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ORIGINAL PAPER

Genetic diversity and structure of *Schistosoma japonicum* within two marshland villages of Anhui, China, prior to schistosome transmission control and elimination

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Abstract Schistosomiasis is caused by the genus *Schistosoma* and affected more than 250 million people worldwide. *Schistosoma japonicum* was once seriously endemic in China and nearly 60 years of efforts has seen great success in disease control. However, due to its zoonotic nature and complex life cycle, the schistosomiasis transmission control and final elimination would require, besides an intersectoral approach, deep understanding of population genetics of the parasite. We therefore performed a snail survey in two marshland villages of Anhui province of China and collected *S. japonicum* cercariae from infected snails. By using the recent developed microsatellite panel comprising seven loci, we genotyped the sampled parasites and analyzed the population genetic diversity and structure. The results showed much lower infection prevalence of *S. japonicum* in snails and low infected snail density in either marshland village. Through population genetic analyses, a considerable genetic diversity of parasites was revealed, whereas a small number of clusters were inferred and the sign of bottleneck effect was detected in each village. For the first time in *S. japonicum* in two villages, we provided estimates of effective population sizes with two different approaches. The results indicated that the parasite in two villages could eventually be eradicated with the ongoing integral control measures, but with potential risk of reinvasion of immigrant parasites through the Yangtze River. Such would be of great importance in assessment of the effects of ongoing control measures and prediction of the transmission capability for *S. japonicum*, thus guiding decisions on the choice of further control work.

Keywords *Schistosoma japonicum* · Genetic diversity · Bottleneck effect · Effective population size · Marshland

Introduction

Schistosomiasis is caused by blood flukes of the genus *Schistosoma* and affected more than 250 million people worldwide (WHO 2015). In China, where *Schistosoma japonicum* was seriously endemic, nearly 60 years of efforts has seen great achievements in schistosomiasis control; therefore, in 2014, the central government proposed the goal of schistosomiasis elimination by 2025 (Lei and Zhou 2015). However, this country still faces great challenges. For example, a nationwide survey of infectious spots with sentinel mice in 2010 revealed that, among 72 sites in 34 counties (cities, districts) from 7 provinces (i.e., Hunan, Hubei, Jiangxi, Anhui, Jiangsu, Yunnan, and Sichuan), 17 infectious sites were identified and the infection rate of *S. japonicum* in sentinel mice was up to 3.69% (90/2346); even in the sites (or their surrounding areas) with no infected snails found within the last 3 years, a proportion of 12.12% sites were infectious (Zheng et al. 2012). The surveillance data of 2012 revealed that, in 60 endemic villages from four provinces (i.e., Hunan, Hubei, Jiangxi, and Anhui), bovines were still raised in 44

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villages with the infection prevalence of the parasite in cattle as high as 4% in one village (Cao et al. 2013), although a comprehensive control strategy to control schistosomiasis involving removing bovines from snail-infested areas has been adopted as the national strategy since 2004 (Engels et al. 2005; Hong et al. 2013). Nearly three million people across China still needed treatment for infections or for preventive chemotherapy (WHO 2014). At the end of 2014, five counties did not meet the criteria for schistosomiasis transmission control and 15 counties had difficulty in reaching their targets of transmission interruption (Lei and Zhou 2015).

Moreover, of schistosomes infecting humans, *S. japonicum* is unique due to its zoonotic nature, and about 46 mammal species could serve as reservoir hosts for this parasite (He et al. 2001). The role of bovines and other domestic animals in the transmission of the disease in China has been acknowledged, and thus, corresponding control measures have been recommended and implemented (Ross et al. 2001). However, our recent work in a hilly region of Anhui province suggested that rodents may have served as main reservoirs for the maintenance of low transmission of the parasite in the local areas (Lu et al. 2010; Lu et al. 2009; Rudge et al. 2013), and the rodent-associated *S. japonicum* have been showed to have the potential capability to spread to near marshland regions through river systems (Wang et al. 2014).

