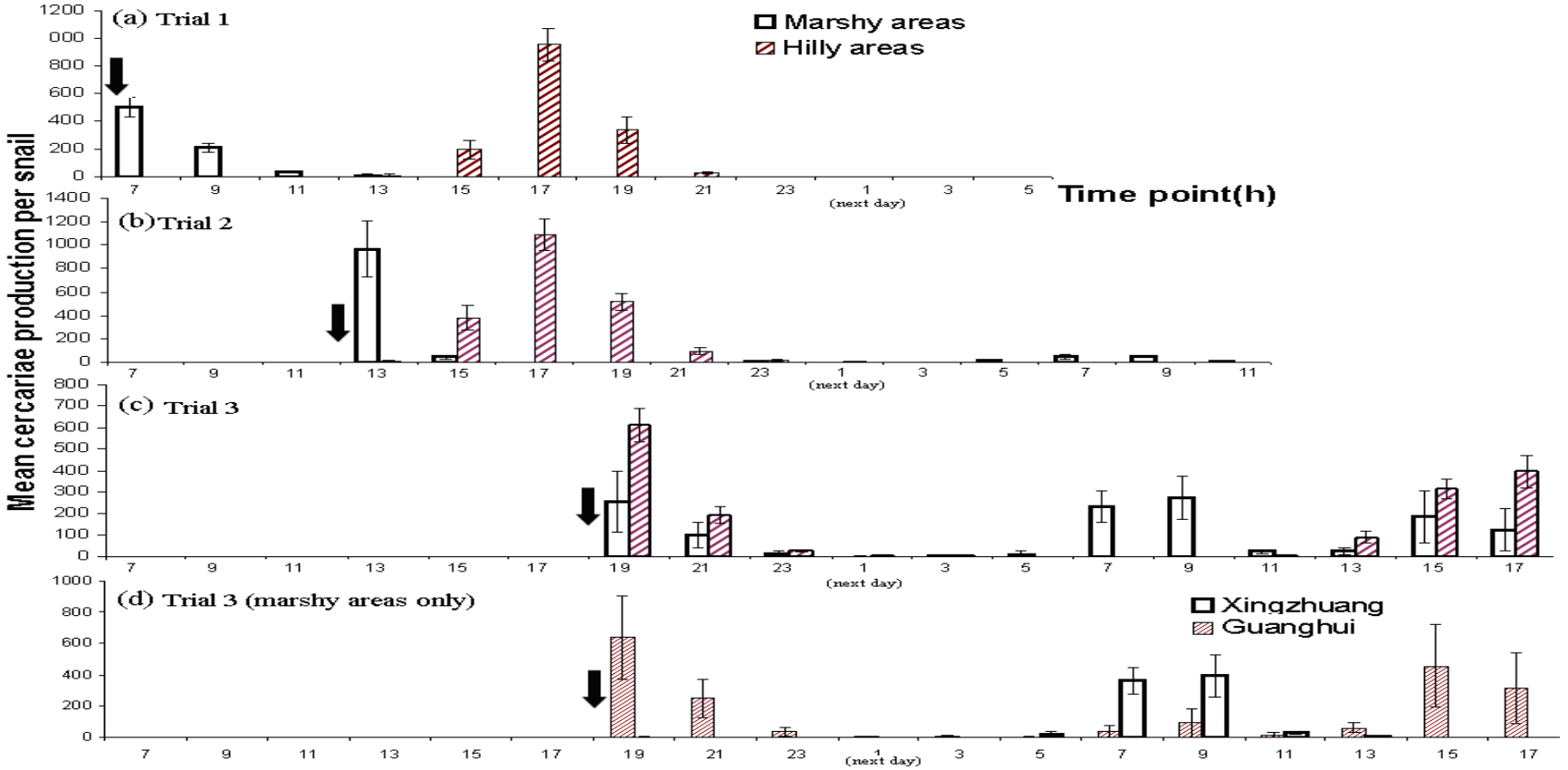
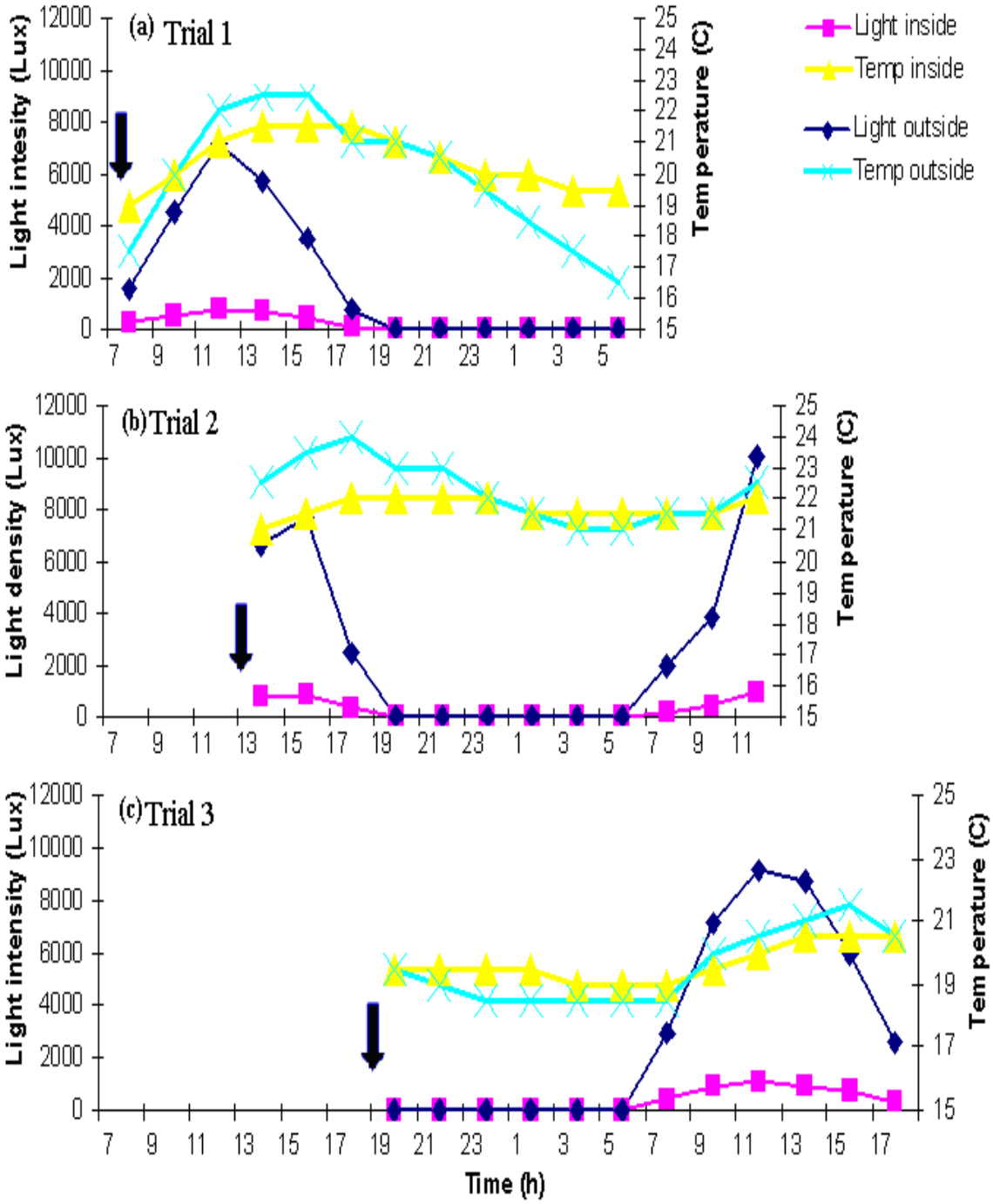


Figure 3.8.2 Daily change in air temperature (˚C) and light intensity (Lux) inside and outside laboratory in three trials. The values were measured only at the beginning of each 2h shedding intervals over 24hr (a, for starting at 7 am; b, for starting at 1pm; c, for starting at 7pm. Arrows refer to the time point when snails were put into water. Temp, for temperature).



Chapter 4: Contrasting reservoirs or transmission patterns for *Schistosoma japonicum* between marshland and hilly regions in Anhui, China: inferred from the genetic composition of parasites within definitive hosts and intermediate hosts

4.1 ABSTRACT

The transmission process of *Schistosoma japonicum*, a multi-host parasite, remains unknown, as over forty species of mammals are suspected of serving as reservoir hosts for the parasite. However, knowledge of population genetic structure of the parasite at two larval stages of its lifecycle will be useful in defining and tracking the transmission pattern between intermediate hosts and definitive hosts. The first two chapters of this thesis have suggested a high possibility of different reservoirs between the marshland and hilly regions. Such may raise the question how the disease transmits in either region or even within each village, as this is mainly relative to the focus of implemented control measures. In this chapter, for each village, genetic diversities of three *S. japonicum* larval populations each collected in either Apr. 2006, Sept.-Oct. 2006, or Apr. 2007, were first calculated and compared, followed by measuring Pairwise F_{ST}s, a short-term genetic distance based on different alleles, of parasites between two larval stages and constructing phylogenetic trees of parasites. Results showed an obvious difference between two geographical regions in genetic diversity of parasites at levels of individual hosts or species, both for miracidia and cercariae. In each marshland village, very low genetic differentiation between miracidia from cattle and cercariae from snails would confirm the main reservoir of cattle in the marshland region; whereas, in the hilly region, besides rodents as the main reservoirs to maintain the disease within a limited territory, dogs with their higher mobility may play a role in transmission of parasites among territories.

4.2 INTRODUCTION

Although population biologists have been very successful in developing a formal understanding of the dynamics and evolution of single-host pathogens, understanding the more complex population biology of multi-host pathogens seems to be one of the major challenges to biomedical science in the 21st century (Woolhouse *et al.*, 2001), as the process or pattern by which definitive hosts acquire infections and spread the parasites remains unknown. This is especially true for *Schistosoma japonicum*, a multi-host parasite infecting over forty species of mammals (He *et al.*, 2001), which may be transmitted or spread through one or more species of hosts. Molecular tools in particular allow such complex transmission patterns or transmission efficiency to be investigated, and such studies will present a valuable opportunity to gain much needed insight into the transmission and epidemiology of the parasite.

S. japonicum exhibits a complex life cycle with obligatory alternation of sexual phase within mammals and asexual reproduction within snails. Transmission between hosts occurs via two larval stages: miracidia and cercariae. A number of factors, including host aspects and environmental heterogeneity, may be predicted to influence such transmission process at the level of the population genetic structure of parasites (Huyse *et al.*, 2005). Definitive host movement, for example, may be an important determinant of parasite distribution, as both miracidia and cercariae have a short life span (hours) in the wild and a limited or passive mobility by water and the intermediate host snails have very low mobility. As reported by Blouin and colleagues (Blouin *et al.*, 1995), by comparing the population genetic structures of five species of parasitic nematodes from three

different hosts: *Ostertagia ostertagi* and *Haemonchus place* from cattle, *H. contortus* and *Teladorsagia circumcincta* from sheep, and *Mazamastrongylus odocoilei* from white-tailed deer, the parasites of sheep and cattle with higher mobility showed a pattern consistent with high gene flow among populations, while the parasite of deer with lower mobility showed a pattern of substantial population subdivision and isolation by distance mainly due to the limited host movement restricting gene exchange of parasites between different host individuals. Moreover, the variance in mobility between either individual hosts or distinct groups within same species could lead to the subdivision of parasites at individual levels or a group-specific population structure, for example, on *S. mansoni* in the marshy forest focus of Guadeloupe (French West Indies), in which parasites from male rats were genetically more diverse than parasites infecting female rats (Caillaud *et al.*, 2006).

Patterns of population subdivision and degrees of relatedness between parasite populations can provide information on transmission process or even transmission efficiency of the parasites. For example, multilocus enzyme electrophoresis was used to determine the clonal identities and genetic relationships of different serogroup strains of *Neisseria meningitidis*, and a group of *N. meningitidis* clones were considered as the source of the meningococcal disease on a focus in northern Norway (Caugant *et al.*, 1986). After comparing patterns of variation in the ribosomal DNA of *Ascaris* collected from humans in assumed non-endemic areas to those from pigs and humans from worldwide locations, a *Hae* III restriction site distinguished two classes of rDNA repeats. Repeats bearing this restriction site were found in >96% of parasites from pig populations

worldwide and in all worms from humans in North America. In contrast, repeats bearing this restriction site were detected in <2% of parasites from humans in endemic areas. Such results clearly suggested pigs as the source of infection in the humans in North America (Anderson, 1995). The investigation of the population genetic structure of *S. mansoni* and its definitive host *Rattus rattus* and intermediate host *Biomphalaria glabrata*, by using microsatellite markers, revealed that molluscs displayed a pattern of isolation by distance whereas such a pattern was found neither in parasites nor in rats, and a further comparison of the distribution of genetic variability in the parasites sampled from the definitive and intermediate hosts strongly suggested that dispersal of *S. mansoni* appears to be determined mainly by definitive host dispersal (Prugnolle *et al.*, 2005). Also in Guadeloupe, the transmission efficiency between definitive and intermediate hosts was estimated using Random Amplified Polymorphic DNA markers (Theron *et al.*, 2004). By investigating the genotypic composition of *S. mansoni* adults within *R. rattus* and cercariae within *B. glabrata,* both from the same transmission site, it appeared that a rat may recruit cercariae originating from an average of 30 infected snails, cercariae from an infected snail may infect three different definitive hosts, and two cercariae from an infected snail may infect the same definitive host.

Whilst there is an ethical and logistical difficulty in sampling *S. japonicum* adult worms from mammals, their progeny miracidia, which are hatched from eggs that are passed in faeces, are, in contrast, available. Moreover, a filter-paper-based, room temperature storage system for schistosome larval DNA has been successfully developed and a single larva DNA is able to be genotyped with up to 7 microsatellite loci in a multi-locus

microsatellite analysis (Gower *et al.*, 2007). Such recent methodologies thereby facilitate the sampling of both miracidia and cercariae populations in field and the subsequent population genetic analyses, thus avoiding the introduction of sampling bias through the loss of genotypes by sampling error or host-induced selection (Bremond *et al.*, 1993; Sire *et al.*, 2001a; Rodrigues *et al.*, 2002; Stohler *et al.*, 2004; Gower *et al.*, 2007).

The results from my previous epidemiological investigation of *S. japonicum* in two contrasting geographical regions (Chapter 2) and chronobiological cercariae emergence from the snails from the same locations (Chapter 3) have suggested that, in the hilly region, rodents may be the most important reservoir, mainly responsible for the re-emergence of the disease in the previously controlled areas; however in the marshland, cattle may have been playing a major part in the transmission of the disease. As female adult worms within their hosts are able to live for five to 30 years (Bush *et al.*, 2001) and the period of egg excretion in faeces post-infection (for infected species of mammals observed in two regions) may last for six months to over one year (He *et al.*, 2001), the miracidia found near the end of one transmission season (mainly from Jun. to Oct. (Li *et al.*, 2009)) could partly be either the result or the cause of the cercariae found at the beginning of the transmission season. However, the cercariae found at the beginning of the next transmission season (in the next year) may mainly result from the population of miracidia found in the first year, as infected snails have only a short life span of months (Liang *et al.*, 2005). Therefore, within each village, it may be predicted that: 1) there would be a host species-related genetic diversity of *S. japonicum* miracidia; and 2) there would be a closer relationship in terms of gene flow between the miracidia population

from the main contributor host species and the cercariae population from intermediate hosts, particularly between the miracidia population sampled this year and the cercariae population sampled in the next year.

To evaluate demographic parameters that are relevant to population genetic structure, parasite populations are ideally studied using neutral genetic markers (Huyse *et al.*, 2005), and the application of microsatellite markers for such studies has been recommended (Curtis & Minchella, 2000). The aims of this chapter were, therefore, by using recently identified *S. japonicum* polymorphic microsatellite loci (Shrivastava *et al.*, 2003), to study the population genetic diversity of *S. japonicum* larvae sampled in three consecutive periods: cercariae from snails in Apr. 2006 (at the beginning of the transmission season), miracidia from their definitive hosts (humans, bovines, goats, dogs, cats and rodents) in Sept.-Oct. 2006 (near the end of the transmission season), and cercariae from snails in Apr. 2007 (at the beginning of the next transmission) from the same areas and then, by tracking the movement of parasite alleles and comparing genetic diversity of parasites between two larval stages, to: 1) determine the main definitive host reservoir species for *S. japonicum*; and 2) elucidate the transmission dynamics within each village.

4.3 MATERIALS AND METHODS

4.3.1 Sampling larvae and genotyping

The investigation of *S. japonicum* infection in intermediate host snails and definitive host mammals in the sampled villages were described in Chapter 2 (See 2.3.2-3). Individual cercariae from infected snails or miracidia hatched from faecal samples from definitive hosts were picked up in laboratory (using a loop for cercariae or a pipette for miracidia) under a binocular microscope and washed twice through a series of transfer to a well on the plate containing autoclaved deionised water, in order to minimize the presence of contaminates. Following that, each larva was transferred to Whatman FTA® indicator cards for storage before use of PCR. DNA extraction from these samples was performed as described by (Gower *et al.*, 2007). Eight previously isolated and characterized *S. japonicum* microsatellite markers (Shrivastava *et al.*, 2003) were used in a novel multiplex PCR assay. For each locus, the 5' end of the forward primer was fluorescently labelled using one of 6-Fam, Vic, Ned and Pet dyes (Applied Biosystems, Cheshire, UK). DNA amplifications using PCR were performed on a PTC-200 Thermal Cycler (MJ Research-UK). Amplifications were performed in 25 µl reactions containing the sample DNA, 0.05µm for each of primers M5A, RRPS and J5, 0.1 µm for each of SMS9-1, MPA, MF1, TS2 and 2AAA, 3mM $MgCI_2$, ultra pure quality dNTP Mix and HotStartTaq DNA Polymerase (QIAGEN Multiplex PCR Kit, West Sussex, UK). Thermal cycling was conducted with a step-down PCR beginning with an initial hot-start activation of 15min at 95°C, followed by 42 cycles of 1min at 94°C, 1.5min at annealing temperature (2 cycles at each temperature from 60 to 55°C followed by 30 cycles at 55°C), and 1min at 72°C, with a final extension at 60°C for 30min. Routine gel electrophoresis on 1%

agarose was used on the PCR products to check for successful marker amplification. Following that the products were diluted in N, N'-dimethyl formamide with GS600LIZ Size Standard and analyzed using an ABI3730 automated sequencer (Applied Biosystems, UK). GeneMapper V4.0 (Applied Biosystems, UK) was used to assign allele sizes for each locus. Six primers (MPA, MF1, TS2, 2AAA, M5A and RRPS) amplified well and were thus used in the analyses of genetic datasets. Ten cercariae per snail for cercariae sampled in each April of 2006 and 2007, and 10-12 miracidia (if available) per individual host for miracidia sampled in Sept.-Oct. 2006 were genotyped and then used in molecular analyses.

4.3.2 Genetic analyses

Prior to the following genetic analyses, the repeated cercariae genotypes within individual snails were reduced to single copies with GENECAP (version 1.2.2) (Michael & Brian, 2004), a software to find matching genotypes by comparing each allele of a cercariae to all other alleles in all other compared cercariae, as at this stage the parasite undergoes asexual reproduction.

A parasite population is compartmentalized between individual hosts, and thus the parasites of an individual host can be considered an infra-population of the total parasite population (Anderson *et al.*, 1993; Fisher & Viney, 1998). The population structure of larval parasites for each species of hosts within each village was defined as containing 2 levels: (1) a parasite infra-population, the population of larvae found within an individual host; and (2) a parasite (total) population, all larvae found within all individuals sampled.

The common indices of diversity of the miracidia population for each species of definitive hosts and of the cercariae population, such as the number of alleles, the observed heterozygosity (**Ho**) and the expected heterozygosity (**He**) were computed with Arlequin (Excoffier *et al.*, 2005). The number of private alleles per locus within each population (exclusive to one population among all compared populations) was calculated with Genetic Data Analysis (Lewis & Zaykin, 2001).

Genetic structures of parasite populations from each species were investigated using Wright's *F*-statistics (Wright, 1965). F_{IS} (*f*) measures the within-infra-population magnitude of departures from Hardy-Weinberg equilibrium expectations while F_{ST} measures the genetic differentiation between infra-populations. Wright suggested the following qualitative levels for the interpretation of F_{ST} genetic differentiation: 0-0.05 'little'; 0.05-0.15 'moderate'; 0.15-0.25 'great'; and >0.25 'very great' genetic differentiation (Wright, 1978). For each species, the estimator *f* within infra-population and the estimator F_{ST} (among hosts) were calculated over all loci according to (Cockerham & Weir, 1984) using Genetic Data Analysis (Lewis & Zaykin, 2001). To assess the significance of the *F*-statistics obtained above, 95% confidence intervals were obtained by bootstrapping over loci with the number of replicates set to 15000.

Two groups of parasite populations were defined for calculation of population Pairwise F_{ST}s, a short-term genetic distance, between pairs of two larval populations, miracidia *vs* cercariae, to examine the relationship of the parasites between two larval stages. For each village, the first group consisted of the cercariae population sampled in April and the

miracidia populations from definitive hosts sampled in Sept.-Oct. 2006; the second group consisted of the miracidia populations sampled in Sept.-Oct. 2006 and the cercariae population sampled in Apr. 2007. Pairwise F_{ST}s were computed with Arlequin (Excoffier *et al.*, 2005) and their significance was tested through 15000 permutations. The null distribution of Pairwise F_{ST} values under the hypothesis of no difference between the populations was obtained by permuting individuals for 15000 times between populations. The p-value of the test was the proportion of permutations leading to a F_{ST} value larger or equal to the observed one. Since Cavalli-Sforza and Edwards's chord distance (Cavalli-Sforza & Edwards, 1967), assuming that there is no mutation and therefore all gene frequency changes are due to genetic drift alone, well reflects the dispersal rate between two populations and has low sensitivity to effective population size (Kalinowski, 2002), it was thus employed here to calculate the genetic distances between all pairs of parasite populations. Based on this genetic distance, UPGMA (Unweighted Pair Group Method with Arithmetic mean) trees were constructed with PowerMarker V3.25 (Liu & Muse, 2005) by bootstrapping with 1000 replications. The reliability of phenogram was assessed through obtaining a consensus UPGMA tree with Phylip V3.67 (Felsenstein, 2005) from the replications. The trees were finally visualized using MEGA version 3.1 (Kumar *et al.*, 2004).

