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Research Article

Retrotransposon-microsatellite amplified polymorphism, an electrophoretic approach for studying genetic variability among *Schistosoma japonicum* geographical isolates

In the present study, retrotransposon-microsatellite amplified polymorphism (REMAP) was used to examine genetic variability among *Schistosoma japonicum* isolates from different endemic provinces in mainland China, using *S. japonicum* from Japan and the Philippines for comparison. Of the 50 primer combinations screened, eight produced highly reproducible REMAP fragments. Using these primers, 190 distinct DNA fragments were generated in total, of which 147 (77.37%) were polymorphic, indicating considerable genetic variation among the 43 *S. japonicum* isolates examined. The percentage of polymorphic bands (PPB) among *S. japonicum* isolates from mainland China, Japan, and the Philippines was 77.37%; PPB values of 18.42% and 53.68% were found among isolates from southwestern (SW) China and the lower Yangtze/Zhejiang province in eastern (E) China, respectively. Based on REMAP profiles, unweighted pair-group method with arithmetic averages (UPGMA) dendrogram analysis revealed that all of the *S. japonicum* samples grouped into three distinct clusters: parasites from mainland China, Japan, and the Philippines were clustered in each individual clade. Within the mainland China cluster, SW China isolates (from Sichuan and Yunnan provinces) grouped together, whereas worms from E China (Zhejiang, Anhui, Jiangxi, Jiangsu, Hunan, and Hubei provinces) grouped together. These results demonstrated that the REMAP marker system provides a reliable electrophoretic technique for studying genetic diversity and population structures of *S. japonicum* isolates from mainland China, and could be applied to other pathogens of human and animal health significance.

Keywords:
China / Genetic diversity / Retrotransposon-microsatellite amplified polymorphism (REMAP) / Retrotransposons / *Schistosoma japonicum*
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1 Introduction

Transposable elements, a ubiquitous class of mobile genetic units that are able to jump into new genomic loci with high copy number, are widely distributed in many eukaryotic genomes [1]. Transposition has been

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Abbreviations: **E**, eastern; **gDNA**, genomic DNA; **IRAP**, inter-retrotransposon amplified polymorphism; **ISSR**, intersimple sequence repeats; **LTR**, long terminal repeat; **PPB**, percentage of polymorphic bands; **RBIP**, retrotransposon-based insertional polymorphism; **REMAP**, retrotransposon-microsatellite amplified polymorphism; **RIVP**, retrotransposon internal variation polymorphisms; **RSAP**, restriction site amplified polymorphism; **SRAP**, sequence-related amplified polymorphism; **SSR**, simple sequence repeat; **SSAP**, sequence-specific amplification polymorphism; **SW**, southwestern

Colour Online: See the article online to view Fig. 1 in colour.

suggested as a creative force in shaping genomes through insertion/deletion/duplication/rearrangement to influence genomic organization, genetic structure, and biological diversity of species [2]. Based on their DNA structure and transposition mechanisms, transposable elements are mainly classified into two categories: (i) DNA transposons, which make DNA as an intermediate to complete the mobile process by a "cut and paste" mechanism; (ii) retrotransposons, which are replicated via an RNA intermediate and reverse transcription into cDNA followed by insertion into the genome [3]. Retrotransposons offer advantages over DNA transposons as molecular markers because their mechanism of duplication most resembles that of retroviruses, keeping the original copies of genes but also and gradually producing dispersed and abundant insertion loci from retrotransposon families, forming rich polymorphisms for genetic analyses [4].

As a result of its defined and conserved long terminal repeat (LTR) domain, the LTR retrotransposon, one of the main groups of retroelements, has been used widely for studying genetic diversity, evolution, and pedigree among individuals, species, or populations in recent years [5–7]. LTR is the direct sequence repeat bounding the internal coding region containing promoters, signals, and motifs necessary for new DNA integrations. Since the elements in LTR–LTR combination would be disrupted and removed when recombination/rearrangement activity occurred in the genome, the transposition of retrotransposons is not linked with removal of the mother element from its locus (Fig. 1). Each retrotransposon family has its own genetic structure and evolutionary lineage, as well as the uniqueness of each LTR from each retrotransposon family [6]. Therefore, LTR retrotransposons can be envisaged as an evolutionary stopwatch generating recording markers in both time and space associated with, or subsequent to, speciation events. Their analysis consequently provides an excellent basis for developing marker systems relying on PCR to generate fingerprints and polymorphisms (Fig. 1).

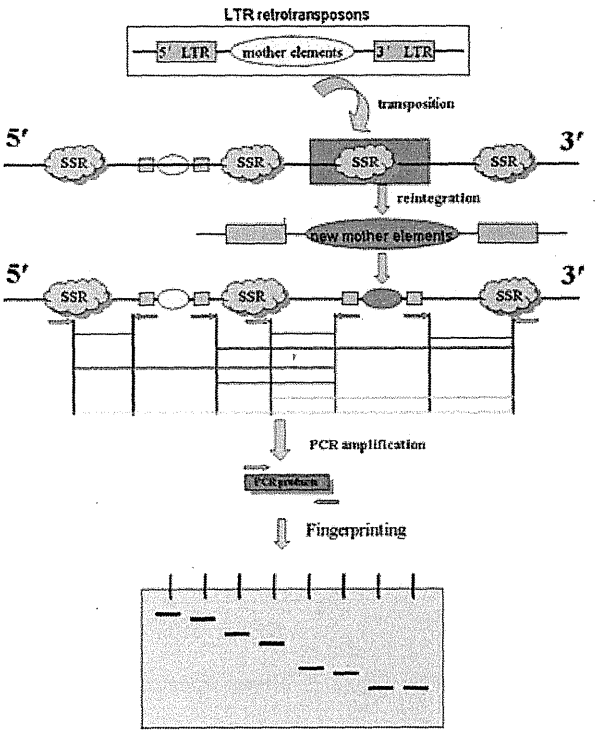

Figure 1. Schematic representation of retrotransposon-microsatellite amplified polymorphism technique. This shows the genomic features, amplification mode, and the positions of the priming sites for the REMAP fingerprinting strategy described in the text. gDNA is shown as a solid black line, with SSR and LTR retrotransposons on it. LTR retrotransposons consisted of 5′ LTR, mother elements, and 3′ LTR. PCR products with different lengths are amplified by different primer binding sites as blue and pink arrows annotate. Fragments amplified between retroelements and microsatellite are shown as red solid lines, while PCR products amplified from two microsatellite loci and two retroelements are shown as light green and dark blue solid lines, respectively.