Schistosomiasis transmission and then elimination will require an intersectoral approach with substantial and continual effort (Rollinson et al. 2013). Beyond that, a deep understanding of the population genetics of the circulating parasites may provide insight into the effect of control measures and the potential of *S. japonicum* transmission, which should be crucial for final stage of elimination. We here used the recent developed microsatellite panel comprising seven loci (Bian et al. 2015) to genotype the parasites (*S. japonicum* cercariae) sampled from two marshland villages of Anhui province and analyze the population genetic diversity and structure. The questions investigated included (1) Is there any bottleneck effect (i.e., reduction in population size) of parasites after several years of integrative control? (2) What is effective population size (i.e., **N**e, the size of an ideal population needed to reproduce the genetic variability and rate of drift present in the actual population) in local parasite populations? (3) What is the extent of individual parasite relationships? Our results suggested that schistosomiasis elimination is feasible in both areas, but migrant parasites (i.e., harbored within an intermediate or final host) are highly possible and should be paid more attentions.

Materials and methods

Research sites

Two research villages were set in Anhui province of China, with Zhongxing village (ZX) (longitude, 117.5929; latitude, 30.8995) from Zongyang County and Muxiahe village (MXH) (longitude, 118.3668; latitude, 31.5750) from Hexian County. Both sites are located on the north bank of the Yangtze River, classified as marshland regions with schistosomiasis persistence. See Fig. 1. According to the local historical recording, at the end of 2009, in ZX, a total of 16 communities reside along the river bank with 1632 residents at high risk of infection; the infection prevalence in humans with an indirect hemagglutination test (IHA) were between 11.94 and 12.68% from 2007 to 2009; 114 bovines were raised and the infection prevalence was 3%; snails were found on three habitats. In MXH village, a total of 15 communities reside along the river bank with a resident population of 1364 at high risk; the infection prevalence in humans with IHA from 2007 to 2009 was from 3.33 to 6.06%; a total of 251 bovines were raised with the infection prevalence of 2.44%; snails were found on four habitats. In both villages, integral control measures have been implemented since 2004.

Infected snail surveys and parasite sampling

Snail surveys were systematically preformed in all reported snail habitats in two villages during Sept. of 2010. Frames (0.11 m^2 each) were set at locations in an equally spaced lattice formation (20 m × 20 m) within snail habitats. All snails found within each frame were collected. The sampled snails from the field were checked in the laboratory the following days for infection with *S. japonicum* by using a shedding method, and infected snails with schistosome cercariae were identified and raised individually. Cercariae shed from each of the infected snails were individually picked up using a tiny loop under a binocular microscope and carefully transferred to a Whatman FTA® indicative card (Whatman International Ltd., Kent, UK). The cards with parasite samples were then stored at 4 °C in a refrigerator before being subjected to PCR.

Genotyping *S. japonicum* cercariae

From the FTA paper, 2 mm diameter discs, each with one single cercaria sample, were punched using Harris micropunch (Whatman, Sweden). Cercariae samples were washed with FTA purification agent and then TE buffer. Each wash was carried out twice. Seven microsatellite markers on *S. japonicum* were used to genotype each cercaria. See (Bian et al. 2015) for details. Ten randomly selected cercariae from each infected snail were genotyped. PCR products were sent to Sangon Biotech (Shanghai, China) and were genotyped with an ABI 3100-automated sequencer (Applied Biosystems). Allele calling were performed with the GeneMarker HID (SoftGenetics LLC). Each allele was carefully visualized and confirmed by two technical persons to avoid any possible errors. Prior to population genetic analyses, the repeated cercarial genotypes within individual snails