4.4 RESULTS

4.4.1 Genetic diversity of cercariae within snails

In both regions over two years, the observed distribution of cercariae genotypes differed significantly from expected abundance values under the assumption of random distribution (Poisson goodness-of-fit tests, all $p<0.001$). Within the hilly region, in Longquan and Yuantou in 2006, the observed distribution of cercariae genotypes showed an over-dispersion (Negative binomial distribution goodness-of-fit tests, $p=0.46$ in Longquan, and $p=0.05$ in Yuantou), indicating higher numbers of *S. japonicum* infections in some snails than expected if infections were randomly distributed. The mean abundance of *S. japonicum* infection (the number of cercariae genotypes per infected snail) was higher in the marshland than in the hilly region in both years (Tables 4.7.1-2). This was also true with the allelic diversity and **He** (expected heterozygosity) at the level of populations (see Table 4.7.3). For example, the mean number of alleles per locus ranged from 11 to 15 among the marshland villages, whereas it was between 4.33 and 7.5 among the hilly ones.

In the marshland, a significant infra-population heterozygosity deficiency (f), namely a departure from Hardy-Weinberg equilibrium expectations, was found in two cases, one in Guanghui and the other in Heping in 2006. With the exception of cercariae from Longshang and Yuantou in 2007, there was a substantial genetic differentiation among infra-populations observed across the marshland (F_{ST} = 0.269-0.471) and the hilly region (F_{ST} = 0.103-0.359) (Table 4.7.3).

4.4.2 Genetic diversity of miracidia at levels of hosts or species

In the marshland, the miracidia populations from cattle exhibited considerable higher allelic diversity than the populations from other species. The mean number of alleles per locus and the mean number of private alleles per locus were higher in parasites from cattle than those from other species in each village. Whereas in the hilly villages, the highest mean numbers of alleles per locus and of private alleles per locus were always found in parasites from humans or dogs, rather than from rodents (Table 4.7.4).

Among the three villages in the marshland, the genetic structures of the parasites from all species (excluding humans) studied here were all very similar with estimates of 'little' or no genetic differentiation among infra-populations ($F_{ST} \leq 0.021$) and of significant infra-population heterozygote deficiency ($f \geq 0.363$). However, within the hilly region, a marked infra-population heterozygote deficiency was only found in parasites from dogs in Longquan ($f = 0.306$) and from humans in Yuantou ($f = 0.175$). For all species of definitive hosts excepting dogs in Longquan, the F_{ST} values of genetic differentiation among infra-populations ranged from 0.094 to 0.274, namely 'moderate' to 'great' differentiation. It was also noted that most of the 95% confidence intervals of the F_{ST} values were much wider in the hilly villages than in the marshy villages (Table 4.7.4 and Figure 4.8.1).

4.4.3 Gene flow of parasites between two larval stages within each village

As shown in Table 4.7.5, across three villages in the marshland, the smallest estimate of F_{ST} was observed between the miracidia population from cattle and the cercariae

population from snails, ranging from 0.002 to 0.017 in 2006 and being 0.008 in 2007, all far less than 0.05, suggesting very little genetic differentiation between the compared larval populations. Such results were confirmed by the consensus UPGMA phenograms, where parasite populations from cattle closely clustered with cercariae populations, but far separated from those from humans or dogs (Figure 4.8.2(a-b)).

In the hilly region, a different profile appeared among the three hilly villages. As displayed in Table 4.7.5, in Longquan, a 'little' genetic differentiation was seen between the cercariae population and the miracidia population from rodents or dogs. In Longshang, such close relationship was seen between the cercariae population and the miracidia from rodents. In Yuantou, the smallest estimate was found between the cercariae population and the parasites from dogs, with the second between the cercariae population and those from humans. The consensus UPGMA phenograms based on another measure of genetic distance also showed the similar results (Figure 4.8.2(c-e)).

4.5 DISCUSSION

4.5.1 Genetic diversity of miracidia relative to definitive hosts

Adult infra-populations (and then subsequent miracidia infra-populations) within definitive hosts constitute the primary level of population parasite fragmentation (Anderson *et al.*, 1995; Fisher & Viney, 1998). An infra-population of parasites may be determined not only by their host's limited and complicated immunity (Chapter 1 for host immune ability against *S. japonicum*) but also mainly by the recruitment of external free-living infective stages. Therefore, a combination of various factors, such as host mobility and spatial distribution of infective forms of parasites in the environment are of major significance in the diversity of the aforementioned infra-populations of parasites.

Davies and colleagues (Davies *et al.*, 1999) demonstrated that populations of the human parasite *S. haematobium* only showed differentiation at a wide geographical scale between two river systems in the Zimbabwean Highveld; whereas, for populations of *S. mansoni* parasitizing wild rats on Guadeloupe island, a significant degree of genetic variation was reported between geographically very close populations (less than 2 km) (Sire *et al.*, 1999), suggesting that host movement (e.g. highly mobile human versus sedentary murine hosts) is more important than geographic distances and differences in landscape ecology in determining the population structures of parasites. In this study, within each village in the marshland, the parasite populations from species of cattle, water buffalo, or goats each showed a pattern consistent with high gene flow among infra-populations (F_{ST}) and a significant heterozygosity deficit of infra-populations (*f*). As water contact activities may vary from species to species, or even from individual to

individual, the observed 'little' genetic differentiation among individual hosts and high within-infra-population inbreeding found for parasites from each host species would suggest that, for all host species, individual hosts each sampled a various number of cercariae originating from the same parasite population, namely the common infection 'foci' shared by all host individuals or species within each marshland village. As seen in Chapter 2, there was one focus in Guanghui and one in Xingzhuang in either year of 2006 and 2007, and two but separated by a river branch in Heping in both years. It would be suspected that these foci each may comprise a large 'well-mixed' parasite population (in terms of components of genotypes).

However, within each hilly village, besides the parasites from rodents, those from dogs or humans with higher mobility all showed a pattern of substantial genetic differentiation among individual hosts, with most showing no significant heterozygosity deficiency of infra-populations. An associated consequence of host moment could be continuous infection with the same genotypes of larvae or self re-infection (a host infected by progeny of its own parasites) (Paterson *et al.*, 2000), both of which may result in high genetic differentiation among infra-populations (among hosts) and high heterozygosity deficit of infra-population parasites (within hosts). Continued infection or self re-infection is very likely to occur in small rodents, as such animals are usually limited to their territory (a geographical area defended by a group of residential rodents against other outsiders) with a high probability of both excreting faeces and contacting water at the same locations. However, no significant heterozygosity deficiency (*f*) of infra-populations, together with the biological character of a short lifespan (less than two years

on average), may not support the possibility of self re-infection for rodents here. It seems highly probable that the high genetic differentiation of parasites between rodents may arise from their different captured sites, under the condition of the existence of several separated 'less-mixed' foci within each of the three villages, namely 5, 3 and 5 infection habitats respectively in 2006, and 5, 10 and 2 infection habitats respectively in 2007 (see Table 2.7.3 in Chapter 2). Such heterozygosity in transmission sites could also explain the observed genetic structure of parasites from other species of definitive hosts.

A large number of private alleles were found in cattle in the marshland, and in humans or dogs in the hilly region. It seems reasonable to infer that, within each marshland village, not all alleles can be transmitted to other host species when cattle are regarded as the main reservoirs. However, within each hilly village, dogs or humans with more private alleles, in relation to rodents, could be associated with their being exposed to the parasites outside their villages due to their higher mobility.

4.5.2 Genetic diversity of cercariae indirectly relative to definitive hosts

The distribution of larval genetic diversity of schistosomes within and between snail hosts has been well documented for *S. mansoni* and *S. haematobium* in various ecological and epidemiological transmission settings (Minchella *et al.*, 1995; Dabo *et al.*, 1997; Davies *et al.*, 1999; Sire *et al.*, 1999; Eppert *et al.*, 2002), from which within-host genetic diversity, measured as the number of larval genotypes per snail on average, ranged from 1.1 to 6.2. In this study, for *S. japonicum*, the higher within-host genetic diversity was found in the marshland than in the hilly region. The higher genetic diversity (expected

heterozygosity) at the level of population in the marshland than in the hilly region was consistent with those observed for miracidia populations. It is worth mentioning that it was more common for snails to harbour multiple parasite genotypes in the marshland than in the hilly region. Over-dispersion of *S. japonicum* infections among snails was found in two hilly villages. As emphasized by Minchella and colleagues (Minchella *et al.*, 1995), the degree of over-dispersion, together with multiple infections within snail intermediate hosts, may facilitate multi-genotype transmission to the vertebrate host and the maintenance of parasite genetic diversity. The extent of over-dispersion of infections among snails could result from heterogeneities in either the exposure, mainly associated with definitive host excreting movements, or the susceptibility of the snails to infection, thus raising further interesting questions on the snails and/or the impact of their ecological habitats on such infections to be investigated.

4.5.3 Main reservoirs based on gene flow between two larval stages

In this study, the observed Pairwise F_{ST}s based on different alleles, coupled by the bootstrapped UPGMA based on Cavalli-Sforza and Edwards's chord distance (Cavalli-Sforza & Edwards, 1967), suggest that the main reservoirs were probably cattle in the marshland. The higher genetic diversity (expected heterozygosity or private alleles) of schistosome in cattle than in other species also supports the hypothesis of cattle as a main reservoir in such areas. Cattle, with high frequency and intensity of water contact and a long life span, seem to be ideal 'genetic mixing bowls' for the parasites, and then may cause the 'well-mixed' infection foci via frequently discharging faeces in snail infested areas for itself and other species of definitive hosts, which was further confirmed by a

closer relationship of the miracidia from cattle to the cercariae sampled in 2007 than to the cercariae sampled in 2006.

However, in the hilly region, a more complicated transmission profile, based on Pairwise F_{ST}s and UPGMA, was seen among villages, with the main reservoirs being dogs and rodents in Longquan, rodents in Longshang, and dogs and humans in Yuantou. Annual selective chemotherapy and improvement in sanitary (Chapter 2) may have been limiting humans' role in the transmission, but it could be possible for dogs as one potential reservoir because of its high mobility and excreting faeces randomly. Therefore, by combining the results from previous prevalence surveys (Chapter 2) and cercariae emergence observation (Chapter 3), the transmission dynamics of *S. japonicum* within each of the hilly villages could be reasonably inferred as follows: 'clonal' rodents maintain the parasite life cycle within their own territory, which may include one or more infection foci; with the existence of such several territories in each village, dogs get infected in one or more and spread the parasites among infection territories and no infection territories due to higher mobility; dogs could also get infected outside the village, acquire private alleles and then spread them; new infection territories would develop when immigrant parasites by dogs infect and establish within local rodents.

To conclude, the results from this chapter clearly demonstrated an obvious difference in population genetic diversity and the structure of *S. japonicum* at levels of individual hosts or species between two regions, marshland and hilly region. Within each marshland village, very low genetic differentiation between miracidia populations from cattle and cercariae populations from snails would confirm the main reservoir of cattle in the

marshland, as expected from previous chapters. However, within the hilly region, it would be suggested that, besides rodents as the main reservoirs to maintain the disease within a limited territory, dogs with higher mobility might play a role in spreading parasites among territories.

4.6 ACKNOWLEDGEMENTS

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4.7 TABLES

Table 4.7.1 Distribution of S. japonicum infections in snail hosts in 2006

Villages	Prevalence (%)	Infected snails	No. of total cercariae genotypes	Mean abundance*	No. parasite genotypes per snail (genotyping 10 cercariae per snail)									p†	p‡
					1	2	3	4	5	6	7	8	9		
Guanghui (M)	5.21	34	85	2.50	15	4	8	4	0	1	1	0	1	<0.001	0.020
Heping (M)	2.77	39	79	2.03	15	13	7	3	1	0	0	0	0	<0.001+	0.003
Xingzhuang (M)	2.70	31	81	2.61	7	10	6	4	4	0	0	0	0	<0.001+	<0.001
Longquan (H)	0.74	27	45	1.67	18	6	0	1	1	1	0	0	0	<0.001+	0.456
Longshang (H)	1.41	27	46	1.70	14	9	2	2	0	0	0	0	0	<0.001+	0.039
Yuantou (H)	1.44	23	31	1.35	16	6	1	0	0	0	0	0	0	<0.001+	0.051+

Note: * mean abundance is the number of cercariae genotypes per infected snail; † p is the p-value of Poisson distribution goodness-of-fit test; ‡ p is the p-value of Negative binomial distribution goodness-of-fit test; +, for one cell with an expected value less than 3 in calculation of the χ^2 value under a given distribution.

M, for marshland; and H, for hilly region

Table 4.7.2 Distribution of S. japonicum infections in snail hosts in 2007

Villages	Prevalence (%)	Infected snails	No. of total cercariae genotypes	Mean abundance*	No. parasite genotypes per snail (genotyping 10 cercariae per snail)									p†	p‡
					1	2	3	4	5	6	7	8	9		
Guanghui (M)	8.11	39	140	3.59	4	10	9	5	3	4	2	2	0	<0.001	<0.001
Heping (M)	0(0/1243)	0													
Xingzhuang (M)	2.55	27	72	2.67	4	12	3	6	1	1	0	0	0	<0.001^{+}	<0.001
Longquan (H)	1.32	30	67	2.23	9	11	5	4	1	0	0	0	0	<0.001^{+}	<0.001
Longshang (H)	1.13	27	53	1.96	7	14	6	0	0	0	0	0	0	<0.001^{+}	<0.001
Yuantou (H)	1.44	34	85	2.50	6	14	7	5	2	0	0	0	0	<0.001^{+}	<0.001

Note: * mean abundance is the number of cercariae genotypes per infected snail; † p is the p-value of Poisson distribution goodness-of-fit test; ‡ p is the p-value of Negative binomial distribution goodness-of-fit test; +, for one cell with an expected value less than 3 in calculation of the χ^2 value under a given distribution.

M, for marshland; and H, for hilly region

Table 4.7.3 Standard diversity indices with F-statistics analysis of cercariae population samples at levels of villages or individual snails (Ho, observed heterozygosity over 6 loci; He, expected heterozygosity over 6 loci)

	No. of infected snails	No of cercariae genotypes	Mean no. of gene copies (SD)	Mean no. of alleles per locus (SD)	Ho (SD)	He (SD)	*f* (95%CI)	F_{ST} (among snails) (95%CI)
Guanghui, marshland								
2006	34	85	148.33(20.28)	11.5(5.94)	0.333(0.145)	0.675(0.201)	0.335(0.051,0.505)	0.269(0.203,0.319)
2007	39	140	268.67(9.78)	15.00(9.73)	0.400(0.148)	0.675(0.198)	0.089(-0.164,0.304)	0.413(0.227,0.559)
Heping, marshland								
2006	39	79	136.67(20.19)	11(7.44)	0.280(0.141)	0.663(0.216)	0.348(0.098,0.576)	0.360(0.292,0.421)
Xingzhuang, marshland								
2006	31	81	135(22.68)	12.67(8.28)	0.330(0.179)	0.700(0.225)	0.314(-0.041,0.604)	0.323(0.258,0.372)
2007	27	72	131.33(9.71)	13.00(6.98)	0.384(0.150)	0.714(0.201)	0.103(-0.110,0.334)	0.471(0.251,0.639)
Longquan, hilly region								
2006	27	45	76(11.43)	6.83(4.98)	0.313(0.219)	0.584(0.250)	0.264(-0.014,0.510)	0.284(0.220,0.372)
2007	30	67	121.00(16.48)	7.50(4.11)	0.391(0.214)	0.603(0.246)	0.023(-0.269,0.289)	0.359(0.120,0.582)
Longshang, hilly region								
2006	27	46	80(11.14)	6(3.27)	0.297(0.259)	0.552(0.244)	0.240(-0.183,0.703)	0.303(0.133,0.396)
2007	27	53	94.67(12.31)	5(2.31)	0.435(0.329)	0.481(0.300)	-0.298(-0.454,0.006)	0.103(-0.057,0.373)
Yuantou, hilly region								
2006	23	31	55(8.62)	4.33(3.20)	0.357(0.293)	0.434(0.304)	0.030(-0.376,0.440)	0.159(0.023,0.246)
2007	34	85	160(6.93)	6.33(4.31)	0.396(0.325)	0.467(0.321)	-0.199(-0.536,0.152)	0.158(-0.210,0.435)

Table 4.7.4 Standard diversity indices with F-statistics analysis of miracidia population samples at levels of species or individual hosts (Ho, observed heterozygosity over 6 loci; He, expected heterozygosity over 6 loci)

	No of hosts	No. of miracidia genotyped	Mean no. of gene copies (SD)	Mean no. of alleles per locus (SD)	Mean no. of private alleles per locus	Ho (SD)	He (SD)	*f* (95%CI)	F_{ST} (among hosts) (95%CI)
Guanghui, marshland									
Buffaloes	2	22	41.33(3.94)	7.17(4.22)	0.17	0.294(0.172)	0.630(0.204)	0.542(0.324,0.739)	-0.007(-0.040,0.028)
Cattle	15	168	321.33(11.47)	16.33(10.19)	6.17	0.387(0.116)	0.643(0.201)	0.387(0.167,0.533)	0.021(0.004,0.034)
Dogs	2	21	39.67(3.35)	8.33(4.31)	0.83	0.402(0.119)	0.642(0.234)	0.383(0.196,0.524)	-0.007(-0.047,0.043)
Heping, marshland									
Cattle	3	30	56.33(2.93)	9.33(5.71)	-	0.414(0.172)	0.652(0.221)	0.363(0.133,0.539)	0.016(-0.023,0.065)
Xingzhuang, marshland									
Cattle	13	150	294.67(5.38)	15.33(10.17)	5	0.395(0.114)	0.664(0.204)	0.396(0.227,0.525)	0.016(0.005,0.031)
Goats	8	83	164.33(2.93)	10.5(6.55)	0.33	0.396(0.085)	0.653(0.202)	0.397(0.253,0.491)	-0.004(-0.018,0.012)
Humans	1	9	17.33(0.94)	2.67(1.11)	0.17	0.519(0.175)	0.517(0.193)	-0.004(-0.247,0.182)	-
Longquan, hilly									
Cats	1	7	13.33(0.94)	1.83(1.07)	0.83	0.333(0.375)	0.294(0.312)	-0.148(-0.333, 0.217)	-
Dogs	8	90	172.67(7.72)	7.33(5.71)	2.67	0.379(0.232)	0.561(0.276)	0.306(0.017, 0.546)	0.034(0.010, 0.057)
Rodents	6	67	113.33(27.63)	4.67(2.49)	0.17	0.429(0.213)	0.558(0.261)	0.094(-0.135,0.510)	0.180(0.121,0.251)
Longshang, hilly									
Dogs	1	1	2	1.5	0.0	0.5	0.5	-1.0	-
Humans	5	47	88.33(6.16)	7.83(4.41)	3.83	0.417(0.069)	0.666(0.191)	0.187(-0.026,0.337)	0.274(0.204,0.345)
Rodents	5	55	100.33(8.44)	5(3.61)	1.67	0.373(0.333)	0.445(0.331)	0.067(-0.133,0.366)	0.124(0.068,0.165)
Yuantou, hilly									
Dogs	4	31	57.67(7.95)	3.83(2.03)	0.33	0.384(0.231)	0.500(0.286)	0.148(-0.146,0.373)	0.138(0.039,0.242)
Humans	3	36	72(0)	6.33(2.81)	3.00	0.426(0.219)	0.551(0.263)	0.175(0.074,0.288)	0.094(0.005,0.164)
Rodents	2	23	43.33(2.49)	2.83(1.57)	0.17	0.447(0.356)	0.425(0.301	-0.226(-0.546,0.014)	0.238(0.059,0.368)

Table 4.7.5 Pairwise F_{ST}s between cercariae populations from snails and miracidia populations from each host species

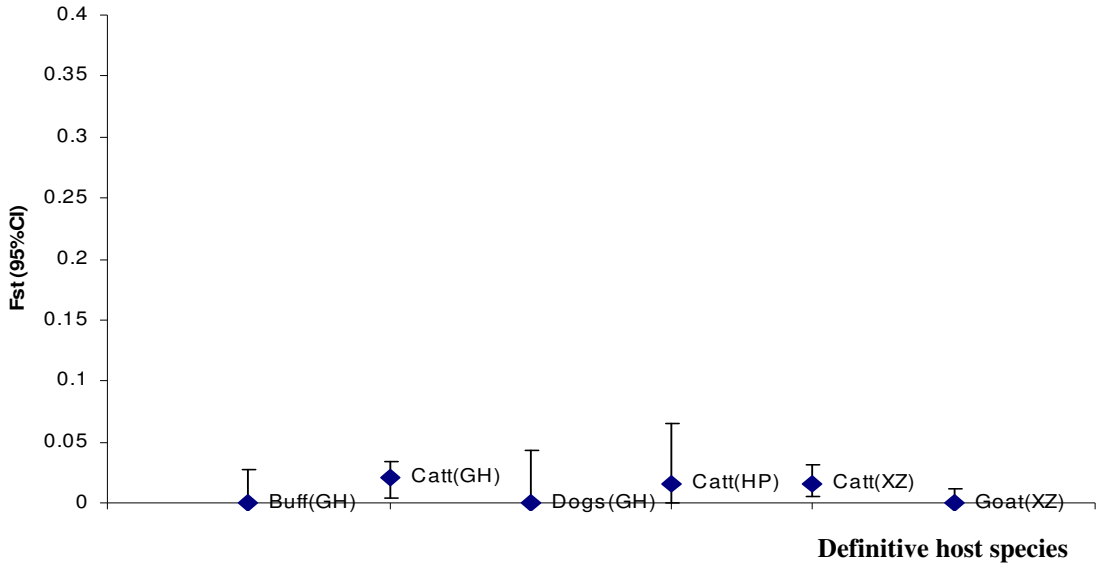
	Humans	Goats	Water buffalo	Cattle	Cats	Dogs	Rodents
Guanghui, marshland							
Snails(2006)			0.010	0.017**		0.041**	
Snails(2007)			0.014	0.008*		0.016	
Heping, marshland							
Snails(2006)				0.011			
Xingzhuang, marshland							
Snails(2006)	0.129**	0.023**		0.002			
Snails(2007)	0.136**	0.012**		0.008*			
Longquan, hilly region							
Snails(2006)					0.459**	-0.010	-0.034
Snails(2007)					0.428**	0.003	0.018**
Longshang, hilly region							
Snails(2006)	0.114**					0.294*	0.043**
Snails(2007)	0.154**					0.381*	-0.006
Yuantou, hilly region							
Snails(2006)	0.054**					0.043**	0.133**
Snails(2007)	0.056**					0.032**	0.115**

Note: * $p<0.05$; ** $p<0.01$

4.8 FIGURES

Figure 4.8.1 The differentiation value F_{ST} (among hosts) (±95% CI) observed for each host species by village (a, for marshland villages, GH, HP and XZ for Guanghui, Heping and Xingzhuang respectively; Buff for water buffalo and Catt for cattle. b, for hilly villages. LQ, LS and YT for Longquan, Longshang and Yuantou respectively; Rode for rodents and H for humans. The same Y scale was used in two plots in order to compare the values between two regions.)