Retrotransposons and microsatellites are the two major genomic factors generating genetic variation in species, population, and subpopulation levels. Retrotransposon-microsatellite amplified polymorphism (REMAP), a simple PCR-based assay targeting LTR retrotransposons and proximal microsatellite regions of the genome, was developed based on such associations and amplified DNA derived from insertions near simple sequence repeats (SSRs) [8, 9]. The technique uses forward primers annealing within the LTR region and reverse primers designed from di-, tri-, or tetra-nucleotide repeat SSR motifs with one or two randomly anchored "selective" bases added at the 3′ or 5′ ends [10]. Compared with retrotransposon-based marker systems such as sequence-specific amplification polymorphism (SSAP) [11], inter-retrotransposon amplified polymorphisms (IRAP) [12], retrotransposon internal variation polymorphisms (RIVP) [13, 14], and retrotransposon-based insertional polymorphism (RBIP) [15], REMAP has distinct advantages: there is no requirement for restriction enzyme digestion and ligation to generate marker bands or for retrotransposon insertion and flanking sequence information for primer design. Regarding this latter point, one of the greatest advantages of REMAP is that the anchored SSR primers of REMAP can be designed without reference to sequence data from the study organism [14]. Therefore, REMAP is regarded as a convenient technique that can be established at low cost, and can generate polymorphism data directly from electrophoretic fingerprints [7, 10, 16]. Furthermore, REMAP overcomes the limitation of IRAP where amplification sites may be dispersed too far apart to produce amplification products with the available thermostable polymerases [16]. Therefore, REMAP is recognized as a ubiquitously useful marker system that displays a broad application potential and has been used successfully for studying biodiversity and phylogenies in barley [12], rice [16], citrus [17], and *Magnaporthe grisea* [18]. Additionally, it has been proven as an efficient marker system for gene mapping and resistance gene selection in barley [4]. These studies showed that REMAP was a reliable

Table 1. Population of *S. japonicum* (SJ) samples used for REMAP analysis

Population	Geographical origins and codes	Sample codes	Gender
SW China[a)]	Yunnan (Eryuan); YEII	SJYEIIF50, SJYEIIF52	Female (F)
		SJYEIIM50, SJYEIIM52	Male (M)
	Sichuan (Tianquan); ST	SJSTM50, SJSTM51	Male (M)
		SJSTM63, SJSTM69	
E China[b)]	Zhejiang (Jiashan); ZJ	SJZJF57, SJZJF58	Female (F)
		SJZJM57, SJZJM58	Male (M)
	Anhui (Guichi); AG	SJAGF57, SJAGF59,	Female (F)
		SJAGM57	Male (M)
	Jiangxi (Yongxiu); JY	SJJYF23, SJJYF52	Female (F)
		SJJYM23, SJJYM52	Male (M)
	Jiangsu (Wuxi); JW	SJJWF32, SJJWF59	Female (F)
		SJJWM11, SJJWM59	Male (M)
	Hunan (Changsha); HC	SJHCM22, SJHCM50	Male (M)
		SJHCM51, SJHCM52	
	Hunan (Junshan); HY	SJHYF57, SJHYF59	Female (F)
		SJHYM57, SJHYM59	Male (M)
	Hunan (Yueyanglou district); HL	SJHLF55, SJHLF57	Female (F)
		SJHLM55, SJHLM57	Male (M)
	Hubei (Wuhan); HW	SJHWF55, SJHWF58	Female (F)
		SJHWM55, SJHWM58	Male (M)
PL	Philippines (Leyte); LEY	SJLEYF7	Female (F)
		SJLEYM7	Male (M)
YY	Japan (Yamanashi); YY	SJYYF4	Female (F)
		SJYYM11	Male (M)

a) SW China includes Yunnan and Sichuan provinces.
b) E China includes the lower Yangtze provinces (Hubei, Hunan, Jiangxi, Anhui, and Jiangsu) and Zhejiang province.

molecular marker system that embraced boarder application with simplicity, moderate throughput ability, and good reproducibility. However, prior to the present study, REMAP has not been used to study genetic diversity in populations of important etiological agents with public health significance for humans and animals, such as *Schistosoma japonicum*.

Schistosoma japonicum is the causative agent of schistosomiasis, a disease that affects over 200 million people in Southeast Asia and is regarded as the second most significant tropical disease in terms of socioeconomic and public health importance [19–21]. China has a long history of endemic schistosomiasis in the provinces south of the Yangtze River. The natural distribution areas of the sole intermediate host—*Oncomelania hupensis*—have spread more widely than previously due to environmental changes, frequent flooding, and population movement, consequently making genetic variation of *S. japonicum* among different epidemic regions complicated [22–25]. A variety of genetic markers such as mitochondrial DNA [26, 27], microsatellite DNA [28, 29], dideoxy fingerprinting [30], sequence-related amplified polymorphism (SRAP) [31], restriction site amplified polymorphism (RSAP) [32], and IRAP [7] have revealed obvious genetic variation among *S. japonicum* from different endemic regions in mainland China.

The objectives of the present study were to assess the usefulness of the REMAP marker system for studying genetic variation and pedigree relationship among *S. japonicum* isolates from different endemic regions in mainland China, using *S. japonicum* from Japan and the Philippines for comparison.

2 Materials and methods

2.1 Parasites and isolation of genomic DNA

In total, 43 adult *S. japonicum* samples representing isolates and populations from mainland China, Japan, and the Philippines were used in the present study. Information regarding populations, geographical origins, sample codes, and genders is listed in Table 1. Isolates of *S. japonicum* were obtained from eight endemic provinces in mainland China, namely Sichuan, and Yunnan provinces in southwestern (SW) China, and the lower Yangtze provinces (Jiangsu, Jiangxi, Hubei, Hunan, Anhui) and Zhejiang province in eastern China (E China).

All of the adult parasites were collected from rabbits experimentally infected with cercariae from infected snails (*O. hupensis*) [7, 27]. The male and female adult parasites were fixed in 70% molecular grade ethanol, and stored at –20°C until extraction of their genomic DNA. The Japanese (YY) and Philippines (PL) *S. japonicum* isolates were collected from Yamanashi Prefecture and Leyte island, respectively. Adults were isolated from mice experimentally infected with cercariae and were preserved at –80°C after separation by gender [33].