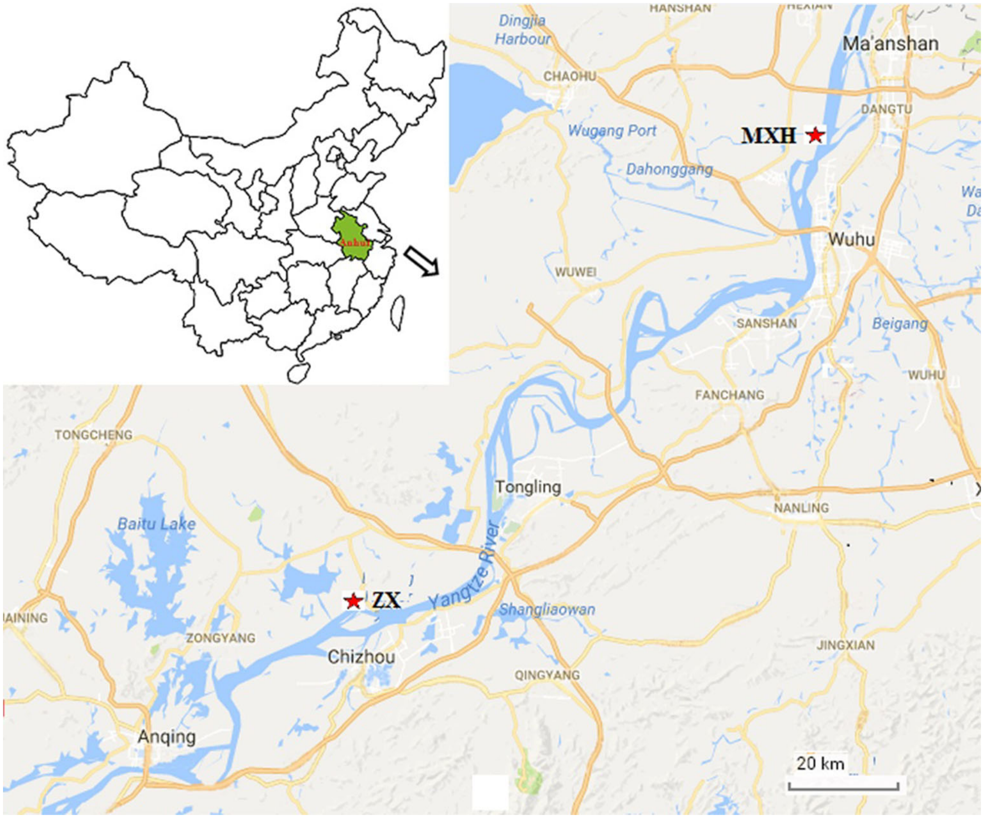

Fig. 1 Geographical locations of the two villages in Anhui province of China. *Pentagon star* refers to village site. The Yangtze River flows from southwest to northeast

were reduced to single copies due to asexual reproduction of the parasite at this stage.

Molecular analyses

Parasite genetic diversity, Hardy–Weinberg equilibrium (HWE), and linkage disequilibrium

Genetic diversity of *S. japonicum* within each village was analyzed in GenAlEx V6.5 (Peakall and Smouse 2012). The diversity parameters calculated include number of observed (Na) and number of effective (Ne) alleles per locus, observed (Ho) and expected heterozygosity (He), unbiased expected heterozygosity (uHe), and the fixation index (F). Deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium between loci within each village were assessed in FSTAT (Goudet 2001). The sequential Bonferroni's procedure was applied to the tests of linkage disequilibrium and HWE to correct for multiple testing (Holm 1979).

Population genetic structure

The Bayesian program STRUCTURE v2.2 (Pritchard et al. 2000) was used to infer the most likely number of genetic clusters (K) present within each village. Simulation series were run with **K** ranging from two to ten and ten iterations per value of **K**. Each run was 2,000,000 generations in length with a burn-in of 500,000 generations. An admixture model with correlated allele frequencies was used and the most probable value of **K** within either village was inferred. Baps 3 (Corander and Marttinen 2006; Corander et al. 2006), a program for Bayesian inference of the genetic structure in a

Table 1 *S. japonicum* infections in snails and infected snail density within two marshland villages of Anhui

Village, county	Snail areas (m^2)	No. frames (0.11 m^2)	No. snails surveyed	No. infected snails (%)	Infected snail density (no./frame)	No. snails with cercariae sampled and genotyped
ZX, Zongyang	1.54 million	3054	2093	50 (2.39%)	0.0164	29
MXH, Hexian	1.50 million	4744	2107	29 (1.38%)	0.0061	21