(a) Marshland



(b) Hilly region

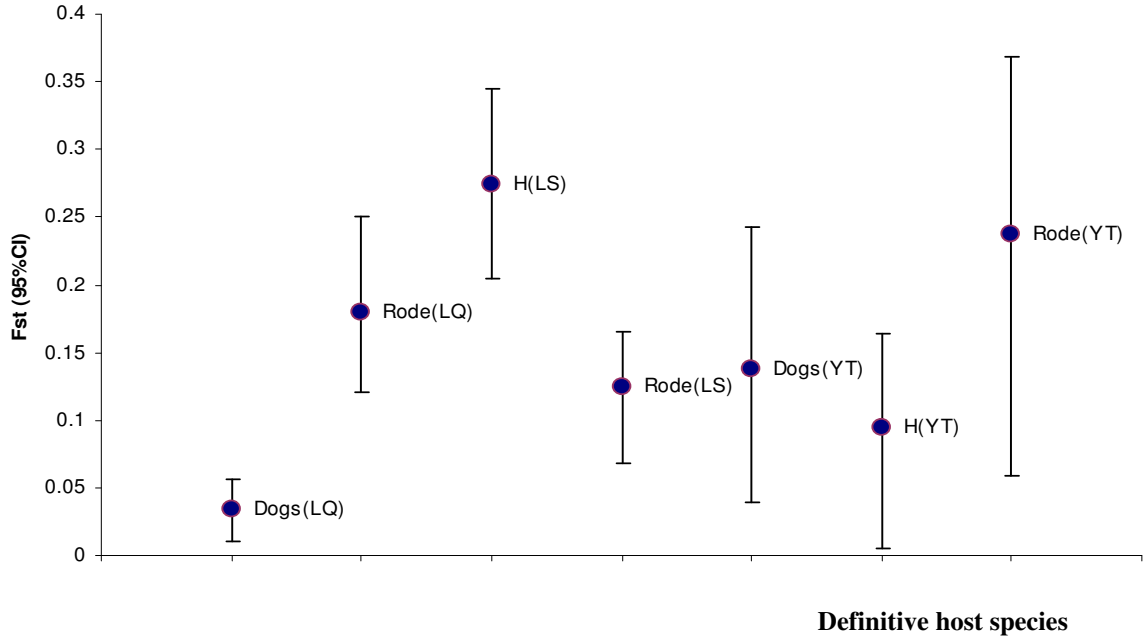
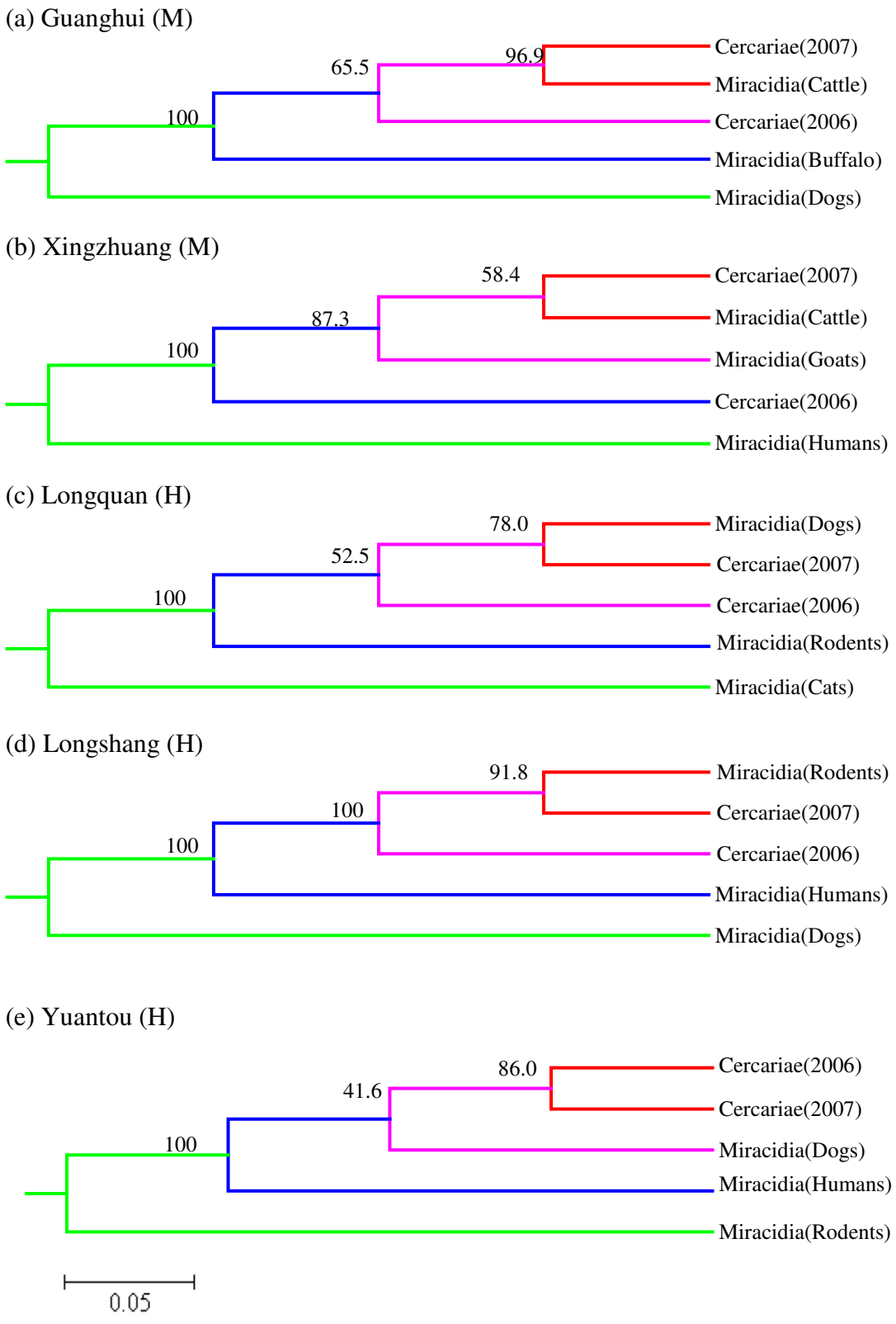


Figure 4.8.2 UPGMA phenograms depicting Cavalli-Sforza and Edwards's (1967) chord distance measured separately for each village between all larval populations (M, marshland; and H, hilly region. Numbers next to branches refer to bootstrap percentages)



Chapter 5: Contrasting reservoirs or transmission patterns for *Schistosoma japonicum* between marshland and hilly regions in Anhui, China: inferred from sibling relationship of parasites between two larval stages

5.1 ABSTRACT

Due to the existence of potentially thousands of clonal *Schistosoma japonicum* cercariae, different definitive host individuals could 'share' one or more adult worm pairs via infection. The distribution of the 'same' adult worm pair, in terms of genotypes, across host species, or the number of genetically unique adult worm pairs within single hosts could be inferred from the familial relationships of their offspring miracidia from the hosts. As most schistosome have a life span of between 5 and 30 years and the period of egg excretion in faeces post-infection may last for a few months to over one year, the successful transmission of parasites from one definitive host to an intermediate host may be inferred from the sib-ship between the cercariae from the snail and the miracidia from the definitive host. In this chapter, sibling relationship analyses of larval samples from each village were performed. The results showed that, in the marshland, cattle harboured a higher number of genetically unique adult worm pairs than any other species, while in the hilly villages dogs or humans did; cross-infection among host species was seen in each village, and the proportion of such infections differed with village; a majority of cercariae may come from cattle in marshland villages, whereas in the hilly areas, they might be from rodents, dogs or humans, varying from village to village. Such findings confirm the main results from Chapter 4 and further suggest a different transmission pattern of the infection between and within two ecological regions.

5.2 INTRODUCTION

Clonal reproduction is the production of new individuals with the original parental genotype. As partially clonal species, schistosomes, exhibit complex life-cycles with obligatory alternation of asexual (within an intermediate mollusc host) and sexual reproduction (within a definitive vertebrate host). This asexual reproduction has significant importance in the maintenance and the distribution of genetic variation in the next generation (Balloux *et al.*, 2003) as well as in the infra-population structure within definitive hosts (Prugnolle *et al.*, 2004). However, few studies have investigated the transmission pattern or process of indirectly transmitted parasitic diseases, such as schistosomiasis, based on the existence of the potentially thousands of genetically identical larvae, which has long been considered only from a demographic point of view, as an adaptation to increase in number to compensate for the considerable random loss of larvae during the free-living transmission phase and thus to increase the probability of meeting the subsequent host (Combes, 2001).

As a single miracidium (male or female) gives rise to thousands of clonal cercariae, capable of infecting different hosts or host species, one pair of adult worms, male and female, found in one definitive host could be genetically identical to a pair found in other hosts. Clonal genotypes of schistosome, for example *S. mansoni*, have been reported to present within the same definitive hosts (Sire *et al.*, 2001b; Steinauer *et al.*, 2008). On the island of Guadeloupe, *S. mansoni* was abundant within the black rat *Rattus rattus* (Theron *et al.*, 1992), and the maximum repetition of an identical genotype, using Random Amplified Polymorphic DNA markers, within a host rat was of 22 out of 122 worms analysed from the host harbouring 343 schistosome (Sire *et al.*, 2001b), with about 68.5% of 200 multilocus genotypes of parasites shared by two

or more rats (Theron *et al.*, 2004). Consequently, the offspring, eggs and subsequent miracidia, from 'duplicated' adult worm pairs from the same or different hosts, could be considered as 'genetic' siblings even though they belong to different biological parents. Therefore, distribution of given pairs of adult worm genotypes across definitive hosts or host species, which we still know little about due to the logistical difficulty in sampling adult worms from the definitive hosts, could be inferred by the sib-ships between their offspring miracidia.

Several methods using molecular markers have been applied to infer familial relationships between individual members in the absence of pedigree data. There are two categories, with the pairwise method based on either the estimation of relatedness or the probability of one given pair of individuals belonging to a defined class of relationships (Boehnke & Cox, 1997; Epstein *et al.*, 2000; McPeek & Sun, 2000), and group approaches, through which the first-generation pedigree relationships among all analyzed individuals are reconstructed (Thomas & Hill, 2000; Smith *et al.*, 2001; Thomas & Hill, 2002). Kinship relationship between individuals could be estimated with high reliability based on the UPGMA (Unweighted Pair Group Method with Arithmetic mean) graphical representation of the inter-individual genetic distances (Blouin *et al.*, 1996; Romo *et al.*, 2006). A new group-likelihood approach, taking into account the typing errors and mutations, which have been ignored by all previous methods, has been developed and has demonstrated to be able to accurately infer full- and half-sib-ships from molecular data with a high error rate (Wang, 2004). Microsatellite markers have been chosen as the molecular tool to investigate such issues as not only are they suitable for genetic diversity studies (Jarne & Lagoda, 1996), but their high polymorphism makes them particularly well suitable to studies

of relatedness and kinship assessment (Queller *et al.*, 1993; Blouin *et al.*, 1996). Even with few loci, microsatellite markers, without *a priori* pedigree information, have been successfully employed to the partitioning of individuals into families (Smith *et al.*, 2001; Danancher *et al.*, 2008).

As most schistosome have a life span of between 5 and 30 years (Bush *et al.*, 2001) and the period of egg excretion in faeces post-infection may last for six months to over one year (He *et al.*, 2001), given an annual transmission season mainly from Jun. to Oct. (Li *et al.*, 2009), a majority of the cercariae sampled in Apr. 2006 could be the sibs to miracidia sampled in Sept.-Oct. 2006, and most of the cercariae sampled in Apr. 2007 may be sibs to miracidia sampled in Sept.-Oct. 2006, thus making possible the investigation of transmission dynamics (or/and even transmission efficiency) of the parasites based on multilocus genotypes. The objective of this chapter, therefore, was to infer the sibling relationships of miracidia collected from definitive hosts, including humans, domestic animals and small rodents, and cercariae from intermediate host snails from the same villages. These were then used to describe the distribution of memberships from the same 'family' (i.e. offspring from one genetically unique adult worm pair) in order to provide evidence for main reservoir hosts or even transmission efficiency for the parasites within each village, which would otherwise be unclear based only on the level of gene flow between two larval stages, as shown in Chapter 4.

5.3 MATERIALS AND METHODS

The sampling and genotyping of larval samples were detailed in Chapter 4 (See 4.3.1) and the repeated cercariae genotypes within individual snails were reduced to single copies before the following molecular analyses.

5.3.1 Full-sib analyses

The estimation of full-sib relationships, without knowing parental information, between the sampled larvae was implemented with the Colony software (V1.1) (Wang, 2004). This software is a Fortran program implementing a maximum likelihood method to assign individuals sampled from a single generation of a population into full-sib families nested within half-sib families (colonies) using data on co-dominant genetic markers. It adopts a fresh iterative procedure for updating allele frequencies with reconstructed sibships taken into account, allows for the use of parental information, and uses efficient algorithms for calculating the likelihood function and searching for the maximum-likelihood configuration, therefore increasing its usefulness (Wang, 2004). Here all cercariae and miracidia sampled from the same village were pooled and then, by running Colony, partitioned simultaneously into family groups of variable size (group-likelihood approach). Different full-sib-ship configurations were constructed and then compared, and the optimal solution of full-sib-ship reconstruction was chosen when the maximum likelihood estimate was obtained. As no information on typing errors or mutation rate of the larval samples was available, a small error rate of 1% at each of six loci, as suggested by (Wang, 2004), for both Class I error, allelic dropout, and Class II error, including mutations, false alleles and miscalling, was chosen here for the sib-ship analysis of larvae, miracidia and cercariae.

5.3.2 Transmission dynamic indices

Infection intensity within a single host was defined as the number of genetically unique adult worm pairs, based on the number of inferred families, as it is impossible to estimate the number of pairs of biological parents due to the possibility of the same genetically identical worm pairs repeated within the host. The mean infection intensity and standard deviation were calculated for each species, and the significant difference was tested with the non-parametric Kruskal-Wallis (for over two independent samples) or the Mann-Whitney U test (for two samples) using SPSS 11.0 software (**SPSS Inc.**, 2002). Cross infection of the parasites between and among host species was defined as the presence of one genetically unique adult worm pair estimated in more than two host species. The potential role of each definitive host species in the transmission was measured as the proportion of the total cercariae coming from that host species based on sibling relationship between cercariae from intermediate host snails and miracidia from that species.

5.4 RESULTS

5.4.1 Infection intensity for each species of definitive hosts

The infection prevalence of each species is shown in Table 2.7.4 in Chapter 2. The average sample size of genotyped larvae per host for each species is displayed in Tables 5.7.1-2. In the marshland, as demonstrated in Table 5.7.1, the higher infection intensity, here based on the number of genetically unique adult worm pairs estimated within single hosts, was observed in cattle than in any other species in each village, with the exception of Heping where only infected cattle were found.

From Table 5.7.2, in Longquan, in the hilly region, dogs harboured multiple families of parasites with a mean number of eight pairs of adult worms per host, higher than that observed within rodents. This was also true in Yuantou, in which dogs, plus humans, appeared to be infected with more unique genotypes of parasites than rodents. No significance in the infection intensity among species was observed in two hilly villages (Longshang and Yuantou).

When the data were combined at the level of species in either region (county), an obvious difference in infection intensity among species was seen in the hilly region (χ^2=7.68, d.f.=3, p=0.053) rather than in the marshland (χ^2=4.44, d.f.=4, p=0.349). Generally, a significant difference in infection intensity between two regions (Mann-Whitney U test, p<0.001, 2 tailed) revealed that the definitive hosts seemed to harbour more multiple parasite genotypes in the marshland than in the hilly region. The highest infection intensity, however, defined as the number of hatched miracidia per gram of faeces or EPG (Chapter 2), was observed in rodents in the hilly region. No correlation was seen between the number of genetically unique adult worm pairs and

the egg-based infection intensity. This is in agreement with a study on pigs, in which the reduced excretion of eggs into the faeces did not correlate to reduced parasite numbers in the chronic phase of schistosomiasis (Watanabe *et al.*, 2004).

5.4.2 Corresponding parasite genotypes across host species

As shown in Figure 5.8.1(a), out of a total of 82 genetically unique adult worm pairs estimated from miracidial samples from cattle, water buffalo and dogs in Guanghui nearly 59.8% were found in cattle only. The proportions of the genetically unique worm pairs 'shared' among species (i.e. cross-infections among species), observed in cattle and water buffalo, cattle and dogs, and cattle, water buffalo and dogs combined, were 13.4, 17.1 and 2.4%, respectively, totalling 33.0%. Such results suggest that a majority of parasites circulating in the locality were from cattle. In Xingzhuang, a total of 88 genetically unique adult worm pairs were estimated from miracidia sampled from cattle, goats and humans. As illustrated in Figure 5.8.1(b), the percentage of the total pairs in relation to cattle was 85.2%, with 60.2% found in cattle only, 23.9% in cattle and goats, and 1.1% in cattle and humans. In Heping, 20 unique pairs of adult worms were identified in cattle, the only host species found in the village.

In the hilly village Longquan, 41 genetically unique adult worm pairs were inferred from miracidia samples obtained from dogs, cats and rodents. Thirty six pairs (87.8%) were relative to dogs, of which 51.2% were inferred to be cross-infections between dogs and rodents. In Longshang, only 24 pairs were estimated from miracidia from humans, dogs and rodents. Nine and 10 pairs were harboured in humans only and in rodents only, respectively. The proportion of cross-infections between the above two

species was up to 16.7%. In Yuantou, only 19 genetically unique adult worm pairs were estimated from miracidia from humans, dogs and rodents. Out of these unique pairs, the number of pairs estimated in humans, dogs, and rodents, each combined with 'shared' pairs between and among corresponding species, were 14, 14 and 6, respectively (See Figure 5.8.2(a-c)).

In general, without considering sample sizes, higher numbers of genetically unique adult worm pairs were inferred in marshland villages, with the exception of Heping where only a small number of infected hosts and then of larvae were available, than in the hilly villages. The frequency of cross-infection was observed to vary from village to village.

5.4.3 Transmission from definitive hosts to intermediate hosts

The sample size of cercariae genotypes from each village is displayed in Tables 4.7.1-2 (Chapter 4). As displayed in Figure 5.8.3 (a-b), of the 85 and 140 cercariae genotypes identified from the infected snails, collected in Guanghui in 2006 and 2007 respectively, 44 (51.8%) and 67 (47.9%) were estimated to be from cattle only. It was also noted that it was not possible to trace the resource reservoirs of 27.0% in 2006 and 31.4% in 2007, the latter of which included a few cercariae with late emergence. This was also true in the cases of Xingzhuang and Heping. In Xingzhuang, the percentage of cercariae without inferred resources was 27.2% in 2006 and 13.9% in 2007. In Heping, only 36 out of 79 cercariae sampled in 2006 were inferred from cattle.

As seen in Figure 5.8.4(a-c), in the hilly village Longquan, there was evidence that parasites may be transmitted primarily from dogs and/or rodents to intermediate host

snails. It remains unclear which species played a more important role at this stage. In Longshang, 54.3% (25/46 cercariae) and 69.8% (37/53 cercariae) were inferred from rodents in 2006 and 2007 respectively, but 28.8% in 2006 and 13.2% in 2007 remained unknown. However, in Yuantou up to 48.4% of 31 cercariae collected in 2006 and 51.8% of 85 cercariae collected in 2007 were not traced back to their original reservoirs, with the remainder mainly accredited to dogs or/and humans. Such results observed in Longquan and Longshang were consistent with those from Chapter 4, but in Yuantou they were not completely in agreement with the previous findings based on gene flow.