Table 2. Sequences of primers used for REMAP analysis in the present study

LTR primers	Anchored SSR primers
LTR4: 5′-TCCTGTTCGTGTGTTCCCACGTGTC-3′	UBC803: 5′-$(AT)_8$C-3′
LTR6: 5′-CGCGACTAGTCAAGCATCAAGTACG-3′	UBC807: 5′-$(AG)_8$T-3′
LTR7: 5′-TAGCTTTCAGTCGGCTGATTTGAC-3′	UBC824: 5′-$(TC)_8$G-3′
LTR11: 5′-GGTAGTGTTCCGTGGCCGCCACA-3′	UBC825: 5′-$(AC)_8$T-3′
LTR12: 5′-GGTTATCTCGGTGTCACCATAAACTC-3′	UBC833: 5′-$(AT)_8$YG-3′
LTR13: 5′-AGAATCCAAACCACGCGTCACAA-3′	UBC848: 5′-$(CA)_8$RG-3′
LTR15: 5′-GCTCTTATCCTATACTACTGTTTAAGGCAT-3′	UBC853: 5′-$(TC)_8$RT-3′
LTR16: 5′-TAATACACTACTGTAAGGGCAAGGC-3′	UBC855: 5′-$(AC)_8$YT-3′
LTR17: 5′-AAGAGACAGACAGAAAGGAGACTAGATTT-3′	UBC857: 5′-$(AC)_8$YG-3′
LTR18: 5′-TTCCGGTGAATCTAACGCTATGTCA-3′	UBC890: 5′-VHV$(GT)_7$-3′

Y = C/T, R = A/G, V = not T, H = not G

Total genomic DNA (gDNA) was extracted from individual samples by SDS/proteinase K treatment, column-purified (Wizard® SV Genomic DNA Purification System, Promega, Madison, USA) and eluted into 60 μL H_2O according to the manufacturer's recommendations [7,27,31,32]. The integrity of the parasite gDNA extracted in this way was confirmed by amplification of the first internal transcribed spacer of ribosomal DNA [34].

2.2 REMAP analysis

For REMAP analysis, we used ten LTR primers designed using the sequence of the conserved regions of LTR retrotransposons of *S. japonicum* and ten anchored SSR primers randomly selected from University of British Columbia (UBC) primer set #9 (Table 2). Nine SSR primers (UBC803, 807, 824, 825, 833, 848, 853, 855, and 857) were anchored at the 3′ ends of the microsatellite repeats, while UBC 890 was anchored at the 5′ end. In total, 50 different combinations of LTR–SSR primers were screened using gDNA from five randomly selected *S. japonicum* individuals, and ultimately only eight sets of LTR–SSR primers that produced clear and reproducible bands (Table 3) were selected for REMAP analysis. No bands were shared with the IRAP and intersimple sequence repeat (ISSR) patterns revealed by our previous studies (data not shown) [7,27], indicating that all bands spanned intervening domains between LTRs and microsatellites in the genome. The PCR conditions were optimized for specificity and efficiency by varying the annealing temperatures and magnesium concentrations. Eventually, PCR reactions (25 μL in volume) were performed in 2.5 μL 10× Taq buffer, 0.2 mM of dNTPs, 2.5 mM of $MgCl_2$, 2.5 μM of each primer, 1.5 units of Taq polymerase (TAKARA, Dalian, China), and 1 μL of genomic DNA in a thermocycler (Biometra, Goettingen, Germany) under the following conditions: 5 min of initial denaturation at 94°C, followed by 35 cycles of 94°C for 1 min, 47°C, 50°C or 52°C (see Table 3 for detail) for 30 s, 72°C for 2 min, and 8 min at 72°C for final extension. Separation of amplified fragments was accomplished on 6% denaturing polyacrylamide gels (acrylamide-bisacrylamide (19:1), 1× TBE) at 90 V for 2.5 h [32, 35]. The gel was stained with 0.1% $AgNO_3$ solution, and then photographed using a digital camera. Sizes of amplification products were estimated using DNA marker DL2000 (TAKARA). REMAP banding patterns that were difficult to score and those primers that failed to amplify consistently in all samples were excluded.

2.3 Data analysis

Following a visual examination of REMAP gel profiles, all clearly detectable polymorphic and monomorphic bands were digitalized into a binary data matrix as present "1" or

Table 3. Attributes of LTR–SSR primer combinations used for REMAP amplification and number of bands per combination

Primer name	Combination	T_a (°C)	No. of bands scored	No. of polymorphic bands
LTR-SSR-6	LTR6 + UBC825	47	29	24
LTR-SSR-7	LTR6 + UBC857	50	25	18
LTR-SSR-11	LTR7 + UBC857	50	22	14
LTR-SSR-26	LTR15 + UBC825	52	18	15
LTR-SSR-30	LTR16 + UBC825	50	23	17
LTR-SSR-42	LTR6 + UBC855	47	23	19
LTR-SSR-44	LTR11 + UBC855	52	25	21
LTR-SSR-46	LTR13 + UBC855	52	25	19
Total			190	147
Mean			23.75	18.38

absent "0," assuming that each band represents a single locus. Each primer combination was performed to amplify all the samples in triplicate within 1 day or consecutively to verify reproducibility. Where the same fragments appeared in all samples, these were referred to as monomorphic bands while those which were different among the samples were referred to as polymorphic bands [35]. The binary matrix consisting of all scores was processed for statistical analyses by using program POPGENE (version 1.31) [36], including the calculation of percentage of polymorphic bands (PPB), the effective number of alleles per locus (A_e), and observed number of alleles per locus (A_o). A clustering analysis and dendrogram of all individuals were constructed using the Numerical Taxonomy Multivariate Analysis System (NTSYS-pc) version 2.10e software package [37], in which the FIND module was used to identify all trees that could result from different choices of tied similarity of dissimilarity values, and the Nei and Li [38] coefficient for measuring pairwise band similarities between individuals was also calculated using the SIMQUAL module. Finally, the phylogeny trees were compiled using the CONSEN module and bootstrap analyses (using 1000 replicates) were carried out by PAUP* 4.0b10 [39] to assess the robustness of the findings, with values above 50% reported.