Table 2 Genetic diversity of *S. japonicum* within two marshland villages of Anhui

Village		Number	Na	Ne	*I*	Ho	He	uHe	*F*
ZX, Zhongyang	Mean	38.29	12.71	7.37	2.15	0.64	0.85	0.87	0.27
	SE	5.74	1.89	0.90	0.16	0.11	0.02	0.02	0.12
MXH, Hexian	Mean	28.14	10.43	5.92	1.97	0.54	0.82	0.84	0.35
	SE	2.94	1.04	0.62	0.10	0.04	0.02	0.02	0.04

Na no. of different alleles, *Ne* no. of effective alleles, *I* Shannon's information index, *Ho* observed heterozygosity, *He* expected heterozygosity, *uHe* unbiased expected heterozygosity, *F* fixation index

population by using stochastic optimization, was then used to test if there is a significant admixture for each parasite individual. GenAlEx V6.5 was used to generate a matrix of genetic distances between parasite individuals within each village and then a principal component analysis (PCoA) was conducted for visualization of genetic relatedness of individuals.

Population bottleneck tests and effective population size

For each village, tests for bottleneck events of parasites were performed using the program BOTTLENECK V1.2 (Piry et al. 1999). By comparing the expected heterozygosity for a sample (H_E) with the heterozygosity that would be expected for a sample taken in a population at mutation/drift equilibrium with the same size and allele number (H_{EQ}), a bottleneck effect is revealed by the existence of $H_E > H_{EQ}$ in subsequent generations (Cornuet and Luikart 1996) as the number of alleles decreases faster than heterozygosity. Tests were performed under an IAM (infinite allele model), a SMM (stepwise mutation model) or a TPM (two phase model). In TPM, we assumed 70% of mutations at one step and 30% at a multiple-step with a variance of 30 (default in the software). One tailed Wilcoxon sign-rank tests were used to assess whether observed heterozygosity deviate from expectations at mutation/drift equilibrium (Cornuet and Luikart 1996). Estimations were based on 100,000 replications. Reductions in population size were also visualized using the "mode shift" indicator of the distortion of allele frequency classes' distributions (Luikart et al. 1998).

The effective population size (**N**e) is a measure of the rate at which a population looses genetic diversity by drift and roughly represent the number of adults that effectively contribute to the next generation. Two software NeEstimator (Peel et al. 2004) and LDNe (Waples and Do 2008) were used to estimate the values of **N**e and their 95% confidence intervals (CIs). The computation was performed under the assumption of negligible selection, migration or mutation in changing allelic frequencies when compared to drift. In running LDNe, values obtained for alleles with frequency ≥0.05 were chosen.

Results

Infected snails and parasites sampled

As seen in Table 1, out of 2093 snails sampled in ZX village, 50 were identified with *S. japonicum* infection. In MXH village, 29 out of 2107 snails were infected with schistosome. Both villages showed a very low infected snail density. *S. japonicum* cercariae were successfully collected and genotyped from 29 infected snails in ZX and from 21 in MXH.