5.5 DISCUSSION

In this chapter, the kinship analyses of parasites at two larval stages indicate that, in the marshland cattle harboured the highest number of genetically unique adult worm pairs, whilst in the hilly region dogs or humans did. Cross-infection of given genotypes of parasites among species of hosts was seen in both ecological regions, and the proportion of such patterns to the total infections differed from village to village. The cercariae identified within intermediate host snails in the former, marshland, areas appeared to mostly come from cattle, whereas in the latter, hilly, areas, they were varied between villages, coming from rodents, dogs or humans. Such results suggest different transmission dynamics of the infection between and within two ecological regions.

Although the concept of estimating the number of breeding adult worms infecting a host with their offspring was once mentioned by Criscione and colleagues (Criscione *et al.*, 2005), this is the first time that infection intensity has been estimated as the potential number of genetically unique adult worm pairs within mammal hosts using the multilocus genotypes of miracidia. A difference in the total duration of exposure to *S. japonicum* cercariae over months or years among species of mammals could have an impact on such an estimate of infection intensity. Species with longer life-spans, for example humans and bovines, are likely to obtain more genetically diverse infections then species with shorter life-spans such as small rodents. This may either be due to a greater explored infective area over time, or with cercariae of given infected areas differing from time to time. The results from this study support the existence of such a scenario. In the marshland, the highest estimated infection intensity was observed in cattle with an average of more than 10 years of lifespan,

whereas in the hilly region the lowest in rodents with an average of less than 2 years of lifespan. Although humans may have been exposed over an even longer time period than the cattle, a lower index in humans at the time point of the survey could be reduced because of annual test-treatment.

The observed difference in the estimated infection intensities among species could also be explained by the difference in their mobility patterns or home ranges. If *S. japonicum* cercariae are spatially genetically structured, definitive hosts from the more dispersing species or the species with a larger home range, for example, dogs, cattle and water buffalo in relative to rodents, could harvest a higher number of genetically distinct larval parasites. Within this thesis, Chapter 2 showed very low snail infection prevalence, ranging from 0.15 to 2.22%, among the sampled villages and Chapter 4 displayed multiple infections of parasites within individual snails, suggesting the high probability of the aggregation of parasites at the levels of habitats or individual snail hosts, particularly in the hilly villages where separated ditches probably lead to the infective cercariae being patchily distributed. In terms of variation in home ranges among species, rodents may generally engage in their activities within their home territory, whereas bovines may have extended areas within villages, which they frequent, depending on the grassland. Dogs and humans could, in theory, go wherever bovines have been or even beyond village boundaries, but infection with the extant or circulating parasites are more greatly determined by their water contact patterns and frequencies. As expected, the results of this study did show that definitive hosts with high movement, such as cattle in the marshland and dogs and humans in the hilly region, appeared to harbour the highest numbers of genetically unique adult worm pairs. Therefore, species-relative mobility and home

range, as well as longevity seem to be likely hypotheses to explain such differences in the estimates of infection intensity.

Definitive hosts often develop immunity against schistosome after infection (Taylor, 1996) and such induced immune responses, for example, in bovines to *Schistosoma*, is of major importance in the regulation of infection intensities in the field (Vercruysse & Gabriel, 2005). However, the acquired resistance may differ, in terms of the quality and quantity, among species of hosts, and even vary within species with host age, sex and duration or intensity of infection (Gryseels, 1994) (see Chapter 1). In some cases, the immunity against *S. japonicum* is strain-specific (McManus & Bartley, 2004) and strains (or genotypes) that are not the same as or related to the previously established ones within the host may have a higher probability of successful penetration and following development. For example, irradiated Chinese *S. japonicum* cercariae vaccines were protective only against homologous challenge in mice (Hope *et al.*, 1996). Such strain-specific acquired immunity against *S. japonicum* may inflict differential selection pressure on various *S. japonicum* strains within host individuals. From this study, in Guanghui (marshland), the infection intensity was higher in cattle than in water buffalo but with no significant difference, partly due to differential immunity against *S. japonicum* between them. Within either of two hilly villages (Longquan and Yuantou), the obvious difference in the infection intensity between rodents and dogs, however, could be owing to their contrasting mobility and home range rather than any potential immune ability. Nevertheless, species-associated complicated immunocompetence and moreover parasite strain-specific immunity could be a part in the explanation of the differences among host species in the

estimated infection intensities or within-host parasite population growth (Anderson, 1998), particularly for species of mammals with a longer life span.

Infection of given genotypes of parasites across host species, cross-infection, could be due to a common infection focus, 'hot spot', shared among them. *S. japonicum* cercariae from a given infection 'hot spot' may not vary, in terms of genotypes or genotype composition, rapidly within a short period, for example a few weeks or months, due to the existence of the infected snails (Liang *et al.*, 2005; Ishikawa *et al.*, 2006). Therefore, cross–infection of the parasites among host species could occur via water contact and the extent of which would be determined on part by the type and frequency of contacts of the species and accessibility of the 'hot spot'. In this study, large environmental differences existed among the hilly villages as well as between the marshland and the hilly regions. In the marshland, bovines, goats and dogs may have free access to grasslands and shallow water bodies along the banks, with bovines and goats usually grazing in the morning and dogs roaming randomly there without fixed point of time. In the hilly areas, infected snail habitats are distributed along ditches around patched rice fields, which each belong to a different farmer. It is thus very likely that, in the hilly region, the owners, their dogs and nearby rodents, may contact the same pool of cercariae. These different settings between the two regions and even among the hilly villages may explain the observed different frequency of cross infection among species between villages.

From the epidemiological point of view, the definitive hosts from which most of the cercariae genotypes or 'strains' are derived from could be regarded as the main reservoirs in each area. Indeed, the results from this study revealed that, in the

marshland, cattle may be responsible for the establishment of the vast majority of cercariae within snails, whereas in the hilly region, dogs or rodents in Longquan, rodents in Longshang, and dogs, humans or an unknown source in Yuantou appeared to be key reservoirs. In one marshland village, Guanghui, cercariae with late emergence were not able to trace back to any infected diurnal mammals identified there, potentially suggesting additional reservoirs, for example, rodents.

There is one inherent limitation in this study. As each female *S. japonicum* produces an average of over 1,000 eggs/day with one third to one-half of the eggs being passed in its host's faeces (Cheever *et al.*, 1994), only ten to 12 miracidia, due to limited resources and samples available, from each infected host were genotyped and then included into the analyses, potentially therefore leading to sample bias. However, this study is, as far as I am aware, the first to elucidate the transmission dynamics based on sibling relationship analyses. Although the results from this study did not appear to provide enough strong evidence for the hypothesis particularly regarding the main reservoirs in the hilly region, it did indeed display that the mean infection intensity, based on the estimated genetically unique adult worm pairs per host, varied with species, and that the proportion of infection with given parasites across species differed from village to village. Such results may be useful for the improved understanding of differences in transmission dynamics between and within these two regions.

5.6 ACKNOWLEDGEMENTS

Many thanks go to Jaya and Lynsey for her teaching and training on PCR analyses. Special thanks go to James for his optimization of PCR reaction conditions and running PCR on most of the miracidia samples. Many thanks also go to Poppy and Lottie for their critical reading and comments of this chapter. This research was funded by grants from the Royal Society (to JPW), the Kwok Foundation (to DBL, CAD and JPW), and the Medical Research Council (to JWR).

5.7 TABLES

Table 5.7.1 Estimated genetically unique adult worm pairs within individual definitive hosts in the marshland

Hosts	Analyzed miracidia	Adult worms (pairs)	Hosts	Analyzed miracidia	Adult worms (pairs)	Hosts	Analyzed miracidia	Adult worms (pairs)
Guanghui, marshland								
Water buffalo 1	11	10	Cattle 1	10	7	Dog 1	10	9
Water buffalo 2	11	9	Cattle 2	12	9	Dog 2	11	11
			Cattle 3	11	11			
			Cattle 4	12	10			
			Cattle 5	12	11			
			Cattle 6	10	8			
			Cattle 7	12	10			
			Cattle 8	11	8			
			Cattle 9	10	10			
			Cattle10	10	8			
			Cattle11	12	11			
			Cattle12	11	11			
			Cattle13	11	7			
			Cattle14	12	11			
			Cattle15	9	9			
Mean(SD)	11 (0)	9.5 (0.71)		11 (1)	11 (1.50)		10.5 (0.71)	10 (1.41)
Kruskal-Wallis Test, χ^2=0.30, df=2, p=0.86								
Heping, marshland								
			Cattle01	8	7			
			Cattle02	10	10			
			Cattle03	10	7			
Mean(SD)				9.3(1.15)	8 (1.73)			
Xingzhuang, marshland								
Goat 1	11	10	Cattle 1	11	9	Human1	9	2
Goat 2	12	7	Cattle 2	11	8			
Goat 3	2	2	Cattle 3	10	10			
Goat 4	11	9	Cattle 4	10	10			
Goat 5	12	10	Cattle 5	12	12			
Goat 6	12	7	Cattle 6	12	9			
Goat 7	12	11	Cattle 7	12	11			
Goat 8	11	10	Cattle 8	12	12			
			Cattle 9	12	11			
			Cattle10	12	9			
			Cattle11	12	10			
			Cattle12	11	10			
			Cattle13	12	12			
Mean(SD)	10.4(3.42)	8.3(2.92)		11.5(0.89)	10.2(1.46)		9	2
Kruskal-Wallis Test, χ^2=5.20, d.f. =2, p=0.07								

Table 5.7.2 Estimated genetically unique adult worm pairs within individual definitive hosts in the hilly region

Hosts	Analyzed miracidia	Adult worms (pairs)	Hosts	Analyzed miracidia	Adult worms (pairs)	Hosts	Analyzed miracidia	Adult worms (pairs)
Longquan, hilly region								
Cat 1	7	1	Dog 1	11	9	Rodent 1	10	2
			Dog 2	10	8	Rodent 2	11	6
			Dog 3	12	8	Rodent 3	11	8
			Dog 4	11	10	Rodent 4	12	9
			Dog 5	11	9	Rodent 5	9	3
			Dog 6	11	8	Rodent 6	10	4
			Dog 7	11	7			
			Dog 8	12	5			
Mean(SD)	7	1		11.13(0.64)	8(1.51)		10.5(1.05)	5.33(2.80)
Kruskal-Wallis Test, χ^2=5.42, d.f. =2, p=0.07								
Longshang, hilly region								
Human 1	10	4	Dog 1	1	1	Rodent 1	11	6
Human 2	10	2				Rodent 2	10	7
Human 3	10	5				Rodent 3	10	5
Human 4	10	4				Rodent 4	11	1
Human 5	6	1				Rodent 5	9	6
Mean(SD)	9.2(1.6)	3.2(1.47)		1	1		10.2(0.75)	5(2.10)
Kruskal-Wallis Test, χ^2=3.77, d.f. =2, p=0.15								
Yuantou, hilly region								
Human 1	11	6	Dog 1	4	2	Rodent 1	11	1
Human 2	12	6	Dog 2	6	4	Rodent 2	11	5
Human 3	13	6	Dog 3	9	7			
			Dog 4	12	9			
Mean(SD)	12(1)	6(0)		7.8(3.50)	5.5(3.11)		11(0)	3(2.83)
Kruskal-Wallis Test, χ^2=2.28, d.f. =2, p=0.32								

5.8 FIGURES

Figure 5.8.1 Percentage of the total estimated genetically unique adult worm pairs, at the level of villages, for host species each or combined in the marshland

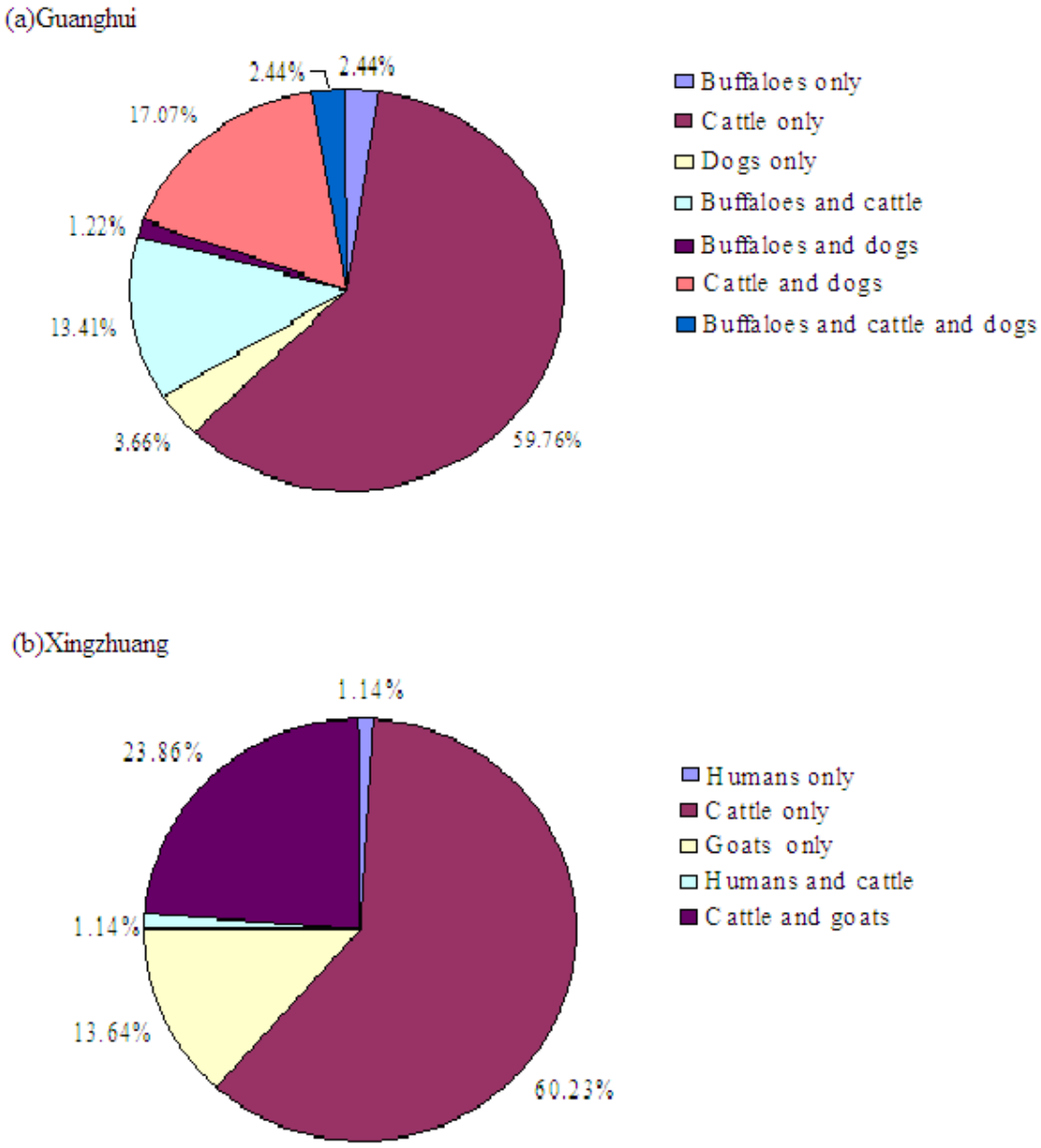


Figure 5.8.2 Percentage of the total estimated genetically unique adult worm pairs, at the level of villages, for host species each or combined in the hilly region

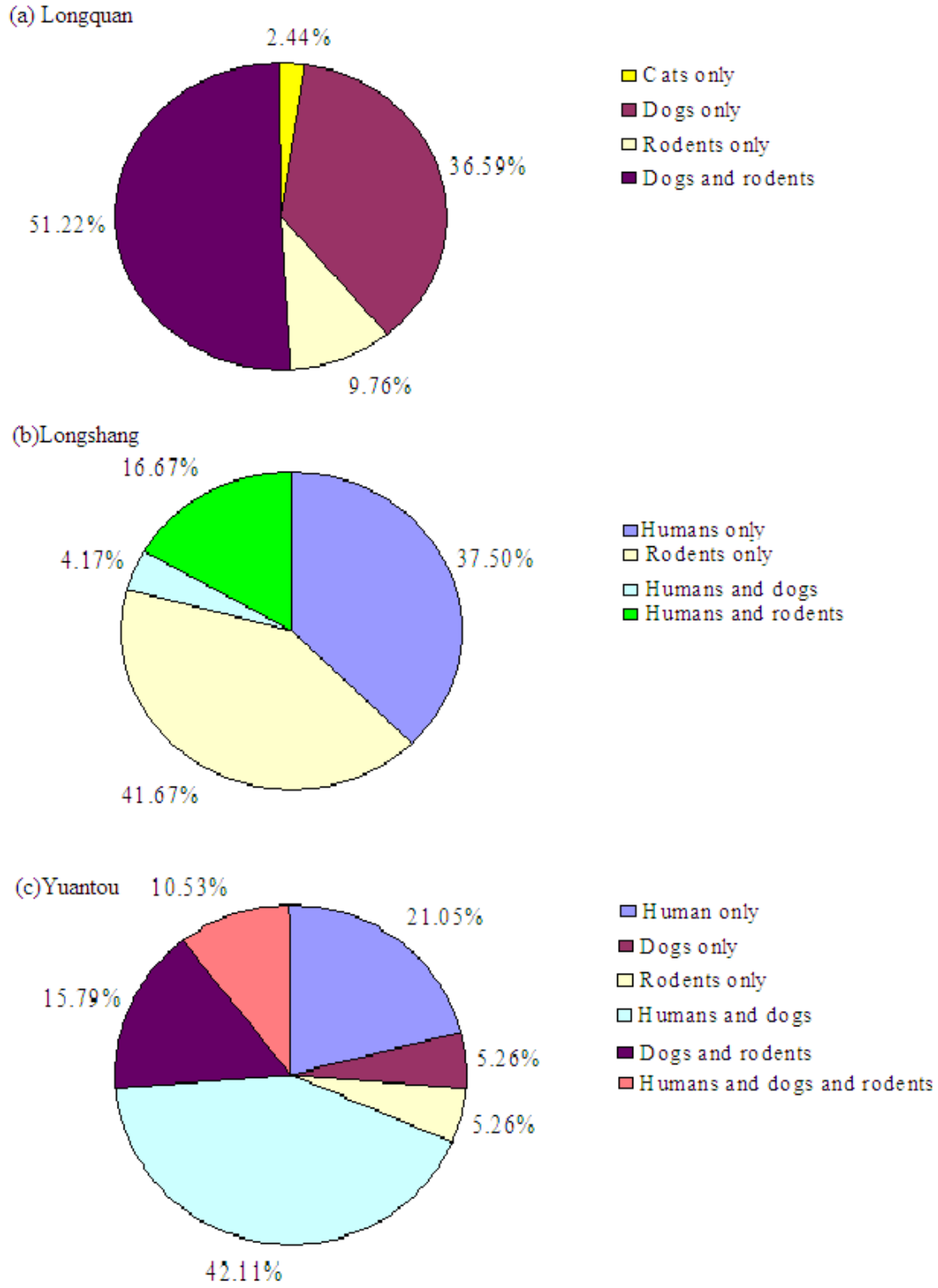
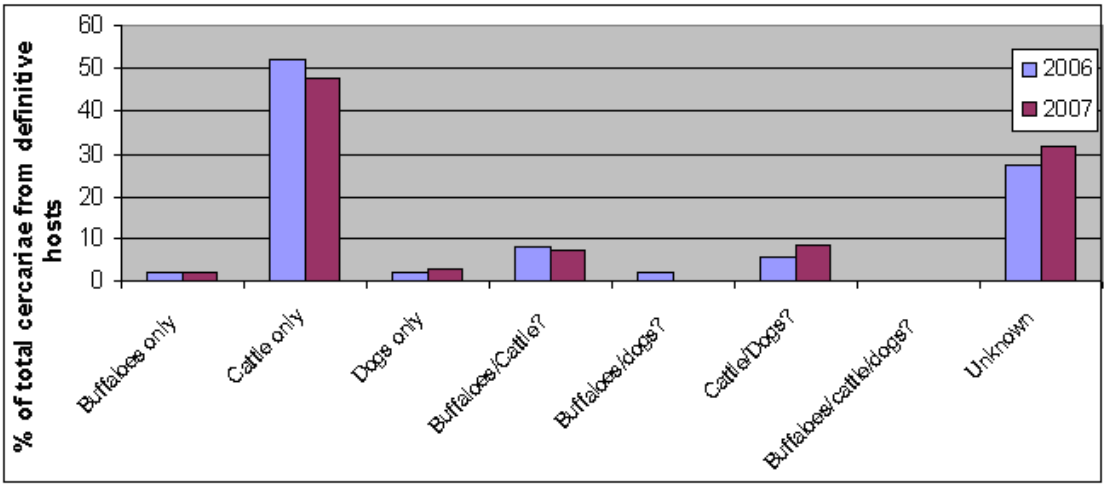


Figure 5.8.3 The estimated proportion of the total cercariae in either year originating from host species within each marshland village.