3 Results and discussion

Of 50 REMAP primer combinations screened, eight displayed significant polymorphisms among *S. japonicum* isolates from China, Japan, and the Philippines, namely LTR-SSR-6, LTR-SSR-7, LTR-SSR-11, LTR-SSR-26, LTR-SSR-30, LTR-SSR-42, LTR-SSR-44, and LTR-SSR-46. The numbers of bands produced using each primer combination ranged from 18 to 29, corresponding to an average of 23.75 bands per primer combination, with product sizes ranging from 100 to 2000 bp (Fig. 2). The primer combinations producing the highest and lowest numbers of bands were LTR–SSR–6 (29 bands) and LTR–SSR–26 (18 bands), respectively (Table 3). Of all bands, 77.37% (147 out of 190) were polymorphic among all the parasite isolates, that is, the PPB among all the *S. japonicum* isolates from mainland China, Japan, and the Phillippines was 77.37%, with a significant level of variability in average effective numbers of alleles per locus (1.37). For isolates from mainland China, the total PPB was 66.32%, being 18.42% for the trematodes from SW China habitat and 53.68% for E China habitat. The average effective numbers of alleles per locus was 1.34, 1.15, and 1.30 for parasites in mainland China, SW China, and E China habitats, respectively. The PPB for a single population ranged from 2.63% to 41.05% with an average of 12.16%. The average effective number of alleles per locus was 1.10 (Table 4).

An unweighted pair-group method with arithmetic averages (UPGMA) dendrogram was constructed based on the binary matrix, and FIND module (part of the NTSYS package) was used to identify all trees that could result from different choices of tied similarity of dissimilarity values (Fig. 3). The isolates grouped into three distinct clusters, namely mainland China, Japan, and the Philippines, with high (72% and 93%) bootstrap values. Within the mainland China cluster, SW China isolates (from Sichuan and Yunnan provinces) grouped together. In the clade representing the E China habitat, worms from the same regions clustered together in Zhejiang (clade III), Hubei (clade VI), Anhui (clade V), Jiangxi (clade VI), and Jiangsu (clade VII) provinces. However, *S. japonicum* isolates from three regions (Changsha city, Junshan county, and Yueyanglou district) in Hunan province were located in different clades. Parasites from Yueyanglou district grouped in a solitary clade distinct from all worms from the E China habitat. Owing to different geographical and ecological environments, previous studies indicated that *S. japonicum* populations in southwestern (SW) China were distinct from those within the lower Yangtze/Zhejiang provinces in E China habitats [28, 40, 41]. Recent empirical studies using mitochondrial markers [26], ISSR [27], SRAP [31], and IRAP [7] have also demonstrated that there is a close relationship between the population genetic structure and geographical distribution of *S. japonicum* in mainland China. The population lineages for this species can thus be influenced by distribution and environmental factors, which revealed a separation between these two regions [7, 26, 27, 31]. Due to the underlying effect of environment and stress on retrotransposon activities, retrotransposon distribution patterns can show eco-geographical gradients [42]. Therefore, these REMAP cluster patterns were consisted with previous studies using other molecular markers [7, 26–28, 31, 40, 41].

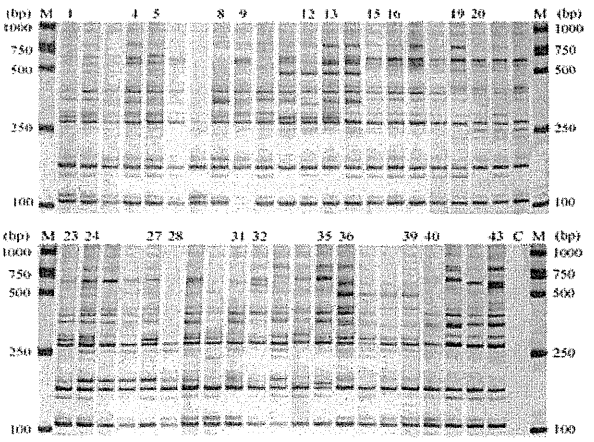

Figure 2. Representative denaturing acrylamide gel displaying sequence variation among *Schistosoma japonicum* samples revealed by the REMAP technique using the primer LTR-SSR-42. Lanes 1–43 represent *S. japonicum* samples SJYEIIF50, SJYEIIM50, SJYEIIF52, SJYEIIM52, SJSTM50, SJSTM51, SJSTM63, SJSTM69, SJZJF57, SJZJM57, SJZJF58, SJZJM58, SJAGF57, SJAGM57, SJAGF59, SJJYF23, SJJYM23, SJJYF52, SJJYM52, SJJWF32, SJJWM11, SJJWF59, SJJWM59, SJHCM22, SJHCM50, SJHCM51, SJHCM52, SJHYF57, SJHYM57, SJHYF59, SJHYM59, SJHLF55, SJHLM55, SJHLF57, SJHLM57, SJHWF55, SJHWM55, SJHWF58, SJHWM58, SJLEYF7, SJLEYM7, SJYYF4, and SJYYM11, respectively. Lane C represents no-DNA control. M represents a DNA size marker 2000 (ordinate values in bp). Refer to Table 1 for sample information.

Table 4. Genetic variability within and among populations, in endemic provinces of *Schistosoma japonicum* in China, Japan (YY), and the Philippines (PL) by REMAP analysis

Population	No. of PB	PPB (%)	A_o	A_e
Yunnan	12	6.32	1.06 ± 0.23	1.05 ± 0.21
Sichuan	5	2.63	1.02 ± 0.16	1.02 ± 0.11
Zhejiang	21	11.05	1.11 ± 0.31	1.08 ± 0.23
Anhui	20	10.53	1.10 ± 0.31	1.08 ± 0.25
Jiangxi	34	17.89	1.18 ± 0.38	1.14 ± 0.31
Jiangsu	25	13.16	1.13 ± 0.34	1.09 ± 0.25
Hunan	78	41.05	1.41 ± 0.49	1.29 ± 0.38
Hubei	21	11.05	1.11 ± 0.31	1.08 ± 0.24
YY	8	4.21	1.04 ± 0.20	1.04 ± 0.20
PL	7	3.68	1.04 ± 0.19	1.04 ± 0.19
SW China[a)]	35	18.42	1.18 ± 0.39	1.15 ± 0.38
E China[b)]	102	53.68	1.54 ± 0.50	1.30 ± 0.35
Between SW China[a)] and E China[b)]	126	66.32	1.66 ± 0.47	1.34 ± 0.33
Among populations	147	77.37	1.77 ± 0.42	1.37 ± 0.32

PB, polymorphic bands; PPB, percentage of polymorphic bands; A_o, number of alleles per locus; A_e, effective number of alleles per locus.

a) SW China includes Yunnan and Sichuan provinces.

b) E China includes the lower Yangtze provinces (Hubei, Hunan, Jiangxi, Anhui, and Jiangsu) and Zhejiang province.