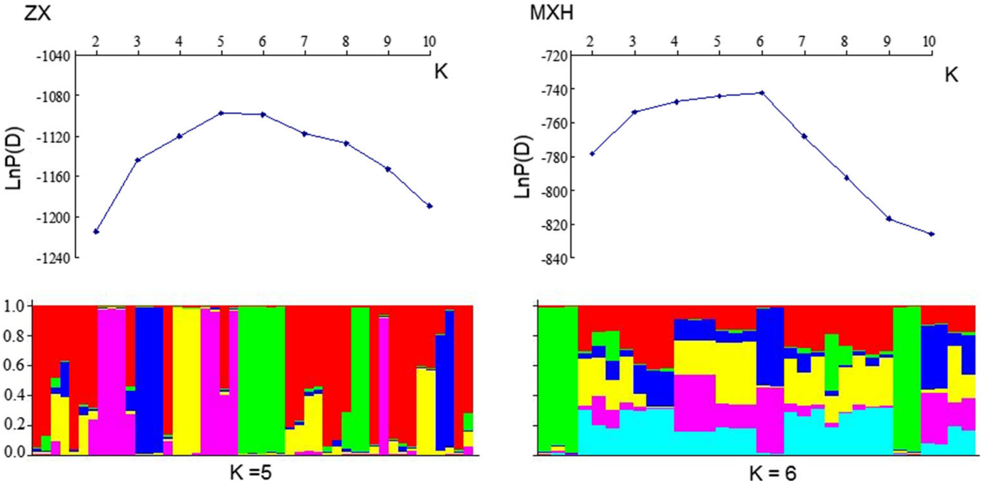

Fig. 2 Estimated numbers of clusters and population structure of *S. japonicum* in ZX and MXH with the structure. LnP(D), mean natural logarithm of the likelihood of the data over 10 replicated runs for each value of assumed *K*. In the bottom, each individual is represented by a vertical line divided into 5 in ZX and 6 in MXH colored segments that represent the individual's estimated membership fractions in assumed clusters

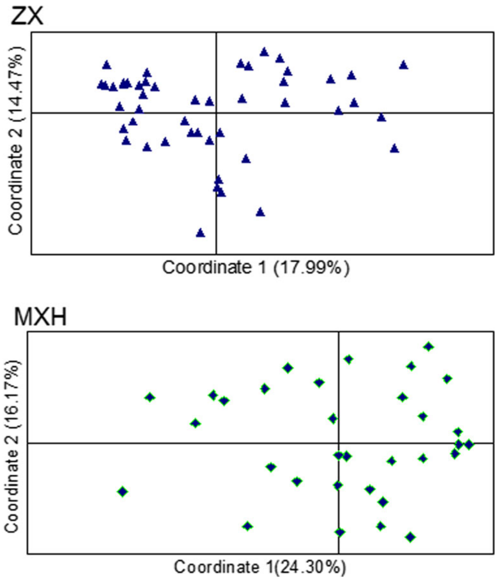

Fig. 3 Principal components analysis (PCoA) of *S. japonicum* in ZX and MXH. Each point (*triangle* or *diamond*) represents one individual with their relative proximity to each other on the graph representing genetic relatedness

Cercarial genotypes and genetic diversity

A total of 79 genetically unique *S. japonicum* cercariae, with 47 from ZX and 32 from MXH, were obtained and analyzed. The numbers of different alleles and of effective alleles in ZX were 12.71 and 7.37, respectively, and in MXH were 10.43 and 5.92, respectively. The values of He (expected heterozygosity) and unbiased He were 0.85 and 0.87 in ZX, and 0.82 and 0.84 in MXH village, see Table 2. At the adjusted *P* value for 5% level of significance, no pairs of loci displayed a significant linkage nor did deviation for each locus from Hardy–Weinberg equilibrium.

Population genetic structure

As demonstrated in Fig. 2 with the software STRUCTURE, the most likely number of genetic clusters (K) could be 5 in ZX and 6 in MXH according to the highest likelihood of the data. In either village, there were a number of individuals which had partial membership in multiple clusters, indicative of the possibility of introgression of parasites from other endemic areas. With the software Baps, 29 cercariae in ZX and 24 in MXH showed admixture, but only one parasite from ZX showed significant admixture ($P = 0.04$). See Online Resource 1. The PCoA graph (Fig. 3) shows genetic relatedness in two dimensions between individuals within villages. The first two axes explained 32.46% in ZX and 40.46% in MXH of cumulative variation. The parasites in ZX seemed to be divided by PCoA into five groups, the same as inferred by the structure; however, parasites in MXH did not form into separated tight clusters.

Bottleneck tests and effective population size

As seen in Table 3, in ZX, significant heterozygosity excess was observed at two loci (Sjp42 and Sjp60) with the modal IAM and at the locus Sjp42 with TPM; whereas in MXH only with IAM two loci (Sjp22 and Sjp58) showed significance in heterozygosity excess. Wilcoxon sign-rank tests for heterozygosity excess revealed the effect of recent genetic bottleneck in both villages ($P = 0.0039$ in ZX and $P = 0.0273$ in MXH) with IAM and in ZX ($P = 0.0273$) with TPM. However, no overt sign of bottleneck effect was displayed by a normal L-shaped distribution of allele frequencies for either village (Fig. 4), partially due to not big sample size.