(a)Guanghui



(b)Xingzhuang

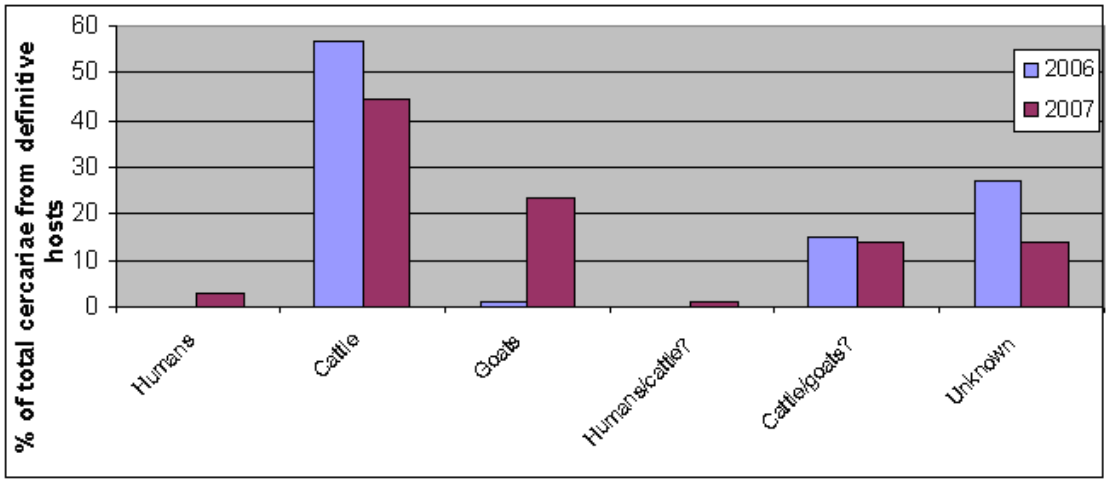
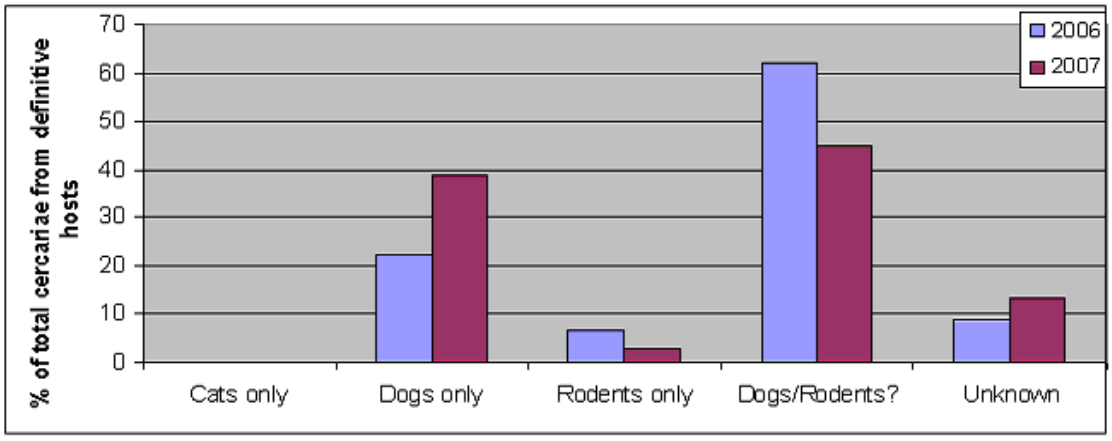
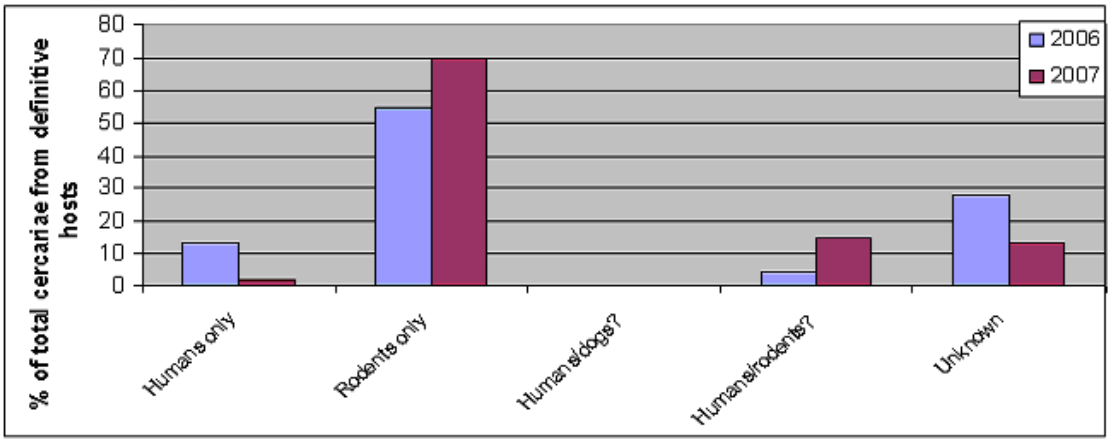


Figure 5.8.4 The estimated proportion of the total cercariae in either year originating from host species within each hilly village.

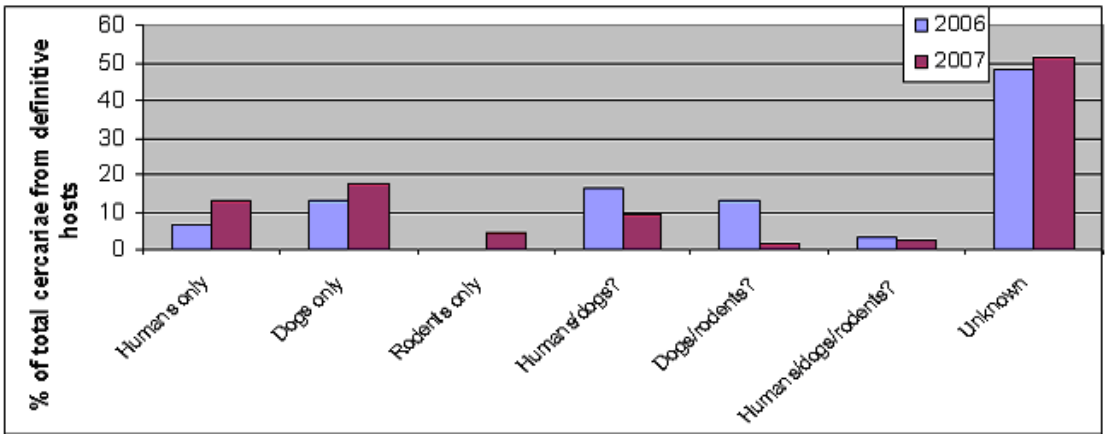
(a)Longquan



(b)Longshang



(c)Yuantou



Chapter 6: Do different main reservoir species mean different 'strains' of *Schistosoma japonicum*?: population genetic substructures of parasites in relation to geographical barriers or host-associated traits

6.1 ABSTRACT

As *Schistosoma japonicum* has been reported to be highly genetically diverse, the observed phenotypic variation (Chapter 3) and/or, more importantly, the heterogeneity of transmission (Chapter 4 & 5) for *S. japonicum* could be predicted to be associated with any genetic diversity and/or potential multi-strains of parasites between and within two piloted regions. Therefore, to test the hypothesis of potential multiple 'strains' of parasites between and within the researched regions, the relationship of larval populations between villages was first examined. Based on multilocus genotypes, population genetic substructures of parasites were further analysed at the levels of provinces (two counties combined), counties or villages. The results revealed that most *S. japonicum* larval populations from two ecological regions, as well as cercariae collected in 2007 with different shedding patterns, were substantially differentiated, thus suggesting a potential existence of multiple 'strains' of the parasite in relation to geographical barriers, main definitive host species, or both. Two sympatric 'strains' of parasites in terms of genotypes here identified within one marshland village and no or less substructure found within cercariae populations from either of two hilly villages (Longshang and Yuantou) further indicate that the potential speciation of local parasites towards their suitable definitive hosts, due to host-selection pressure, may indeed occur regardless of geographical distances.

6.2 INTRODUCTION

The majority of pathogens, including many of medical and veterinary importance, can infect more than one species of host (Woolhouse *et al.*, 2001). One of the main characteristics of parasites which puzzles most parasitologists is the diversity of host spectra: some species are extremely host-specific; whereas other parasites are generalists, infecting hosts from different taxonomic orders or even classes (Taylor *et al.*, 2001). For example, within the Schistosomatidae, *Schistosoma haematobium* is specific to human definitive hosts, whereas *S. japonicum*, with the highest virulence in humans due, primarily, to its relatively high egg output, can successfully infect 46 species of mammals (He *et al.*, 2001). One potential explanation proposed for this difference is that the *S. japonicum*-mammal system is 'old' in terms of evolutionary time (Rollinson *et al.*, 1997; Snyder & Loker, 2000; Attwood *et al.*, 2002). Therefore, the analysis of genetic diversity in *S. japonicum* populations is important for understanding the relationship between the parasites and their multiple hosts, and then associated transmission dynamics of the disease.

S. japonicum has been reported to be highly genetically diverse, and quite a number of studies have described the existence of various geographic strains among mainland China, the Philippines, Japan, Taiwan and Indonesia based on biological, morphological, immunological criteria and molecular analyses (Ruff *et al.*, 1973; Sobhon *et al.*, 1986; Merenlender *et al.*, 1987; Kresina *et al.*, 1991; McManus & Hope, 1993), in spite of no or low level of intra-specific variation of *S. japonicum* occasionally reported (Bowles *et al.*, 1993; Sorensen *et al.*, 1998). Recent studies suggest that, even within mainland China, several potential 'strains' of *S. japonicum* may exist, differing in infectivity to *Oncomelania hupensis hupensis* (He *et al.*,

1991a), prepatency (He *et al.*, 1991b) and other characteristics (He *et al.*, 1994; Chilton *et al.*, 1999). Using the Random Amplified Polymorphic DNA technique, the genetic differences were shown within *S. japonicum* populations from seven provinces within China (Gasser *et al.*, 1996; Anou *et al.*, 2002). Moreover, Shrivastava and her colleagues revealed, with microsatellite markers, high levels of genetic diversity between and within the different provinces and geographical regions (Shrivastava *et al.*, 2005b).

The existence of the above mentioned potential strains of *S. japonicum* within mainland China could be mainly accredited to geographically isolated (allopatric) speciation (Losos & Glor, 2003; Huyse *et al.*, 2005), due to genetic drift or mutation. Genetic drift may change allele frequencies due to stochastic fluctuations, and mutation may create novel alleles in isolated populations, either of which may tend to make local parasite populations different from others. Specifically, such potential strains of *S. japonicum* could be possibly explained by the 'speciation' of the parasites towards their different definitive hosts under host-induced selection pressure. As hosts represent a major part of the ecological needs (i.e. habitats, resources, and etc) of their parasites (Renaud *et al.*, 1996; de Meeus *et al.*, 1998) and then bring about a much larger number of diversifying factors for the parasite, the evolution of parasite genomes therefore may be shaped, to a large extent, by their hosts (Renaud & de Meeus, 1991). For instance, the study on *S. mansoni* with an early and those with a late cercarial shedding pattern, both from the Guadeloupean focus, suggests that the genetic variability between the two groups of parasites seems to be the consequence of the selective pressure exerted by the two different hosts (human and rat) implicated in the life cycle of the parasites (Theron & Combes, 1988). This may be particularly

true for *S. japonicum* with over 40 host species suspected as reservoirs (He *et al.*, 2001). Adaptation for *S. japonicum* to different sympatric hosts could select for intrinsic barriers to reproduction and then result in the separation between the parasite populations, which ultimately leads to speciation. Such process may be facilitated by geographical barriers, if any, between different groups of parasites.

It may be therefore predicted that the potential interactions of *S. japonicum* and its multiple definitive hosts, particularly in the case of nocturnal versus diurnal animals as definitive hosts in two regions within Anhui Province of China, as reported in Chapter 2 to 5, may be a useful system in which to test the hypotheses: 1) whether the local parasites specialize, at molecular levels, towards their each suitable definitive hosts, as indeed two biological 'strains' of *S. japonicum* have been observed between two regions and within the marshland (Chapter 3); and, 2) if there are any relationship between genetic distances of parasite populations and their geographical distances. Therefore, in this chapter, coupled with geographical information on the sampled villages (Figure 2.8.1 in Chapter 2) and the biological traits of cercariae (Chapter 3), the relationship of parasites between villages was first investigated and population genetic substructure of parasites was further examined at different levels. Such knowledge may deepen our further understanding of the differential transmission process between and within two regions in terms of parasites.

6.3 MATERIALS & METHODS

The sampling and genotyping of larval samples were detailed in Chapter 4 (See 4.3.1) and the repeated cercariae genotypes within individual snails were reduced to single copies before the following genetic analyses.

6.3.1 Pairwise F_{ST}s of parasites between villages

To measure a short-term genetic distance of larval populations between villages (or between different shedding patterns for cercariae sampled in 2007), Pairwise F_{ST}*s*, based on different alleles, were calculated with Arlequin 3.11 (Excoffier *et al.*, 2005). Correlation between genetic and geographic distances was implemented using GENEPOP 3.3 (Raymond & Rousset, 1997) and the significance of the correlation coefficient was tested using the Mantel test (Mantel, 1967) at the number of 1000 permutations.

6.3.2 Phylogenetic trees of parasites based on different genetic distances

Phylogenetic trees were constructed based on three (as detailed below) different genetic distances of parasites (miracidia or cercariae) between villages. The robustness of the relationships of parasites was obtained by 1000 bootstrap re-samplings of loci. The first two were carried out with SEQBOOT.EXE, GENDIST.EXE, CONTML.EXE, NEIBOR.EXE, and CONSENSE.EXE within PHYLIP package (Felsenstein, 2005) and the last with POPULATIONS software (Langella, 1997). The trees were visualized using MEGA version 3.1 (Kumar *et al.*, 2004).

(1) Under the assumption that each locus evolves independently only by genetic drift, the phylogenies were estimated by the restricted maximum likelihood method with

Contml program (Felsenstein, 2005). This program square-root-transforms the allele frequencies first and then applies the Brownian motion model (Blum *et al.*, 2004) on the resulting coordinates, in an approximation equivalent to the Cavalli-Sforza and Edwards's chord measure (Cavalli-Sforza & Edwards, 1967).

(2) Since mutation rate for a microsatellite sequence is expected to be higher than for other random or coding sequences (Jarne & Lagoda, 1996), allele frequencies may change not only by genetic drift, but also by mutation. Therefore, based on the infinite allele model of mutation, in which there is a rate of neutral mutation and each mutation introduces a distinct new allele, the Nei genetic distance (Nei, 1972) was computed to measure the relationship of parasite populations between villages.

(3) Due to the possible bias resulting from limited sampling of individual larvae within each parasite population in the calculation of the above Nei genetic distance, an estimation of unbiased Nei's standard genetic distance (Nei, 1978) was implemented.

6.3.3 Population substructures of parasites at levels of province, county or village

To elucidate any patterns of speciation for *S. japonicum*, geographical or host-related, the substructures of parasite populations were inferred at different levels. Several methods, based on various parametric models utilizing information from multilocus genotype data and Bayesian statistical frameworks, have been introduced to infer clustering or subdivision of individuals without the need of *a priori* population information (Pritchard *et al.*, 2000; Dawson & Belkhir, 2001; Falush *et al.*, 2003; Corander *et al.*, 2004; Corander & Marttinen, 2006). The ability of these methods to

infer the correct number of subpopulations has been tested with simulated data and various dispersal scenarios (Evanno *et al.*, 2005; Latch *et al.*, 2006; Waples & Gaggiotti, 2006; Vaha *et al.*, 2007). Such model-based methods have been shown to be able to correctly estimate the number of subpopulations even in scenarios with relatively low level of genetic differentiation among subpopulations (Latch *et al.*, 2006).

Here the program STRUCTURE, a model-based clustering method (Pritchard *et al.*, 2000; Falush *et al.*, 2003), was employed to infer the population substructure of parasites at levels of villages, counties or the province (two counties combined). It is assumed that there are *K* subpopulations (where *K* may be unknown) within a defined parasite population, each of which is characterized by a set of allele frequencies at each locus. Individuals are assigned to subpopulations, or jointly to two or more subpopulations if their genotypes indicate that they are admixed. To estimate the *K* value, the program each time was run with a burn-in length of 15,000 and running length of 10,000, based on the admixture model assuming that individuals may have mixed ancestry. The correlated frequencies model, assuming that allele frequencies in different populations are likely to be similar probably due to migration or shared ancestry, was applied for each given *K* to investigate the posterior probabilities of the inferred population structure (a given *K*). For example, *K* value was set from 2 to 40 when running the program using the whole cercariae populations (two counties combined), and *K* from 2 to 30 for data from each region or village only. The subdivision solution with the highest posterior probability was chosen as the correct partitioning on which the number of subpopulations (*K*) was subsequently inferred.

6.4 RESULTS

6.4.1 Population Pairwise F_{ST}s between villages

As seen in Tables 6.7.1-3, according to the qualitative levels for the interpretation of F_{ST} genetic differentiation (Wright, 1978), 'moderate' to 'very great' genetic differentiation was found both between the two regions (i.e. two counties) nearly 200km apart, and between hilly villages which are from three different townships, and 'little' was found between marshland villages which are from the same township (see locations of six villages in Figure 2.8.1), suggesting a relationship between genetic and geographical distances. It was also noted that 'great' genetic differentiation was found between two biological 'strains' of cercariae populations (i.e. early emergence and late emergence), both from the same village Guanghui, as that between cercariae with a late shedding from Guanghui and those from Longquan or Longshang (see Table 6.7.3).

The Mantel test was applied to assess the correlation between Pairwise (F_{ST}/(1-F_{ST})) and natural logarithm of geographic distance between the sampled villages. A significant trend between both variables was seen for cercariae ($r = 0.04$, $p < 0.01$) and miracidia ($r = 0.03$, $p < 0.01$) sampled in 2006, but not seen for cercariae sampled in 2007 ($r = 0.02$, $p = 0.22$).

6.4.2 Population phylogenetic trees among villages

The genetic relationships of the parasite populations between villages were also inferred based on three different genetic distances. Under the assumption that population genetic differences may result from pure genetic drift, a phylogeny was estimated by the restricted maximum likelihood method (Felsenstein, 2005). In the

trees (Figure 6.8.1(a-c)), a reasonably well-supported cluster (70.6-100%) included all the larval populations from the three hilly villages, largely corresponding to regions (counties). As shown in the trees (Figure 6.8.1(a-b)), however, there was inconsistency between the trees resulted from cercariae and from miracidia. In Figure 6.8.1(b), based on miracidia samples, there appeared to be a closer relationship for Heping (marshland) than any other marshland village to the three hilly villages, the opposite of which was seen in Fig. 6.8.1(a) using cercariae sampled in 2006.

Inconsistency was also seen among the UPGMA (Unweighted Pair Group Method with Arithmetic mean) trees (Figure 6.8.2(a-c)), constructed with Nei (1972) genetic distance taking into account both genetic drift and neutral mutation. Figure 6.8.2(a-b) revealed that Longshang (hilly village) separated from the cluster containing all other villages, and further in the deeper branch of the latter, three marshland villages clustered together and separated from the other two hilly villages. This result may be in slightly conflict with the geographical relationship of the villages. As seen in Figure 2.8.1, Longshang, located in the centre of the county, is closer to the marshland than other hilly villages and also linked to the marshland via a small river.

A better demonstration of the relationship between villages is shown in Figure 6.8.3(a-c), when the phylograms were constructed based on unbiased Nei's standard genetic distance (Nei, 1978) considering genetic drift, mutation and sampling sizes. The consistent results, from either cercariae or miracidia, provided evidence of clustering of larval populations at the level of sampled villages. Parasites sampled from marshland villages always clustered together, and those in two hilly villages (Longquan and Yuantou) always clustered together. Parasites from Longshang

showed a closer relationship than from any other hilly villages to those from the marshland, which was indeed in agreement with geographical locations of the villages.

When cercariae from Guanghui (one marshland village) sampled in 2007 were classified into two groups of populations according to their biological traits, early or late emergence, the node uniting the cercariae population with late shedding from the marshland to the cercariae populations with the same character from the two hilly villages (Yuantou and Longquan) was strongly supported by bootstrap re-sampling with a value ranged from 45 to 91.8% (Figure 6.8.1(d), Figure 6.8.2(d) and Figure 6.8.3(d)) based on three different genetic distance measures, from which the second and third trees appeared to be better in reflection of the relationship between villages.

6.4.3 Population genetic substructure at different levels

In the whole larvae sample (two counties combined), as seen from Figures 6.8.4-6, by $K = 2$, a very clear distinction between parasite populations from the marshland and from the hilly region was seen only in cercariae sampled in 2006, rather than in miracidia in 2006 and cercariae in 2007. Assuming $k = 3$, a clear distinction remained between parasites from the marshland and from the hilly region, with the exception of cercariae and miracidia sampled in Longshang (Hilly village) in 2006 and of a few miracidia sampled from cats in Longquan (Hilly village) which were not completely separated from those from the marshland. When K increased to 4, most cercariae from two regions in either year were separated; however, miracidia sampled from cats in Longquan and those from humans in Longshang were not able to be distinct from those from the marshland. It was also noted that in Fig 6.8.6, a few cercariae with late

emergence from the marshland always clustered with cercariae from two hilly villages (Longquan and Yuantou). According to the posterior probabilities of given different *K* values, there might be approximately 28, 38 and 28 subpopulations within the cercariae (2006), miracidia (2006) and cercariae (2007) populations, respectively (Figures 6.8.4-6).

As there were multiple clear subpopulations between and within two regions, STRUCTURE was then run using the parasites from one county or village only to see whether there is a strong signal as described above. As seen in Figure 6.8.7, within the hilly region (three hilly villages combined) the separated subpopulations of parasites, namely K=3 for cercariae and K=4 for miracidia, were relative to geographical locations. Such subdivision for miracidia samples was also in relation, to a smaller extent, to the species of definitive hosts. For example, in Longquan miracidia from cats were distinct from those from other species, and in Longshang the great proportion of miracidia from humans were separated from those from rodents. Within the hilly region, there appeared to be approximately 9, 19 and 15 subpopulations within the cercariae (2006), miracidia (2006) and cercariae (2007) populations, respectively. However, within the marshland, no distinction was seen between three villages, nor for the miracidia between different species of definitive hosts, as detailed in Figure 6.8.8. There appeared to be 22, 28 and 23 subpopulations within the cercariae (2006), miracidia (2006) and cercariae (2007) populations, respectively (see Figure 6.8.8).

When running the program at the level of villages, 6, 13 and 6 subpopulations were found within Longquan in cercariae (2006), miracidia (2006) and cercariae (2007)

populations, respectively (Figure 6.8.9). Although approximately 9 subpopulations were seen in miracidia sampled from either Longshang or Yuantou, no or less substructure was observed in cercariae, as each cercaria was estimated to have partial membership in multiple subpopulations with similar membership coefficients for all individuals.