Approximately 40% (159 of 397 Mb) of the *S. japonicum* genome appears to be composed of, or derived from, repetitive families/elements, while a total of 13 469 protein-coding genes take up only 4% of the genome [43]. Transposable elements and microsatellites account for 21.84% and 0.63% of the genome, respectively. There are 29 different kinds of retrotransposons (occupying 19.8% of the genome), which contained 19 LTR retrotransposons (occupying 6.16% of the genome) [43]. Because of the high abundance and the greater nondirectionality of retrotransposon integration

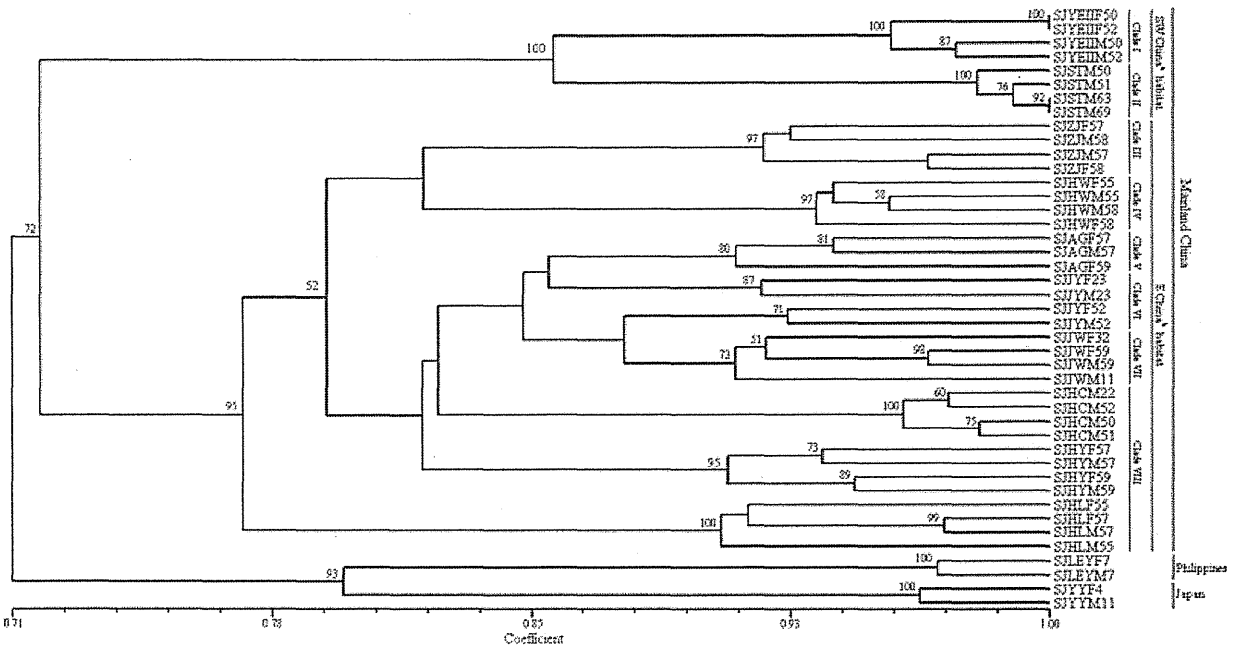

Figure 3. An unweighted pair-group method with arithmetic averages (UPGMA) dendrogram of genetic relationships among representative samples of *Schistosoma japonicum* calculated on the basis of genetic similarity analysis by means of eight REMAP combination primers. Clade I–VIII represents the *S. japonicum* samples from Yunnan (Eryuan, YEII), Sichuan (Tianquan, ST), Zhejiang (Jiashan, ZJ), Hubei (Wuhan, HW), Anhui (Guichi, AG), Jiangxi (Yongxiu, JY), Jiangsu (Wuxi, JW), and Hunan provinces (Changsha, HC; Junshan, HY; Yueyanglou district, HL), respectively. Bootstrap values (in %) above 50% from 1000 pseudo-replicates are shown. Bootstrap values lower than 50 are not given. Refer to Table 1 for sample information.

compared to that of point mutations, or to microsatellite expansion and contraction, the REMAP technique has come into focus recently and confers great advantages in reconstructing pedigrees and phylogenies. REMAP uses primer combinations that are shared by IRAP and ISSR. Therefore, this technology could theoretically produce two other kinds of products in REMAP reaction: fragments amplified between two microsatellite loci, and between retroelements (Fig. 1). However, this is rarely the case in practice due to the genome structure and the competition mechanism within the PCR reactions. Generally, sequences in retrotransposons appear more conserved compared to the relative distribution of SSR regions, and most inter-SSR amplification would be suppressed [10, 44].

In the present study, the numbers of fingerprinting bands detected by the REMAP primer combinations of LTR-6, LTR-7, LTR-11 plus SSR primers were higher than in our previous study using IRAP primers LTR-6, LTR-7, and LTR-11 only [7]. We have therefore demonstrated that the addition of SSR primers enables the amplification of DNA regions that could not be covered by PCR methods using IRAP and better reflected the activities of LTR retrotransposons in the genome. Such a result was consistent with those of previous studies using REMAP in barley and rice [12, 16]. Therefore, REMAP was shown to be an effective molecular marker technique, showing great potential for use in genome assessments for fingerprinting and diversity studies.

4 Concluding remarks

The present study demonstrated that the REMAP marker system provides a simple and useful electrophoretic technique for studying genetic diversity and population genetic structures of *S. japonicum* from different endemic provinces in China. The efficient identification of different *S. japonicum* populations has important implications for effective prevention and control of schistosomiasis, and the enhanced utility of this technique over others might facilitate adaptation of REMAP to a variety of research purposes in studying other living organisms.