Estimates of effective population size are given in Table 4. With the software NeEst under linkage disequilibrium (LD), both villages revealed very small effective population sizes

Table 3 Bottleneck test of *S. japonicum* populations by locus

Locus	ZX, Zongyang						MXH, Hexian					
	IAM		TPM		SMM		IAM		TPM		SMM	
	DH/sd	*P*	DH/sd	*P*	DH/sd	*P*	DH/sd	*P*	DH/sd	*P*	DH/sd	*P*
Sjp4	0.366	0.444	−0.760	0.187	−2.301	*0.020*	−0.817	0.166	−2.565	*0.026*	−5.344	*0.001*
Sjp18	0.809	0.192	0.147	0.489	−0.851	0.110	0.468	0.394	−0.153	0.352	−1.350	0.096
Sjp22	0.821	0.187	0.092	0.468	−1.073	0.113	1.196	*0.034*	0.933	0.145	0.345	0.444
Sjp42	1.467	*0.002*	1.390	*0.017*	0.979	0.145	0.738	0.236	0.174	0.499	−0.940	0.158
Sjp60	1.222	*0.028*	0.912	0.159	0.105	0.495	0.735	0.242	0.173	0.496	−0.900	0.164
Sjp58	1.086	0.152	0.938	0.222	0.803	0.306	1.298	*0.024*	1.198	0.055	1.052	0.116
TS2	0.823	0.211	0.397	0.420	−0.412	0.272	1.116	0.076	0.854	0.182	0.343	0.442

Italicized entries has significance level, 0.05

IAM infinite allele model, *SMM* stepwise mutation model, *TPM* two-phase model, *DH/sd* ratio of deviation to the standard deviation of H_O and H_{EQ} with positive values for heterozygosity excess and negative for heterozygosity deficiency

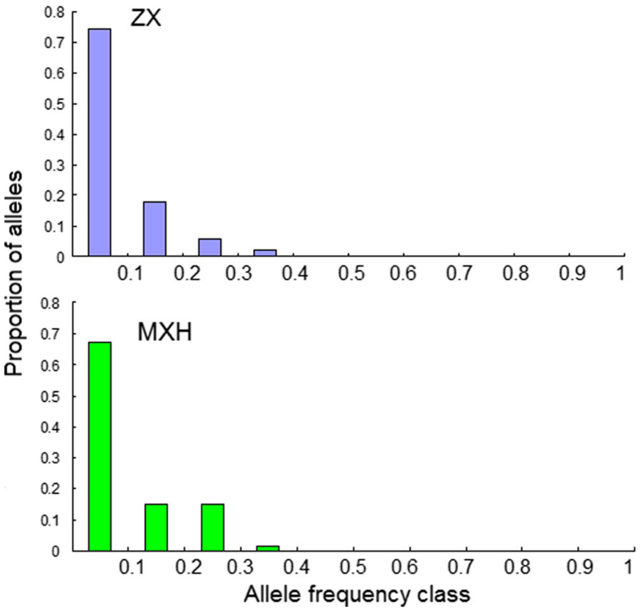

Fig. 4 Distribution of the allele frequency classes based on seven microsatellites of *S. japonicum* in ZX and MXH

(i.e., 18.2 in ZX and 17 in MXH); whereas, under the assumption of heterozygote excess, infinite population sizes were obtained. With LDNe, considerably bigger effective population sizes were estimated for both villages.

Discussions

The results from the epidemiological survey showed much lower infection prevalence of *S. japonicum* in snails and low infected snail density in either marshland village. By using population genetic analyses, although a considerable genetic diversity of parasites was revealed, small numbers of clusters were inferred and the sign of bottleneck effect was detected. For the first time on *S. japonicum*, we provided estimates of effective population sizes in either village. Such would be of great importance in assessing the control effects and in predicting the transmission capability for *S. japonicum*, thus guiding decisions on the choice of further control measures.