Within the marshland, in stark contrast, more complicated substructures were observed within each village. As shown in Figure 6.8.10, the estimated maximum numbers of subpopulations within Guanghui could be 10, 19 and 17 within three larval populations sampled in three consecutive periods. In Xingzhuang, estimates of 9, 21 and 4 subpopulations could be respectively inferred from the corresponding larval samples. However, in Heping such substructure was only seen in the cercariae rather than in the miracidia population, partly due to the small sample size of miracidia.

6.5 DISCUSSION

Mutation, genetic drift due to finite population size and natural selection favouring adaptations to local environmental conditions will all lead to genetic differentiation of local populations, while the movement of gametes (or infra-populations for parasites), individuals, and even entire populations will oppose that differentiation (Slatkin, 1987). In the current chapter, the microsatellite analysis of *S. japonicum* larval populations, cercariae and miracidia directly sampled from the three marshland and three hilly villages within Anhui Province of China during 2006-2007, demonstrated strong genetic divergence between two regions and between parasites with different shedding patterns, which may be associated with geographical distances and/or different main reservoirs (cattle versus rodents) introducing host-selection pressures on *S. japonicum*.

6.5.1 Genetic differentiation between villages relative to geographical distances

The observed substantial genetic differentiation, from 'moderate' to 'very great', between the two regions nearly 200km apart, and between the three hilly villages more than 20km apart or even separated by river systems, and 'little' differentiation found between marshland villages, which are from the same township, showed a strong relationship between gene flow and geographical distances, as reported at the level of provinces within mainland China (Shrivastava *et al.*, 2005b). This was also evident from the correlation analyses of parasites sampled in 2006 between genetic distance and geographical distance. The UPGMA phenograms (Figure 6.8.3) and structure clustering (Figures 6.8.4-6) showed that the parasites from the hilly region branched off quite separately from the marshland, suggesting that the parasites from two regions may be following different lineages, namely host-adapted specialization

of *S. japonicum*, as it was indeed, from previous data chapters, suggested that contrasting main definitive hosts exist between two regions.

River system (flow direction and distance) also appeared to be an important indicator of parasite genetic variation. This was illustrated by lower genetic distance seen between the hilly village Longshang, connected to the marshland via a small river, and the marshland villages than between Longquan, another hilly village located along a separated river, and the marshland villages. Similar evidence was also demonstrated on the phylogenetic trees (Figure 6.8.3), in the deeper branches of which Longshang always clustered closer with the marshland villages than other hilly villages.

The observed difference in subpopulation richness for parasites at the level of villages, from clustering analyses, could be accredited, to some extent, to the geographical distributions of the sampled sites. Three marshland villages, located on the same island of the Yangtze River (see Figure 2.8.1), may be more frequently influenced, in relation to the hilly villages, by their upstream endemic areas via flooding, as the intermediate host snails tend to be swept along from flood prone regions of the river and be deposited in such suitable habitats (Davis *et al.*, 1999a), whereas the hilly villages are more isolated and then less influenced by other endemic areas.

6.5.2 Genetic differentiation between villages associated with main definitive hosts

Schistosomes spend much of their life-cycle within definitive hosts, compared to their intermediate snail hosts, and different species of definitive hosts may represent different kinds of resources and habitats (Renaud *et al.*, 1996; de Meeus *et al.*, 1998).

Therefore, the biological and immunological aspects of their definitive hosts may be predicted to influence parasite population structure, for example, at the levels of individual hosts or species, which has been described in Chapter 4.

Differential host-induced selection on parasites has already been demonstrated on *S. mansoni* according to the species of definitive host used. Laboratory passaged populations of *S. mansoni* experimentally 'switched' from baboons to murine hosts were shown to lose allozyme polymorphism compared to those continuously maintained through a baboon host (LoVerde *et al.*, 1985). Even when originating from a natural murine host (*Rattus rattus*), maintaining *S. mansoni* on an experimental murine host (white mice) increased the frequency of the least frequent allele for the malate dehydrogenase polymorphic locus (Bremond *et al.*, 1993). Differential expression of *S. mansoni* genes due to host sex is also reported. Of the at least 11 differentially expressed genes in female worms, 10 were preferentially expressed in female worms from male mice, whereas of the 134 differentially expressed genes in male parasites, 79 (59%) were preferentially expressed in worms from female mice (Waisberg *et al.*, 2008). In this study, I indeed found, based on multilocus genotypes of parasites, the contrasting population genetic structures at the level of villages between two regions, with larger numbers of subpopulations found within the marshland villages and smaller numbers within the hilly villages. One possible explanation, apart from an obvious difference in life span of the suspected main reservoirs, might be accredited to the differential selection pressure on *S. japonicum* from different host species reservoirs. This may also be, in part, supported by the phylogenetic trees when taking into account not only genetic drift, but also mutation and sampling sizes.

For *S. japonicum*, Shrivastava and her colleagues even showed that miracidial samples did not closely cluster with their potential parents adult worms from the same hosts (Shrivastava *et al.*, 2005a). The results from my study showed that, within either of two hilly villages (Longshang and Yuantou), miracidia from different species of hosts could, to some extent, be differentiated, whereas all cercariae populations showed a very similar pattern of no or less subdivision, suggesting a high probability of rodent-induced selective mutations of parasites, which are passaged onto and maintained within intermediate host snails.

The reason for the observed higher substructures within each miracidia population than within its either previous or followed-up cercariae populations in all villages, with the exception of Heping, could also be, in part, due to the several aspects, including a possible pre-patent mortality of infected snails (Manning *et al.*, 1995), competitive interactions between different genotypes of larvae within snails (Gower & Webster, 2005), or local repeated molluscicide (see Table 2.7.1 in Chapter 2). However, a recent study found evidence potentially indicative of particularly high microsatellite mutation rates occurring during asexual reproduction of *S. japonicum* within snails (Yin *et al.*, 2008), which could result in increase in diversity of cercariae populations and thus more subdivision.

6.5.3 Genetic differentiation relative to biological trait-based 'strains' of parasites

The cercariae collected in 2007 were classified into two biological 'strains', early emergence or late emergence. The results from the genetic analyses of cercariae populations showed strong genetic divergence associated with their polymorphism biological traits, both from population Pairwise F_{ST}s based on different alleles and the

phenograms based on the unbiased Nei's standard genetic distance. Such genetic differentiation observed between cercariae populations with different shedding patterns, may result, in part, from differences in host preference for the parasites, in which cercariae with morning shedding are suitable to diurnal animals while those with late afternoon shedding suitable to nocturnal animals. Such cercarial emergence asynchrony may be able to both initiate and maintain restricted gene flow among schistosome populations from the two contrasting definitive hosts, if they are sympatric as parasites with different shedding patterns were found in the same marshland village, via an assortative-mating system with early-emerging schistosome tending to return and reproduce in a diurnal animal, and late-emerging schistosome tending to return and mate in a rodent, as has been fully illustrated for *S. mansoni* between humans and rodents as reservoirs in Guadeloupe (Theron & Combes, 1995). In addition, the restricted gene flow between the two (cattle *vs* rodents) host-adapted populations of parasites could possibly be caused or further reinforced by geographical barriers, thus increasing the degree of differentiation (Barraclough & Nee, 2001). The reduction of gene flow, or even disruptive selection, may increase the probability of the isolated speciation of *S. japonicum*, particularly in the hilly villages here, as evident from no or less substructure estimated within cercariae populations from two hilly villages.

When the number of subpopulations were set from 2 to 30 in running STRUCTURE for cercariae sampled in 2007 from the marshland village Guanghui, the optimal partition was consistently obtained for 17 subpopulations, regardless of including or excluding cercariae with late shedding from the same village, thereby suggesting a high probability of the same origin of ancestral populations for the two biological

'strains' of parasites found in the marshland. However, cercariae with late shedding pattern from Guanghui (marshland) and those from Yuantou or Longquan (hilly region) always clustered together based on both STRUCTURE and phylogenetic trees, indicating a possibility of either dispersal of the parasites via host migration along the small river from the hilly region to the marshland, or simultaneous rodent-specific specialization for the parasites, although no infected rodents were found in the marshland partly due to a small number of rodents sampled (Chapter 2).

Overall, the results revealed that most *S. japonicum* larval populations from two ecological regions, as well as cercariae collected in 2007 with different shedding patterns, were substantially differentiated, thus suggesting a potential existence of multiple 'strains' of the parasite in relation to main definitive host species, geographical barriers, or both. Two sympatric 'strains' of parasites in terms of genotypes here and phenotypes (Chapter 3) found in the marshland and no or less substructure found within cercariae populations from either of two hilly villages (Longshang and Yuantou) further indicated that the potential speciation of local parasites towards their suitable definitive hosts may indeed occur due to host-selection pressure, regardless of geographical distances.

6.6 ACKNOWLEDGEMENTS

I wish to thank Jaya and Lynsey for her teaching and training on PCR analyses. Special thanks go to James for his optimization of PCR reaction conditions and running PCR on most of the miracidia samples. This research was funded by grants from the Royal Society (to JPW), the Kwok Foundation (to DBL, CAD and JPW), and the Medical Research Council (to JWR).

6.7 TABLES

Table 6.7.1 Pairwise F_{ST}s of cercariae populations between villages in 2006

	Guanghui (M)	Heping (M)	Xingzhuang (M)	Longquan (H)	Longshang (H)	Yuantou (H)
Guanghui(M)	0					
Heping(M)	0.0368*	0				
Xingzhuang(M)	0.0088	0.0207*	0			
Longquan(H)	0.1914*	0.1891*	0.1753*	0		
Longshang(H)	0.1294*	0.1281*	0.1216*	0.1610*	0	
Yuantou(H)	0.2597*	0.2665*	0.2514*	0.0762*	0.2907*	0

Distance method: different alleles; *, for $p<0.01$; number of permutations=15000.

M, for marshland; H, for hilly region

Table 6.7.2 Pairwise F_{ST}s of miracidia populations between villages in 2006

	Guanghui (M)	Heping (M)	Xingzhuang (M)	Longquan (H)	Longshang (H)	Yuantou (H)
Guanghui(M)	0					
Heping(M)	0.0315	0				
Xingzhuang(M)	0.0097	0.0365	0			
Longquan(H)	0.1752	0.1881	0.1688	0		
Longshang(H)	0.0645	0.0624	0.0647	0.1570	0	
Yuantou(H)	0.2006	0.2258	0.1939	0.0753	0.2069	0

Distance method: different alleles; all $p<0.01$; the number of permutations=15000

M, for marshland; and H, for hilly region

Table 6.7.3 Pairwise F_{ST}s of cercariae populations between villages or between different biological traits in 2007

	Guanghui (M)(E)	Guanghui (M)(L)	Xingzhuang (M)(E)	Longquan (H)(L)	Longshang (H) (L)	Yuantou (H)(L)
Guanghui(M)(E)	0					
Guanghui(M)(L)	0.2077	0				
Xingzhuang(M)(E)	0.0181	0.2233	0			
Longquan (H)(L)	0.1592	0.1609	0.1658	0		
Longshang (H)(L)	0.1235	0.3613	0.1576	0.2159	0	
Yuantou (H)(L)	0.2159	0.0974	0.2271	0.0837	0.3231	0

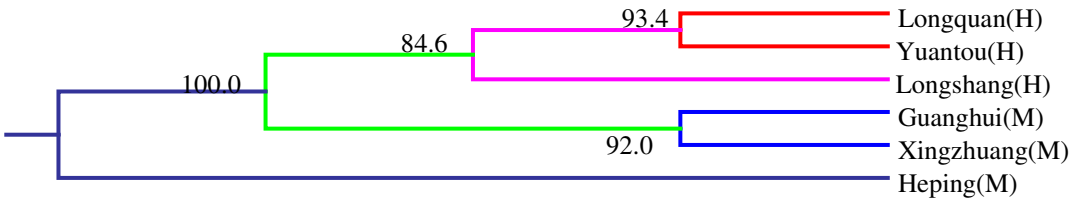
Distance method: different alleles; all $p<0.01$; the number of permutations=15000

M, for marshland; H, for hilly region; E, for cercariae with early emergence, and L, for cercariae with late emergence.

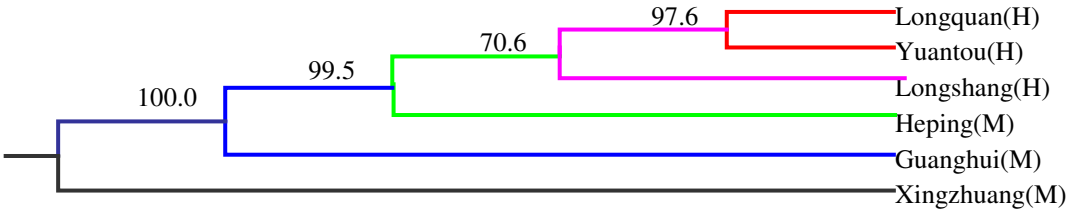
6.8 FIGURES

Figure 6.8.1 Phenograms depicting the relationship of parasites between villages using maximum likelihood analysis (H, hilly; M, marshland; L, cercariae with late emergence; and E, cercariae with early emergence). Numbers (percentage) next to branches indicate bootstrap support. Branch lengths are scaled according to genetic distance.

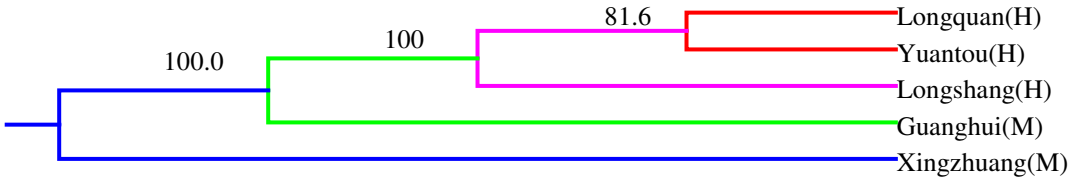
(a) Cercariae (2006)



(b) Miracidia (2006)



(c) Cercariae (2007)



(d) Cercariae with different shedding patterns (2007)

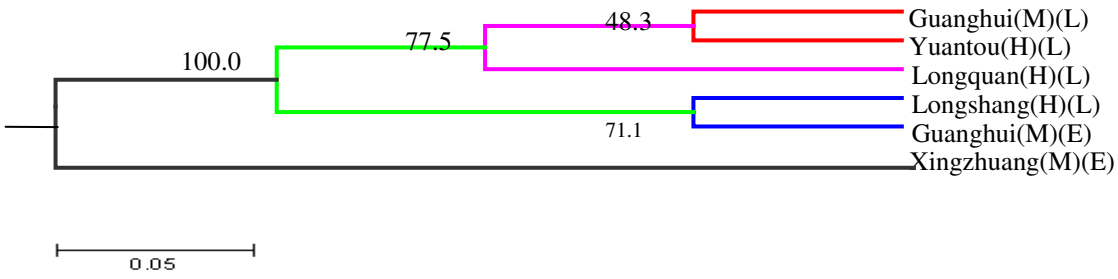
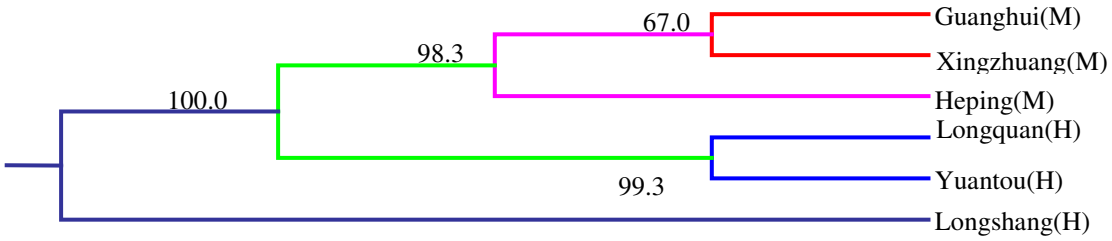
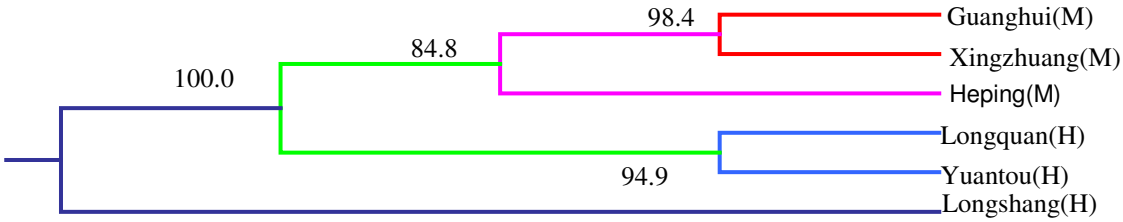


Figure 6.8.2 UPGMA Phenograms depicting the relationship of parasites between villages based on Nei (1972) genetic distance (M, marshland; H, hilly; L, cercariae with late emergence; and E, cercariae with early emergence). Numbers (percentage) next to branches indicate bootstrap support. Branch lengths are scaled according to genetic distance.

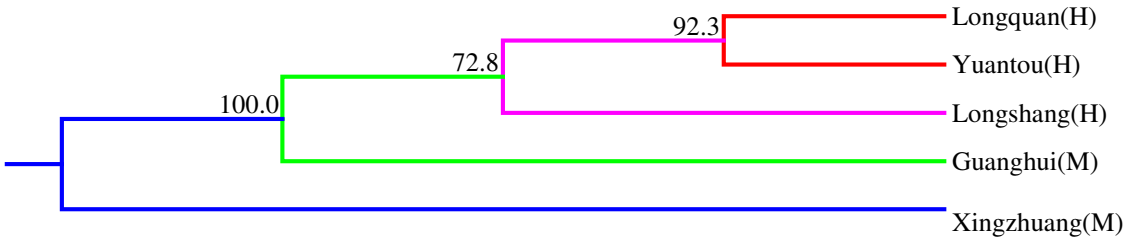
(a) Cercariae (2006)



(b) Miracidia (2006)



(c) Cercariae (2007)



(d) Cercariae with different shedding patterns (2007)

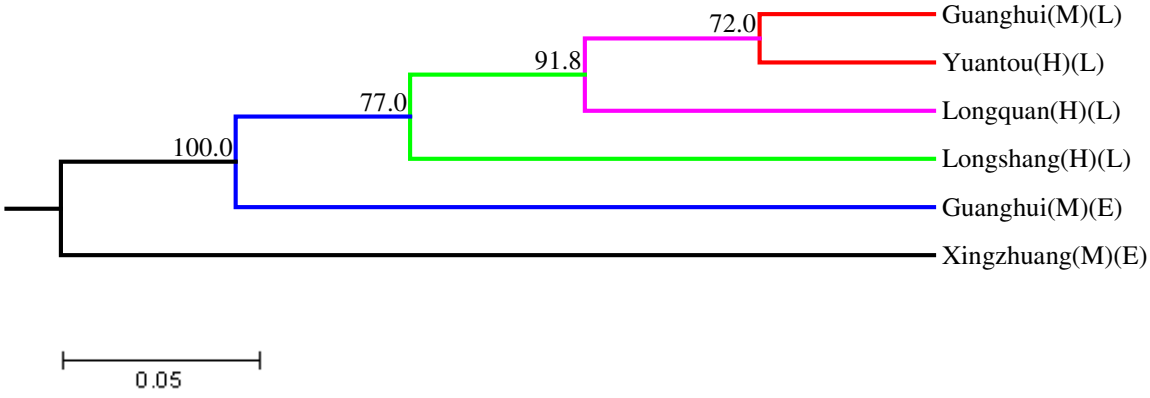
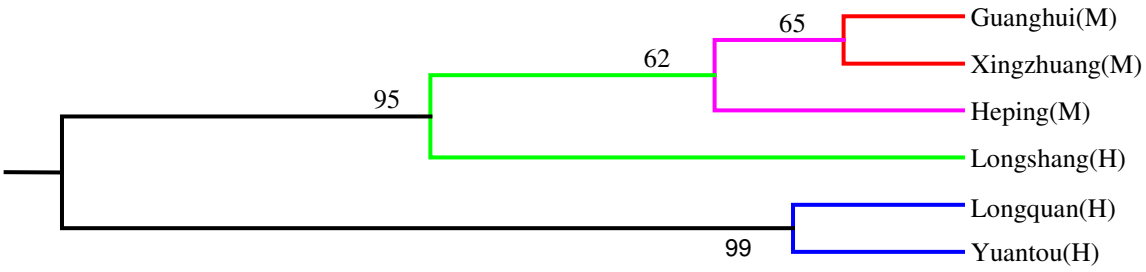
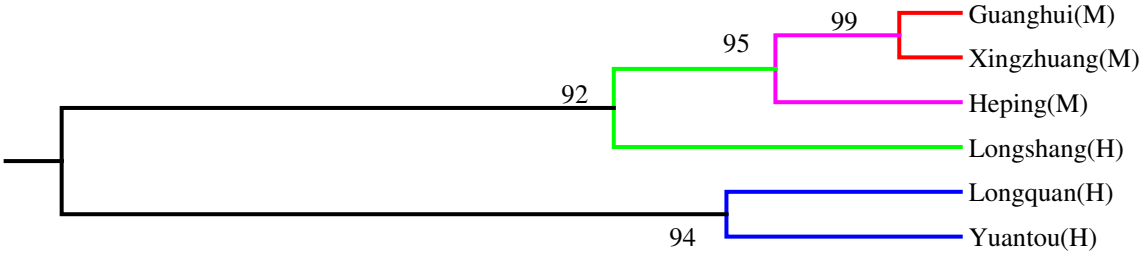


Figure 6.8.3 UPGMA Phenograms depicting the relationship of parasites between villages based on unbiased Nei's standard genetic distance (M, marshland; H, hilly; L, cercariae with late emergence; and E, cercariae with early emergence). Numbers (percentage) next to branches indicate bootstrap support. Branch lengths are scaled according to genetic distance.