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中国のタチウオから検出されたアニサキスⅠ型幼虫の分子同定

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Molecular diagnosis of *Anisakis* type I larvae from hairtail fish captured off China

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我が国で水揚げされたタチウオには、アニサキスⅠ型の幼虫として、*Anisakis simplex* sensu stricto（狭義の *A. simplex* で本邦人体症例の主要病原種、以下 As（s.s.））および *Anisakis pegreffii*（以下 Ap）が寄生していた。一方で台湾のタチウオからは、アニサキスⅠ型の幼虫として、*Anisakis typica*（以下 At）が優占的に検出された（Umehara *et al.*, 2010）。そこで今回は中国のタチウオを対象に、同様の検討を試みた。

【材料と方法】東シナ海に面する浙江省寧波市において、舟山群島近海で漁獲されたタチウオ（7尾）を購入し、アニサキスⅠ型幼虫の検出を試みた。検出された虫体からDNAを調製し、リボソームDNA·ITS領域（ITS1+5.8S+ITS2）をPCR増幅して、塩基配列を解読した。

【結果】検査した7尾のタチウオのうち、陽性魚は2尾で、計6隻のアニサキスⅠ型幼虫が検出された。配列解読の結果、虫体は総てAtであることが明らかとなった（表1）。

【考察】中国・台湾ではタチウオが最も人気のある海産魚で、漁獲量も多く、季節を問わずに市場で入手できる。今回、タチウオから検出されたAtは、熱帯・暖海域に分布する虫種とされる。魚介に寄生するAtの第3期幼虫は、As（s.s.）/Apとの形態鑑別が困難で、アニサキスⅠ型として取り扱われている。一方で第4期にまで発育すると、AtはAs（s.s.）/Apと形態鑑別が可能とされる（陰門の位置が指標）。第4期幼虫が、時にアニサキスによる人体症例から検出されるが、このような事例の中に、原因虫種をAtと形態同定した報告も認める（影井、1993）。従って、アニサキスⅠ型の幼虫が検出された場合には、Atの存在も視野に入れて、慎重に種同定を試みる必要がある。このような対応は、特に台湾や中国でのアニサキス症の原因種の調査で、重要であると考えられた。

Key words：*Anisakis typica*, hairtail fish, China

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Table 1. *Anisakis type* Ⅰ larvae from hairtail fish

Locality		Date of collection	No. of fish Infected /examined	No. of parasites Collected	identified as At[a]	Ap[b]	As[c]	Reference
China	Ningbo	22 Jul 2011	2/7	6	6	0	0	this study
Taiwan	Taichung	14 Oct 2008	6/7	110	93	15	2	Umehara *et al.*, 2010
Japan	Nagasaki	3 Dec 2008	5/7	20	0	20	0	
	Shizuoka	12 Jul 2010	7/7	33	1	0	32	
	Wakayama	18 Jul 2010	2/7	6	0	0	6	
	Kochi	13 Jul 2010	1/7	2	0	0	2	

a：*A. typica*　b：*A. pegreffii*　c：*A. simplex* sensu stricto

肺吸虫の感染を予防するためのサワガニ冷凍条件の検討

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Key Words: ウェステルマン肺吸虫，サワガニ，メタセルカリア，温度感受性，感染予防

はじめに

東京の鮮魚店で食用として販売されたサワガニの約20%から，人体寄生性肺吸虫（ウェステルマンと宮崎）のメタセルカリアが検出されている[1)]．この市販の食用サワガニを食材に利用し，出身地固有の料理を調理・喫食して，肺吸虫に感染した外国人の事例が報告されている[2),3)]．肺吸虫の感染予防法の確立が必要であり，その1つとして加熱によるサワガニの前処理が有効と考えられた．そこで，ウェステルマン肺吸虫陽性地区由来のサワガニを55℃で5分間加熱処理し，虫体を回収して，マウスへ実験的に投与した．その結果，感染が成立しなくなることが明らかとなった[4)]．肺吸虫感染予防のための前処理として，冷凍も同様に有効と考えられたので，今回はその条件を検討した．

材料と方法

ウェステルマン肺吸虫（2 倍体型）陽性のサワガニは，三重県伊賀市の流行地で採集した[5)]．実験に当たっては，活発に運動するサワガニを選び，20×30 cm のネット（ポリエチレン製，メッシュサイズ 16）に入れ，庫内の平均温度を-18℃に設定した冷凍庫（容量 334 リットル）内で，所定の時間，冷凍処理した．処理後のサワガニは，ネットに入れたまま流水（水道水）に 1 分間浸漬して解凍した．そして速やかに解剖用はさみで細切し，多量の水道水で洗浄した．洗浄水を静置した後，沈渣を実体顕微鏡下に精査し，虫体（幼虫）を回収した．得られた虫体は形態を観察すると共に，マウス（ddY 系，雄，5 週齢，各群 5 頭）に各 10 個ずつ，経口的に感染させた．マウスは感染後

Effect of freezing on the infectivity of *Paragonimus westermani* metacercariae in intermediate host crabs

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22-28 日に剖検し，既報に準じて全身からの虫体回収を試みた[6]．

結果

1. 形態所見

(1) 非冷凍（未処理）サワガニ由来のメタセルカリア[4]

メタセルカリアはほぼ球形を呈した．嚢内の幼虫は体全体を回転させる，あるいは体肉の一部を波動させるなど，活発に運動した．幼虫は，体の中央部にI字状に伸びる排泄嚢を有し，その中には排泄顆粒が充満していた．また排泄嚢の両側には，腸管が明瞭であった（図1A）．

(2) 冷凍サワガニ由来のメタセルカリア

A. -18℃・50 分間の処理

幼虫は嚢内に留まり，やや不明瞭ながらも腸管を特定し得たメタセルカリアが大部分を占めた（図1B）．しかし，一部は嚢壁に欠損を認め，更にこの欠損部から虫体の一部あるいは大部分が嚢外に脱出していた．嚢壁に欠損を認めた個体では，幼虫の体肉は混濁し，腸管の特定が困難で，運動性は著しく減弱していた．

B. -18℃・100 分間あるいは 150 分間の処理

ほぼ総てのメタセルカリアが嚢壁に欠損を認め，この欠損部から虫体の一部あるいは大部分が嚢外に脱出していた．完全に脱嚢した個体も少数ながら認めた．幼虫は総て体肉が混濁し，腸管の特定が困難で，運動性を欠いていた（図1C）．

2. マウスへの感染試験

(1) 非冷凍（未処理）サワガニ由来のメタセルカリア[4]

総ての試験マウス（5頭）で感染が成立した（表1）．回収数は1頭あたり平均5.8虫体（1頭あたり5-8虫体）であった．部位別の回収数では骨格筋が最も多く，1頭あたり平均4.8虫体（1頭あたり4-7虫体），次いで体腔から平均1虫体（0-2虫体）が回収された．横隔膜・肝・肺は陰性であった．