Regarding the genetic diversity, the research by Yin and his colleagues in two similar ecological regions (i.e., Yueyang of Hunan province and Shashi of Hubei province) demonstrated that 8 years of disease control effort did not see a reduction of the genetic diversity in *S. japonicum* populations (Yin et al. 2016). The values of **H**o and **H**s observed in our work were similar to those reported in a previous studies on *S. japonicum* at two sites within the same province (Yin et al. 2008), although in the later smaller numbers of infected snails were sampled. Indeed, different sampling strategies for *S. japonicum* could have potential effect on the inferred population structure (Huo et al. 2016). In the present work in each village snail surveys were systematically performed and then snails and parasites were sampled. Therefore, the sampled parasites should be more representative of the local parasite populations. In addition, over seven loci no significant deviation from H-W equilibrium was found, which may provide sound support for computation of those indexes based on the assumption. The inferred small number of clusters (**K**) of parasites within either village, although only in ZX which was confirmed by PCoA, could be explained by existence of different snail inhabitants but each with a less restricted availability for final hosts. We also noted that in either village, not all parasite individuals showed more or less similar pattern of division by the inferred clusters (**K**). This may lead us to suspect that some parasites (probably harbored within snails or final hosts) might migrant along the River from the upstream. Indeed, annual flooding in such regions along the River could bring great numbers of snails into previously control or elimination areas (Davis et al. 1995; Wilke et al. 2000), and infections in animals in downstream regions, even in highly effective control region, have been reported (Yang et al. 2013). If this is the case, this would threaten the success of the ongoing control and elimination program, as high variation in *S. japonicum* exists among regions in mainland China (Young et al. 2015).

It is very well known that integrated control measures are effective in schistosomiasis control (Hong et al. 2011; Wang et al. 2009). Here indeed the signature for a bottleneck in the parasite isolates was detected in either village. One of our previous works revealed that in one hilly region where *S. japonicum* transmission was maintained in the rodent-snail system, by using the same microsatellites we did not detect any sign of bottleneck effect (Bian et al. 2015). Reduction in effective population size (**N**e) can occur in the case of successful disease control. **N**e has been used as a predictor of extinction (Frankham 2005) with a variance

Table 4 Effective population size (**N**e) of *S. japonicum* samples with 95% confidence intervals (CIs)

Village, county	Under LD with NeEst	Under heterozygote excess with NeEst	Under random mating with LDNe[a]
ZX, Zongyang	18.2 (15.6, 21.6)	Infinite (NA)	809.6 (54.6, infinite)
MXH, Hexian	17.0 (14.1, 21.0)	Infinite (NA)	54.1 (21.2, infinite)

NA not available

[a] Lowest allele frequency used was 0.05

Ne <50 indicating an extinction in the short-term (Palstra and Ruzzante 2008; Reed and Bryant 2000). In two rural Brazilian communities after three successive rounds of community-wide treatment, **N**e was reduced to less than 1000 in the *S. mansoni* populations (Barbosa et al. 2016). We here estimated the much lower estimates of **N**e (under LD) with the software NeEst, an optimistic sign of the trend for parasite elimination. However, it is noteworthy that under the assumption of heterozygote excess or using LDNe, infinite or considerably bigger effective population sizes were estimated, particularly in ZX. We observed that quite a number of bovines were still raised in either village. This suggests that in such areas local parasite populations may be actually more complex, and elimination of the parasite requires a strong commitment of control efforts including effective bovine management (Hong et al. 2013).

In all, the results suggested that the parasite in two villages could eventually be eradicated with the ongoing integral control measures, but with potential risk of reinvasion of immigrant parasites through the River system present. Further analyses should be conducted on the interactions of local *S. japonicum* with the possible immigrant parasites through the River system, the epidemiology of which is rarely known. We also look forward that, within the context of the national schistosomiasis control and eliminations programs that have been launched by the central government, more studies would be conducted on population genetics of both *S. japonicum* (if samples available) and its intermediate host *Oncomelania hupensis*. Indeed, control programs are beginning to recognize the importance of such molecular data in monitoring parasite spread reduction and assessing control strategies.

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