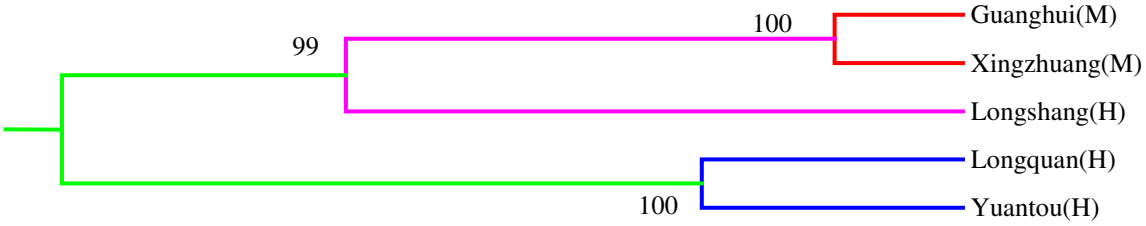
(a) Cercariae (2006)



(b) Miracidia (2006)



(c) Cercariae (2007)



(d) Cercariae with different shedding patterns (2007)

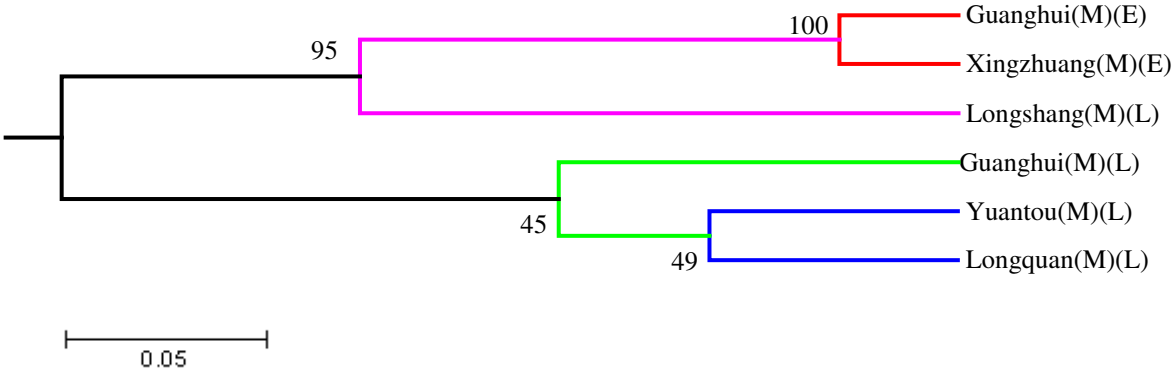


Figure 6.8.4 Estimated population structures of cercariae from two regions combined in 2006. Figure shows that each cercaria is represented by a thin vertical line, which is partitioned into a number of coloured segments that represent the individual's estimated membership fractions in the relative subpopulations (K, estimated number of sub-populations), such as 2, 3 and 4 respectively. The highest posterior probability of a given K was obtained when K was set at 28. The Length of Burn-in period and the number of Markov chain Monte Carlo (MCMC) replicates after Burn-in when running STRUCTURE, were set at 15,000 and 10,000, respectively. The cercariae populations were labelled above the figure with villages.

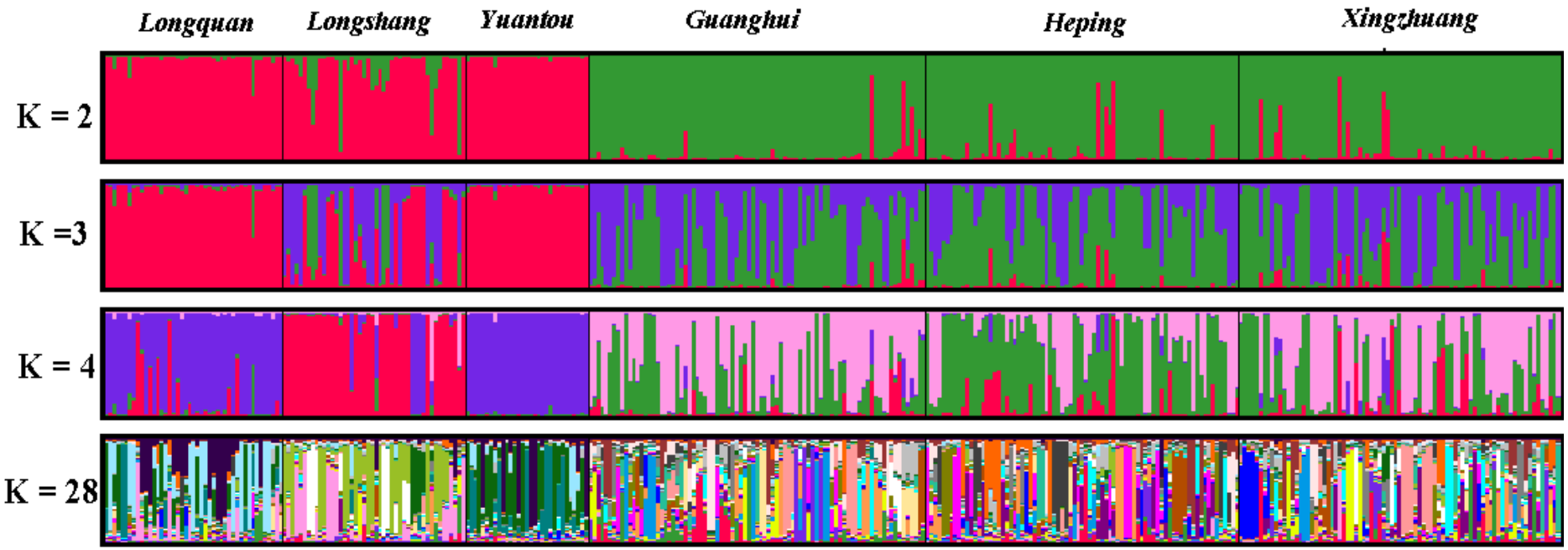


Figure 6.8.5 Estimated population structures of miracidia from two regions combined in 2006. Figure shows that each miracidia is represented by a thin vertical line, which is partitioned into a number of coloured segments that represent the individual's estimated membership fractions in the relative subpopulations (K, estimated number of sub-populations), such as 2, 3 and 4 respectively. The highest posterior probability of a given K was obtained when K was set at 38. The Length of Burn-in period and the number of MCMC replicates after Burn-in when running STRUCTURE, were set at 15,000 and 10,000, respectively. The miracidia populations were labelled below the figure with host species and above the figure with villages.

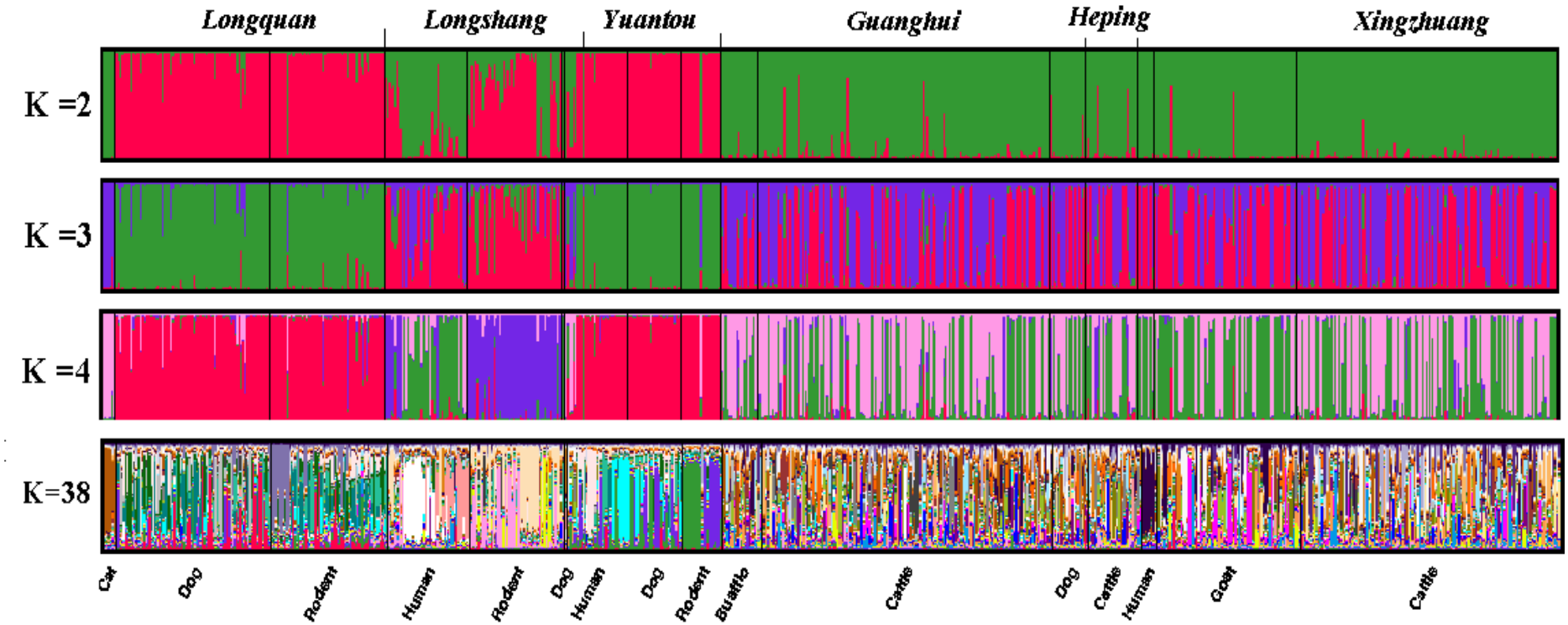


Figure 6.8.6 Estimated population structures of cercariae from two regions combined in 2007. Figure shows that each cercaria is represented by a thin vertical line, which is partitioned into a number of coloured segments that represent the individual's estimated membership fractions in the relative subpopulations (K, estimated number of sub-populations), such as 2, 3 and 4 respectively. The highest posterior probability of a given K was obtained when K was set at 28. The Length of Burn-in period and the number of MCMC replicates after Burn-in when running STRUCTURE, were set at 15,000 and 10,000, respectively. The cercariae populations were labelled above the figure with villages. Guanghui (L) indicats a cercariae population with late emergence, distinct from Guanghui representing cercariae with early emergence.

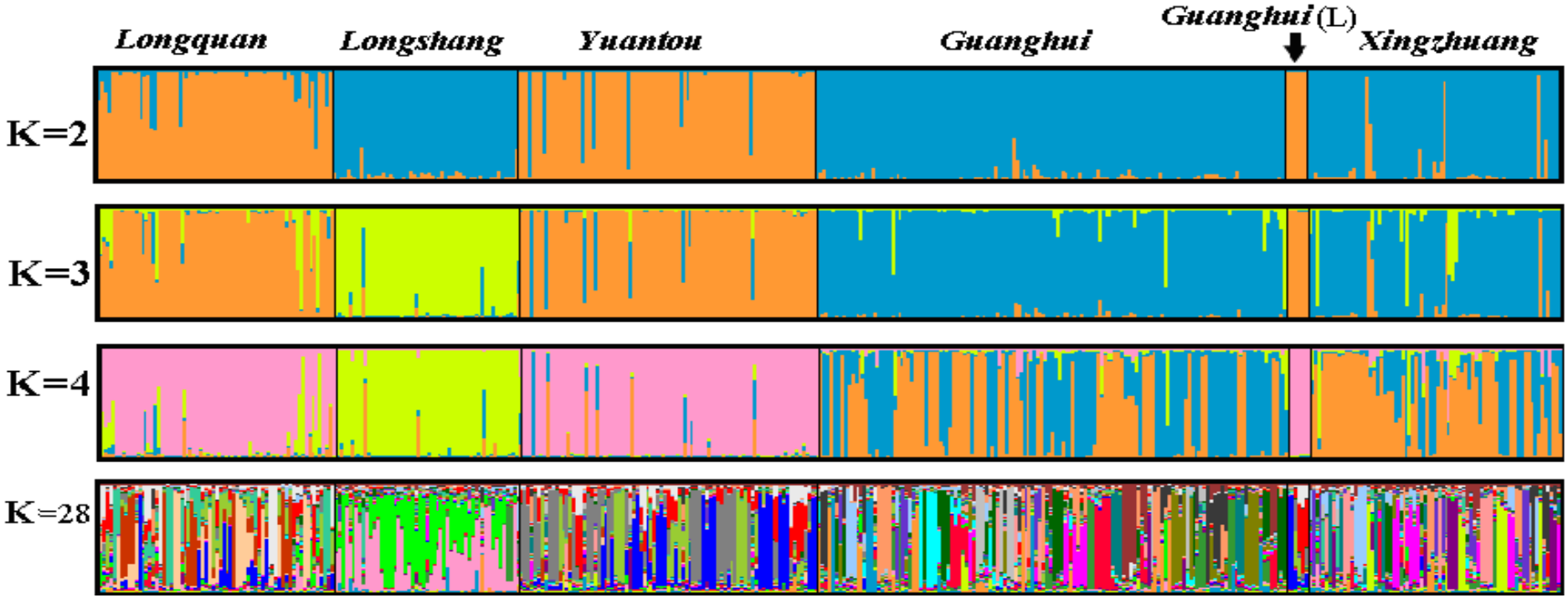


Figure 6.8.7 Estimated population structures of cercariae (2006), miracidia (2006) and cercariae (2007) within the hilly region. Figure shows that each individual larva is represented by a thin vertical line, which is partitioned into a number of coloured segments that represent the individual's estimated membership fractions in the relative subpopulations (K, estimated number of sub-populations), such as 2, 3 and 4 respectively. The highest posterior probability of a given K was obtained when K was set at 9 for cercariae (2006), 19 for miracidia (2006) and 15 for cercariae (2007). The Length of Burn-in period and the number of MCMC replicates after Burn-in when running STRUCTURE, were set at 15,000 and 10,000, respectively. The parasite populations were labelled above the figure with villages and below the figure with species of hosts (miracidia populations only).

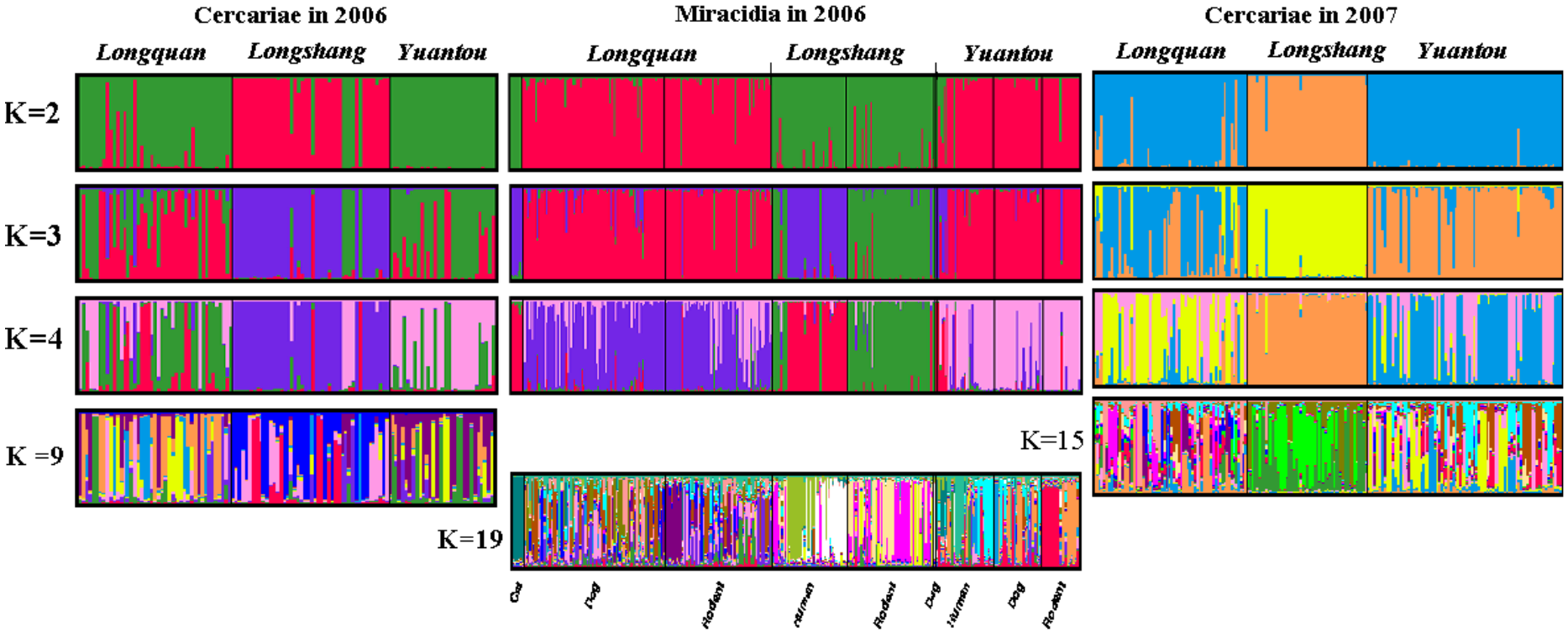


Figure 6.8.8 Estimated population structures of cercariae (2006), miracidia (2006) and cercariae (2007) within the marshland. Figure shows that each individual larva is represented by a thin vertical line, which is partitioned into a number of coloured segments that represent the individual's estimated membership fractions in the relative subpopulations (K, estimated number of sub-populations), such as 2 and 3, respectively. The highest posterior probability of a given K was obtained when K was set at 22 for cercariae (2006), 28 for miracidia (2006) and 23 for cercariae (2007). The Length of Burn-in period and the number of MCMC replicates after Burn-in when running STRUCTURE, were set at 15,000 and 10,000, respectively. The parasite populations were labelled above the figure with villages and below the figure with species of hosts (miracidia populations only).

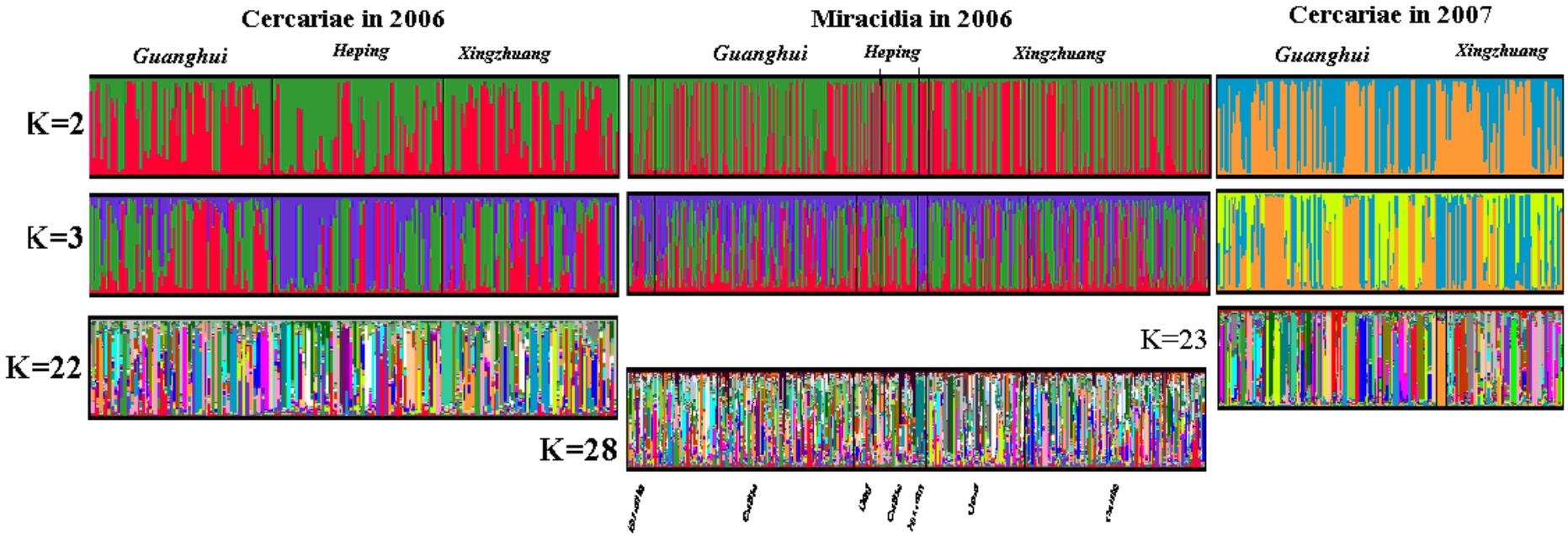
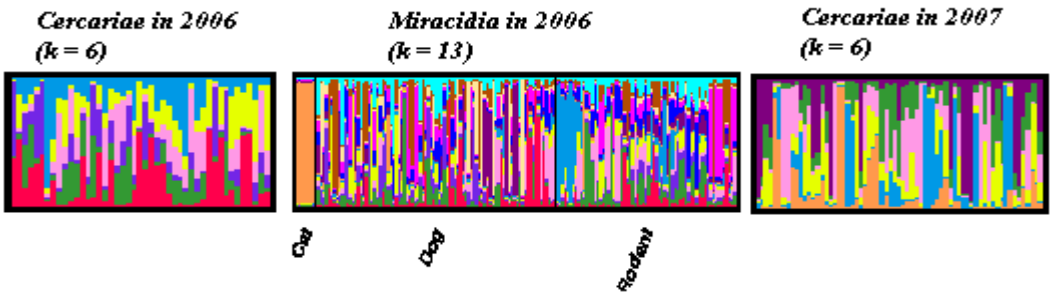
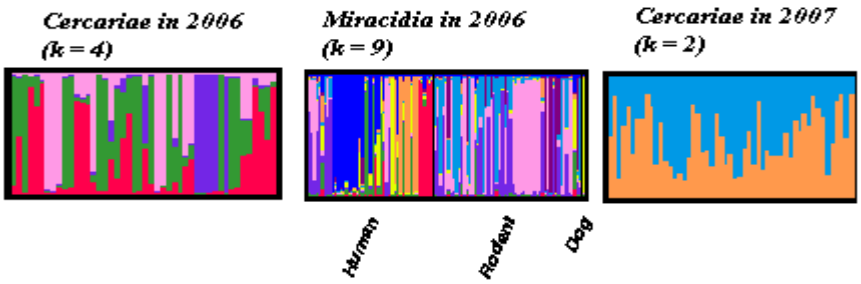


Figure 6.8.9 Estimated maximum numbers of sub-populations of cercariae (2006), miracidia (2006) and cercariae (2007) within each hilly village (a, Longquan; b, Longshang; and c, Yuantou). Figure shows that each larva is represented by a thin vertical line, which is partitioned into a number of coloured segments that represent the individual's estimated membership fractions in the relative subpopulations (K, the number of sub-populations according to the highest posterior probability of a given K). The Length of Burn-in period and the number of MCMC replicates after Burn-in when running STRUCTURE, were set at 15,000 and 10,000, respectively. The parasite populations were labelled below the figure with host species (miracidia populations only).