(2) 冷凍サワガニ由来のメタセルカリア

A. -18℃・50 分間の処理

総ての試験マウス（5頭）で感染が成立した．回収数は1頭あたり平均4.2虫体（1頭あたり1-7虫体）であった．部位別の回収数は骨格筋が最も多く，1頭あたり平均3.2虫体（1頭あたり1-5虫体），次いで体腔から平均1虫体（0-2虫体）が回収された．横隔膜・肝・肺は陰性であった（表1）．

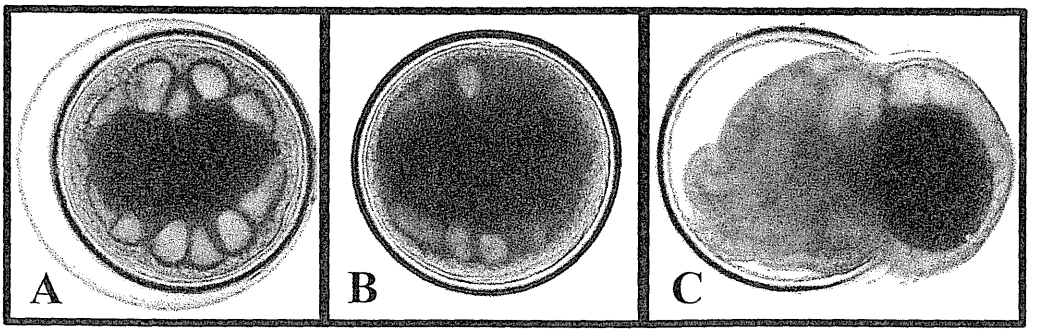

図1　ウェステルマン肺吸虫メタセルカリアの形態所見．非冷凍（A），および-18℃で50分間（B），あるいは同100分間（C）の冷凍による前処理を施したサワガニ由来のメタセルカリア・顕微鏡写真（中拡大像）．冷凍処理により幼虫は体肉が混濁し（B, C），腸管は不明瞭となり，嚢壁の欠損部から虫体が嚢外に脱出した（C）．

B. -18℃・100 分間あるいは 150 分間の処理

虫体は全く回収されなかった（表 1）.

表 1　冷凍・非冷凍のサワガニ由来のウェステルマン肺吸虫メタセルカリアを用いたマウスへの感染試験

群[a]	サワガニ処理		回収虫体数[b] (1 頭平均)			回収率 (%)
	温度 (℃)	時間 (分)	体腔	筋	合計	
1	NF[c]		1	4.8	5.8	58
2	-18	50	1	3.2	4.2	42
3	-18	100	0	0	0	0
3	-18	150	0	0	0	0

[a] ddY 系，雄，5 週齢のマウスを各群 5 頭使用
[b] 試験マウスはメタセルカリア（幼虫）を各 10 個ずつ経口的に投与し，投与後 22-28 日に剖検して，体腔・全身の骨格筋・横隔膜・肝・肺を対象に，虫体の回収を試みた.
[c] NF: 非冷凍

考察

本研究の結果，肺吸虫の感染源となるサワガニ（第 2 中間宿主）を-18℃で 100 分間冷凍すれば，サワガニ体内に寄生するメタセルカリアは，マウスへの感染能力を消失することが分かった．すなわち冷凍は，加熱（サワガニを 55℃で 5 分間前処理）[4]と同様に，肺吸虫の感染予防の手段として有効であった．一方で，-18℃でも 50 分間の冷凍では，総ての試験マウスが感染した．従って，肺吸虫の感染を確実に予防するには，冷凍の温度だけでなく，その時間も厳守されているか，確認する必要があると考えられた.

ウェステルマン肺吸虫の感染源として，サワガニと同様に重要なモクズガニからメタセルカリアを分離し，感染予防に資する条件が検討され，-40℃で 30 分間の冷凍が有効だと報告されている（形態観察に基づく効果判定）[7]．今回我々が検討した-18℃より低い温度，すなわち-40℃では，より短い時間でのサワガニの冷凍で，感染予防が成立する可能性を示唆した成績と考えられた．従って今後更に，種々の温度・時間でサワガニを冷凍し，感染試験を行ない，感染予防に有効な諸条件を明らかにしたいと考えている.

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特発性好酸球増多症候群としてステロイド投与中に両肺多発空洞陰影を呈したウェステルマン肺吸虫症の一例

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Key Words: *Paragonimus westermani,* steroid, triploidy, eosinophilia, Japanese freshwater crab

緒言

ウェステルマン肺吸虫症はサワガニ，モクズガニ，イノシシ肉等の非加熱摂食により感染する寄生虫症である．近年，海外からの移住者で，出身地固有の食習慣により感染したとみられる例も散発しており，他疾患として治療される例もあることから，類症との鑑別に注意すべき疾患である．今回，胸部異常陰影と好酸球増多が認められ，食生活等の問診を契機に肺吸虫症との診断に至っ

A case of *Paragonimus westermani* infection showing multiple cavities in bilateral lungs during the course of steroid treatment for suspected idiopathic hypereosinophilic syndrome

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た外国人の症例を経験したので報告する.

症例

40 歳代女性　帰化中国人（上海出身）
【発症時の主訴】全身の疼痛，腹痛
【既往歴/アレルギー/喫煙歴/家族歴】なし
【職業】飲食関係
【渡航歴】2 回/年中国に帰国

経過

2010 年 9 月頃から全身の疼痛，移動性の腹痛を自覚．同年 9 月 27 日に某総合病院を受診した．WBC14,300 /mm^3（Eosino 58.0 %）と上昇を認め，便虫卵検査陰性等から特発性好酸球増多症（hypereosinophilic syndrome: HES）を疑い，プレドニゾロンを 50mg/日として治療を開始したが，症状に改善はなく，約 1 年後（2011 年 8 月 24 日）の胸部 CT 写真で，右 S2 および左 S1+2 に空洞を伴う結節の出現を認めた．抗真菌薬が投与されたが，改善を認めず，11 月 15 日に呼吸器内科を紹介受診．再度，詳しく問診した結果，発症前の 2010 年 8 月頃，横浜市内のスーパーにて購入したサワガニで「酔蟹」を調理，非加熱で食したことが判明．肺吸虫を強く疑ったが，真菌・抗酸菌感染症および悪性腫瘍等との鑑別のため，気管支鏡精査を行った．

検査所見

WBC 10,600 /mm^3（Band 2.0 %，Seg 64.0 %，Eosino 10.0 %，Baso 1.0 %，Mono 9.0 %，Lymph 13.0 %），Hb 12.0 g/dl，Plt 28.6 万/mm^3，HbA1c 5.0%，CRP 0.4 mg/dl，β-D-glucan 3.6 Pg/ml，アスペルギルス抗原（−），MPO-ANCA <10 EU，PR3-ANCA <10 EU，IgE 45 IU/ml，抗寄生虫抗体:宮崎肺吸虫クラス 3，ウェステルマン肺吸虫クラス 3，喀痰検査：虫卵（−）

画像所見

胸部 CT で胸膜近傍の右 S2，左 S1+2 に空洞を伴う結節（Fig.1）．胸水（−）

気管支鏡検査

経気管支肺生検：気管支壁に好酸球浸潤を認めたが，虫卵は確認できず，また気管支洗浄液でも虫卵は認められなかった．

胸腔鏡下肺生検と病理組織所見

左上葉の肺生検が施行し，20×18×13 mm の空洞を伴う結節（嚢胞）を確認した．組織標本では嚢胞壁に，好酸球・リンパ球・組織球の強い浸潤と異物巨細胞の出現を認め，一部は壊死していた．

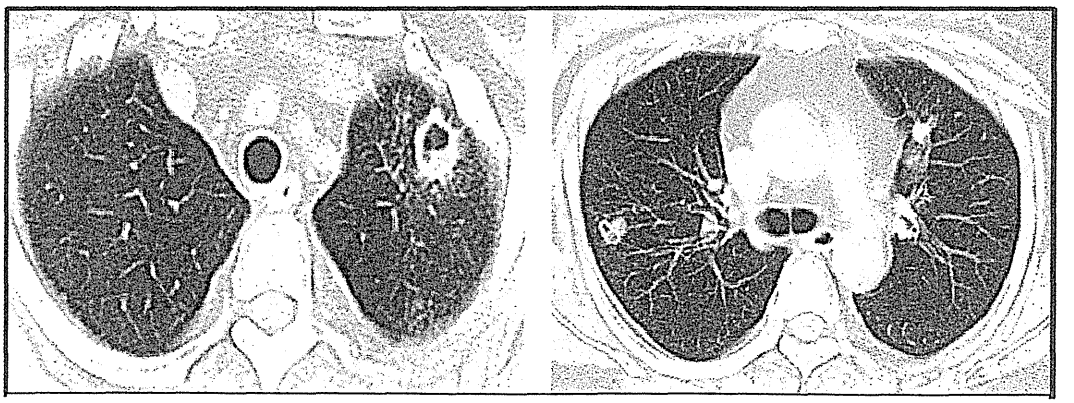

Fig. 1 Chest computed tomography scan revealed multiple well defined solitary nodules with cavitation in the both lungs.

また，径が 0.05－0.1mm の円形・楕円形の卵殻様の構造物を認めた（Fig.2）．形態学的特徴から，構造物を肺吸虫卵と判断した．

原因肺吸虫の分子同定

組織標本中の虫卵から DNA を調製し，PCR 増幅して，産物の塩基配列（rDNA・ITS2 領域およびミトコンドリア DNA・16S rDNA 配列）を解読した．ウェステルマン肺吸虫（3 倍体型）と同定した．

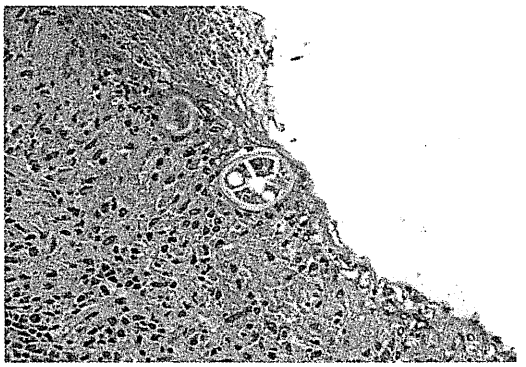

Fig. 2 Histopathological findings of a pulmonary nodule with cavitation in the left upper lobe. Note *Paragonimus* egg surrounded by inflammatory infiltrates which mainly consisted of eosinophils and lymphocytes. (HE stain, × 400)

治療

2011 年 12 月 12 日にプラジカンテル 65mg/kg/日を 3 日間内服開始した．胸部 CT で，左上葉の結節の縮小を確認したものの，結節内の空洞は残存した．WBC 4,500 /mm^3, Eosino 19.0 %と上昇し，腸蠕動音亢進，腹鳴，倦怠感のため，2012 年 4 月 5 日にプラジカンテル 75mg/kg/日 を 3 日間再服した．5 月 9 日に WBC 7,400 /mm^3, Eosino 8.0 %に低下し，それに伴い腹部症状，倦怠感も改善した．

考察

川中らの報告[1]によると 1995 年～2002 年に血清診断で肺吸虫症が強く疑われた症例は 76 例であり，在日外国人の陽性例は 23 例（30%）にのぼる．患者の出身国は，韓国 10 例，タイ 8 例，中国 4 例，ラオス 1 例であった．出身国での食生活を本邦に持ち込むことにより，感染・発症する外国人の肺吸虫症例は多い．本症例は帰化中国人であったが，川蟹を日常的に生食する習慣はないものの，横浜市内のスーパーで購買したサワガニを酔蟹として摂食したことで感染したと思われた．同じ酔蟹を食べた同胞の友人は発症しなかった．食用として販売されるサワガニから，ウェステルマン肺吸虫（2 倍体型・3 倍体型）および宮崎肺吸虫のメタセルカリアが検出されており[2]，サワガニは肺吸虫の感染源として注意する必要がある．

ウェステルマン肺吸虫の画像所見として 62%に肺癌，肺結核，真菌症と類似した結節が見られるとの報告[3]がある．また宮崎肺吸虫感染から HES に発展したと考えられる症例も報告[4]されており，TBLB で好酸球浸潤が認められる例も散見される．本症例では HES としてステロイド治療を開始されたが，好酸球浸潤を伴う炎症像が修飾され，虫卵周囲に孤在性の結節が形成され，空洞陰影を呈したとも考えられた．治療により肺吸虫症は臨床症状が速やかに改善し，好酸球数も低下すると報告されている[5]．治療によっても好酸球が低下しない場合は，2 クール目の治療を検討すべきと考えられた．

結語

ステロイドを長期使用中に，食歴に関する詳しい問診で加熱不十分なサワガニの摂取が判明し，胸腔鏡下肺生検により肺吸虫卵を確認，その遺伝子検査によりウェステルマン肺吸虫（3 倍体型）

と確定診断された1症例を報告した.

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