(a) Longquan



(b) Longshang



(c) Yuantou

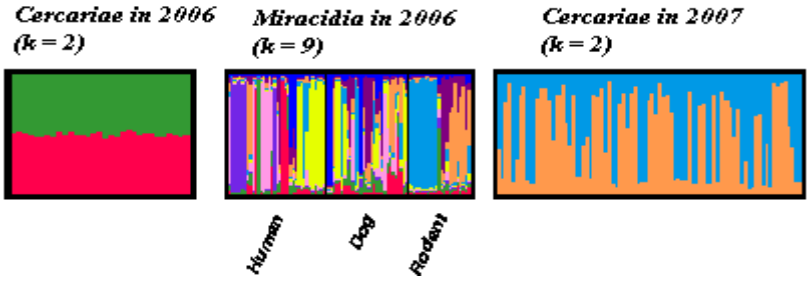
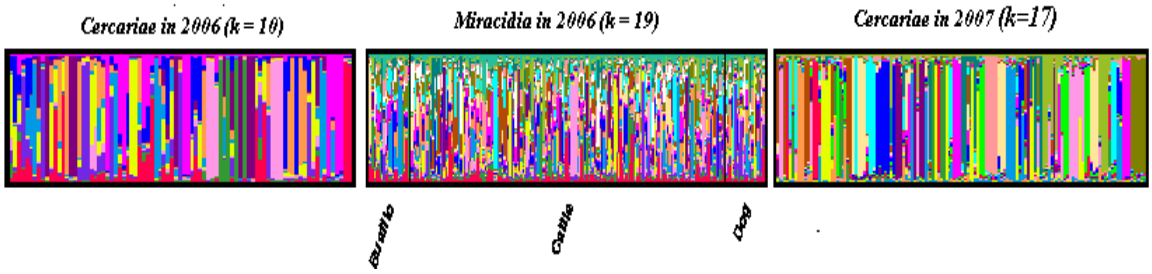
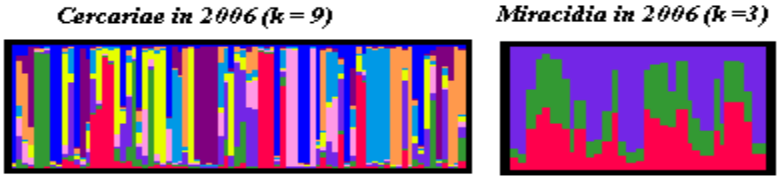


Figure 6.8.10 Estimated maximum numbers of sub-populations of cercariae (2006), miracidia (2006) and cercariae (2007) within each hilly village (a, Guanghui; b, Heping; and C, Xingzhuang). Figure shows that each larva is represented by a thin vertical line, which is partitioned into a number of coloured segments that represent the individual's estimated membership fractions in the relative subpopulations (K, the number of sub-populations according to the highest posterior probability of a given K). The Length of Burn-in period and the number of MCMC replicates after Burn-in when running STRUCTURE, were set at 15,000 and 10,000, respectively. The parasite populations were labelled below the figure with host species (miracidia populations only).

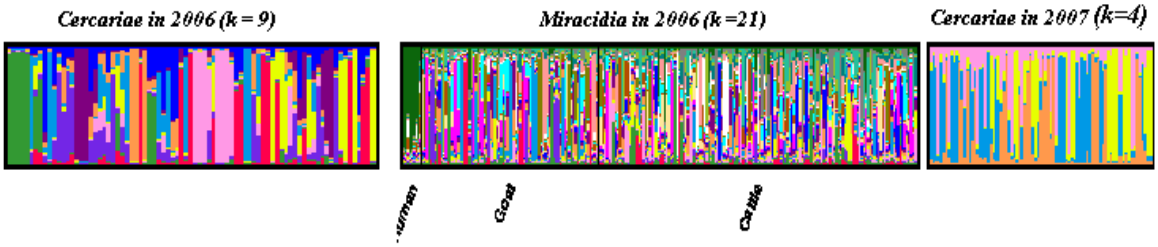
(a) Guanghui



(b) Heping



(c) Xingzhuang



Chapter 7: General discussion

Schistosoma japonicum is a zoonotic species with more than 40 species of domestic and wild mammals serving as potential reservoirs (He *et al.*, 2001). The results presented within this thesis aimed to contribute to our better understanding of the transmission dynamics of this parasite across two contrasting geographical regions, the marshland, where the disease persists, and the hilly region, where the disease was once controlled, in Anhui Province of China. Through five independent data chapters focusing upon the infection prevalence in definitive hosts (Chapter 2), the chronobiology of cercarial emergence (Chapter 3) and the population genetic structures of local parasites (Chapter 4 to 6), main reservoirs in each region and associated transmission dynamic differences between regions or villages were characterised, and their implications, in terms of both control strategy and evolutionary theory, were discussed.

7.1 Contrasting reservoirs inferred from epidemiological surveys

Cattle and water buffalo are the most important reservoir hosts for human schistosomiasis in most endemic areas of China (see Chapter 1 and Section 2.2 in Chapter 2). Indeed, the results of two years of longitudinal surveys, as displayed in Chapter 2, strongly corroborated the hypothesis of cattle as the leading role in the transmission in the marshland, in contrast to that in the hilly areas with small rodents as the most important reservoir. The role of dogs (and cats) should not be ignored in both regions, as considerably high infection prevalence in such species was observed, particularly in the hilly region. The contrasting infection profiles of the parasites in mammals between the two ecological regions would mean different potential for transmission dynamics between the two regions.

7.2 Contrasting reservoirs confirmed from biological traits of parasites and gene flow or sibling relationship of parasites between two larval stages

The application of population genetics to investigating transmission processes of macroparasites has been successfully demonstrated through analysing the amount and partitioning of genetic diversity within and between hosts and within and between populations (Nadler, 1990; Simpson *et al.*, 1993; Nadler *et al.*, 1995). This thesis showed that, with a thorough comparison of the genetic diversity of the parasites at the two larval stages, cercariae and miracidia, both very low genetic differentiation (from Pairwise F_{ST}s) and close relationship (from phylogenetic trees) between miracidia populations from cattle and cercariae populations from intermediate host snails confirmed the main reservoir of cattle in the marshland (Chapter 4). A further sibling relationship analysis (Chapter 5) displayed that, in the marshland, the cercariae identified within intermediate host snails mostly came from cattle, further confirming the probability of cattle as the leading role in such areas.

Although both the highest infection prevalence and infection intensity (the number of miracidia per gram of stool or EPG) were observed in rodents across the three hilly villages over two years (Chapter 2), molecular analyses, based on either gene-flow of parasites (Chapter 4) or sibling relationship of parasites (Chapter 5) between two larval stages, were not able to testify the hypothesis of rodents as main reservoirs within the hilly region. However, the results from chronobiology of cercarial emergence (Chapter 3) indeed suggested that rodents may be responsible for the maintenance of transmission.

Schistosome cercarial emergence patterns, either inter-specific differentiation, for example, between *S. mansoni* and *S. rodhaini* (Theron, 1989), *S. mansoni* and *S. haematobium* (Nojima & Sato, 1982), and between *S. haematobium*, *S. intercalatum* and *S. bovis* (Pages & Theron, 1990b), or intra-specific differentiation, for example, within either *S. mansoni* (Chasse & Theron, 1988; Theron *et al.*, 1997) or *S. haematobium* (N'Goran *et al.*, 1997), have been well documented, and are considered adaptive behavioural traits for the parasites, associated with different definitive host species, or with habitats (for *S. haematobium*). The results presented within this thesis showed two distinct modes of cercarial emergence from field-collected snails. The late afternoon emergence, as once reported in the Philippines (Kawashima *et al.*, 1985), was mainly observed in the snails from the hilly region, compatible with a nocturnal rodent reservoir. The morning emergence (with an initial shedding) was found from the marshland, consistent with a diurnal cattle reservoir. Therefore, it would be inferred that, based on the results from Chapter 2, combined with the results from population genetic analyses (Chapter 4 & 5) and nocturnal activities of rodents, in the hilly region, rodents may be considered as the main reservoirs to maintain the disease within their home territory. Mammals such as dogs (or even humans, see Chapter 5) with high mobility may have been spreading parasites between and among home territories or even between villages. Indeed, as estimated from sibling relationship analyses (Chapter 5), cross-infections of given parasites among host species were seen in such areas and the proportion of such pattern differed from village to village. However, it was noted that such cross-infections were also seen in marshland villages, leading to the possibility that, besides cattle as the leading reservoirs, other diurnal mammals (water buffalo, goats, dogs or cats) could play

a less important role in the spreading and/or maintenance of the parasites. Indeed in Chapter 5, in Xingzhuang quite a number of cercariae sampled in 2007 were estimated to come from goats.

7.3 Population genetic substructures of parasites relative to contrasting reservoirs

This thesis demonstrated an obvious difference in population genetic structures of either miracidia or cercariae relative to main definitive hosts, at the levels of hosts, species or villages (Chapter 4 & 6). For example, clustering analyses (Chapter 6) showed that subpopulation richness varied widely between and within regions from each larval population, with larger numbers of subpopulations found within the marshland and smaller numbers within the hilly region. At the level of villages, no or less substructure was found within cercariae collected from either of two hilly villages (Longshang and Yuantou). One main explanation might be accredited to an obvious difference in the lifespan and mobility of the suspected main reservoirs cattle in the marshland versus small rodents in the hilly, which largely determines the duration and opportunities for recruitment of various parasites.

7.4 Multiple 'strains' of parasites relative to contrasting reservoirs

Many researchers, if not all, believe in the existence of the multiple 'strains' of *S. japonicum* across mainland China due to geographical and topographical isolation. It is true that the two piloted regions within this thesis are separated by nearly 200km, with three hilly villages more isolated, and three marshland villages more influenced by their upstream endemic areas via flooding (Davis *et al.*, 1999a). Accordingly, 'moderate' to

'very great' genetic distance of parasites was observed between two regions and between villages within the hilly region (Chapter 6).

However, the phylogenetic trees and clustering analyses (Chapter 6) revealed that a few cercariae with late emergence found in the marshland always clustered with the cercariae from the hilly region, but not with the cercariae from the same marshland village, suggesting that the geographical barrier only may not be able to explain the observations. Several factors in relation to definitive hosts could explain such scenario: two 'strains' of parasites between two regions and within the marshland. First, selection pressure induced by contrasting definitive hosts throughout the maintenance of the transmission would promote the development of the diversity of the parasite populations both in terms of phenotypes and genotypes. Second, the observed cercarial emergence asynchrony for *S. japonicum* may be able to both initiate and maintain restricted gene flow among parasite populations from the two contrasting definitive hosts (cattle *vs* rodents) via an assortative-mating system, thus increasing the diversity of populations or even strengthening the probability of the local speciation. Indeed, in one marshland village where *S. japonicum* cercariae with different shedding patterns were found, cercariae with late emergence were not able to trace back to their resource reservoirs there (Chapter 4). Finally, geographical barriers could possibly further reinforce such process and then increase the degree of differentiation (Barraclough & Nee, 2001) or facilitate the formation of potential multiple 'strains' of parasites. The observed differentiation in the sensitivity for the parasites to environmental changes, such as temperature and light

intensity, and in the cercariae production per snail (Chapter 3), may indeed reflect the existence of a possible 'strain' complex.

7.5 Future control strategy and new challenges

Although the future eradication of schistosomiasis in China will rely on a combination of various control options, it would be recommended that, due to the limitation of available resources, in the case of the piloted regions where my thesis focused, there should be different focus in each area, with more efforts on controlling infected snails in the hilly region while on controlling bovine infection in the marshland.

There are several issues in relation to such control strategy. The implementation of integrated snail control is an important measure that gives leverage to further accelerate the control process (Yuan *et al.*, 2005). However, such measure has been confirmed to be highly expensive and highly damaging to environment and ecological system. For example, during the first three years of the World Bank Loan Project for schistosomiasis control in China, out of more than 21 million US dollars spent on field activities, 43% was invested in molluscicide (Guo *et al.*, 1998). Moreover, variability in environment reduced the efficiency of snail control with chemicals (Lu *et al.*, 2003). As demonstrated in Chapter 2, although intensive snail killing with chemicals was performed every year in each hilly village, infected snails were still found every time, even on the same habitats. Finally, such control measure targeted at 'hot spots' contributing most to transmission can be very efficient but, on the contrary, will be ineffective if any of these sites are missed (Woolhouse *et al.*, 1998).

In the marshland where bovines may act as the main reservoir hosts, any control measures in bovines should benefit humans, as predicted by mathematical modeling (Williams *et al.*, 2002). Indeed, after the highest infection prevalence in bovines and goats in the marshland had been revealed during the first year of epidemiological surveys, the raising of such domestic animals has been banned since Jan. 2007 by the local government. However, it is well worth mentioning that other infected species of hosts were also found in both years, often at high prevalence levels. From the epidemiological point of view, this would lead to doubting the possibility of eliminating the disease in the marshland through the eradication of bovines (and goats) alone.

As demonstrated in this thesis, considerably high levels of potential cross-infection of parasites between or among species of definitive hosts were observed in both regions. Such would indicate that, first, it seems to be impossible to reach control or elimination level if control methods focus on the main suspected reservoirs only; second, compared to cattle in the marshland or rodents in the hilly region, the long ignored species such as dogs with high mobility may play a role in resulting in the epidemics of *S. japonicum*, particularly in the hilly region and if so, an effective way in preventing dogs against infections or chemotherapy on them is also worth exploring in such areas.

7.6 Raised interests

Host- or local-adapted speciation of *S. japonicum*, leading to the potential existence of two (based on biological traits only) or more 'strains' of this parasite, was suggested to occur in the piloted regions. The timing differences in cercariae shedding patterns has

been demonstrated to be genetically controlled and inherited for *S. mansoni* (Theron & Combes, 1988), and *S. haematobium, S. intercalatum* and *S. bovis* (Pages & Theron, 1990b). Therefore, one important relative issue is, given the previous evidence of hybrids even between species, for example between *S. bovis* and *S. curassoni* in cattle or sheep (Rollinson *et al.*, 1990), between *S. haematobium* and S. *mattheei* (Kruger & Evans, 1990; Southgate *et al.*, 1995), and between *S. haematobium, S. intercalatum* and *S. bovis* (Pages & Theron, 1990b), whether there are any 'transition' parasites, in terms of phenotypes, between early and late schistosome in the marshland where the two 'strains' were found. Does it mean that all diurnal mammals (including humans) infected in the hilly region could be explained by occasional or temporary infection? If so, what would be further influence of such events on the evolution of local parasite populations in such regions, as a pathogen population can not evolve in response to selection pressure from one host population without affecting the selection pressures imposed by other host populations (Woolhouse *et al.*, 2002)? If not, how many generations would the parasites within diurnal animals take in development of the trait of the morning emergence for *S. japonicum* in the hilly region?

Given the potential co-evolution between *S. japonicum* and *Oncomelania* throughout Asia operating at the local and even population level (Davis, 1980; Davis, 1993; Davis *et al.*, 1999b), and the higher aggregation of genetically diverse individual snails along the Yangtze River flood plains than in peripheral areas not affected by annual flooding (Wilke *et al.*, 2000), it would merit further investigation of whether there is genetic differentiation in *Oncomelania hupensis hupensis* snails between and within regions in correspondence with that observed in *S. japonicum* populations. Such studies would

further deepen our understanding of host-parasite interactions, evolution and epidemiology.

7.7 Inherent limitations

The population genetic analyses within this thesis partly relied on samples of miracidia. As one pair of worms within hosts may lay over 1000 eggs per day with one-third to one-half of the eggs being passed in the faeces (Cheever *et al.*, 1994), it is inevitable to sample multiple family members when collecting miracidia from individual hosts, thus resulting in an possible inflation of F_{ST}s (Criscione *et al.*, 2005) (Chapter 4 & 6) and an overestimation of *K* subpopulations (Pritchard & Wen, 2004) (Chapter 6) due to chance allele frequency differences among families. Another is with regard to sample size (Jarne & Theron, 2001) and associated effective population size (Criscione & Blouin, 2005) when comparing genetic diversities or substructures between different populations. Some software, for example, FSTAT (Goudet, 2001), does implement estimation by adjusting sample size via adapting the rarefaction index (Hurlbert, 1971). However, this is also somewhat problematic when one population size is very small. Therefore, it seems essential to obtain appropriate samples in such kind of analyses, and more effort and time would be needed in creative sampling practices (Jones & Ardren, 2003).

As the accuracy of relatedness estimates from sibling analyses is affected by the number of loci (Smith *et al.*, 2001), the number of alleles per locus, and true full-sib family sizes (Thomas & Hill, 2002), considering the factor of only six markers, indeed an economically affordable and moderate number of markers, applied in Chapter 5 for

inferring familial relationship of parasites, it would be recommended that more highly polymorphic microsatellite markers should be developed and employed in such analyses. Finally, one looming problem in genotyping individual eggs or larvae, which has recently found quite a number of applications in population genetic analyses (Shrivastava *et al.*, 2005a; Sorensen *et al.*, 2006; Wang *et al.*, 2006a; Gower *et al.*, 2007; Rudge *et al.*, 2008) and also is applied in this thesis, is the limited amount of template DNA from single larva, thus enabling it impossible to detect genotyping errors by using the additional DNA from the same individual (Pompanon *et al.*, 2005). Using a modified HotSHOT method (Truett et al., 2000) to prepare DNA from individual miracidia, one larva was allowed to be amplified with up to 21 microsatellite markers (Steinauer *et al.*, 2008). Valentim and colleagues (Valentim *et al.*, 2009), however, demonstrated the use of Phi-29 DNA polymerase to replicate DNA extracted from a single miracidium, in which large amounts of DNA was able to be generated from a single *S. mansoni* miracidium. Such approach may enable a single larva to be genotyped with hundreds of microsatellites or other genetic markers, thus not only overcoming the above mentioned demerit, but also greatly expanding the scope of population genetic studies on this kind of parasites.

To a great extent, the transmission of *S. japonicum* depends on the availability and abundance of permissive hosts (He *et al.*, 2001). The results presented within this thesis here provided evidence that contrasting reservoirs existed between two ecological regions, with cattle as main reservoirs in the marshland and rodents in the hilly. Dogs, with their higher mobility, may also play a significant role in *S. japonicum* transmission particularly in the spreading of parasites within the hilly region. *S. japonicum* may potentially be specializing towards their different definitive host reservoirs, both in terms

of phenotypes and genotypes, through the maintenance in diurnal mammals (cattle) in the marshland versus in nocturnal animals (rodents) in the hilly, and, moreover, such potential for diversification in this and related multi-host parasite species may vary between villages. Furthermore, such differential host reservoirs and corresponding multi-strains of *S. japonicum* could have substantial practical implications for schistosomiasis epidemiology and control. This is particularly the case for putative rodent-adapted *S. japonicum*, found both in the marshland and hilly regions, which may raise future concerns as to the possibility of transmission persistence in the marshland or re-emergence in the previously controlled regions due to the potential switching of parasitic 'strains' to humans and domestic animals, even if the parasite in humans and domestic animal definitive host species is eliminated.

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Appendix: Publications from this work

1. Lu, D-B., Wang, T-P., Rudge, J. W., Donnelly, C. A., Fang,G-R., & Webster, J. P.(Corresponding author) (2009). Evolution in a multi-host parasite?: Chronobiological circadian rhythm and population genetics of *Schistosoma japonicum* cercariae indicates contrasting definitive host reservoirs by habitat. *International Journal for Parasitology (in press)*

2. Lu, D-B. (Corresponding author), Wang, T-P., Rudge, J. W., Donnelly, C. A., Fang,G-R., & Webster, J. P.(Corresponding author) (2009). Contrasting reservoirs for *Schistosoma japonicum* between marshland and hilly regions in Anhui, China: inferred from a two-year longitudinal parasitological survey. *Parasitology (in press)*

3. Rudge, J. W., Carabin, H., Balolong, E., Tallo, V., Shrivastava, J., Lu, D-B., Basanez, M. G., Olveda, R., McGarvey, S. T. & Webster, J. P. (2008). Population Genetics of *Schistosoma japonicum* within the Philippines Suggest High Levels of Transmission between Humans and Dogs. *PLoS Neglected Tropical Diseases* 2, e340.

4. Rudge, J.W., Lu, D-B, Feng, G-W, Wang, T-P, Basáñez, M-G & Webster, J.P. (2009). Parasite genetic differentiation by habitat type and host species: molecular epidemiology of *Schistosoma japonicum* in hilly and marshland areas of Anhui Province, China. *Molecular Ecology* 18, 2134-2137.