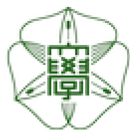
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Analysis for genetic loci controlling protoscolex development in the *Echinococcus multilocularis* infection using congenic mice

(コンジェニックマウスを用いた多包条虫感染における原頭節形成をコントロールする遺伝子座の解析)

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Abbreviations

AE	Alveolar echinococcosis
B6	C57BL/6 (C57BL/6NSlc)
CCL2	The chemokine (C-C motif) ligand 2
Chr	Chromosome
cM	Centimorgan
D2	DBA/2 (DBA/2CrSlc)
IFN-α	Interferon alfa
IFN-β	Interferon beta
Mb	Mega base pair
MGI	Mouse Genome Informatics
NCBI	National Center for Biotechnology Information
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
QTL	Quantitative trait locus
SEM	Standard error of the mean

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1. Introduction

In general, laboratory animals are very important in many aspects such as biomedical research, safety testing for pharmaceutical products, education and contributing to the improvement of knowledge in the scientific community. Laboratory animals are used by biomedical researchers to extend the understanding of the physiological functions and the processes of disease and health. They are widely used to develop new vaccines and treatments for various diseases not only for human benefit, but also helping to develop veterinary techniques. Animal research does not only benefit humans, but also benefits animals, either directly because animal health is the subject of research or indirectly because the same procedures and treatments used in humans can be used in animals (Institute of Medicine and National Research Council, 1988). The mouse is one of the most popular and commonly used laboratory animals all over the world, because humans and mice share many common genetic features. The laboratory animal model permits investigators to pursue the research hypotheses which are not possible to conduct in humans. Understanding of these animal studies could pave the pathway to study the types of diseases and health effects and to look for therapeutics in case of humans. Laboratory mice, particularly the most commonly used inbred strains (e.g., B6 and D2), were persistently produced by generations of brother-sister mating to create homogenous lines to know the pathophysiological conditions in several aspects (Heideman, 2004). Therefore, the laboratory mouse has been proven to be enormously useful in the dissection of the genetic architecture of the host defense mechanism against many infectious

diseases. Mice are susceptible to a similar range of microbial infections as well as humans. However, as there are marked differences in their response to pathogen infection among inbred strains, mice provide the opportunity to analyze the genetic basis of the mechanisms of infection and immunity (Buer et al., 2003). For underlying the phenotypic differences between two independent inbred strains for a given trait, forward genetics is an experimental approach, in which gene mapping and positional cloning are used to elucidate the molecular mechanisms. This strategy has been highly successful in the study of inbred mouse strains that could disclose the differences in innate susceptibility to bacterial, parasitic, fungal, and viral infections (Kielczewska et al., 2006). The major advantage of this so-called forward genetics approach is that the effect of the gene(s) on susceptibility is already validated *in vivo*. An obvious disadvantage of this approach is that the genetic effect may be complex, with individual monogenic contributions that are perhaps difficult to address. Nevertheless, this approach has been extremely successful in the study of infectious diseases and has revealed a treasure trove of genes, proteins, and signaling pathways that play critical roles in the immune response to a large number of infectious agents (Vidal et al., 2008)

The current study will address the role of laboratory inbred mice to unveil the host parasitic interplay in case of alveolar echinococcosis (AE). The aim of this research is to verify genetic loci responsible for controlling the development of AE by using congenic and subcongenic mice. AE is a zoonotic disease caused by the metacestode stage of tapeworm *Echinococcus multilocularis* characterized by a severe hepatic disorder in many parts of the northern

hemisphere (Craig et al., 1996). Infiltrating parasitic growth (hematologous spread) can cause severe organ damage. Long term treatment is one of the expansive issues and causing health problems (Kern at al., 2003; Reuter et al., 2000). Mainly rodents and occasionally humans become the intermediate hosts being infected by oral ingestion of eggs excreted from contaminated feces of the definitive host, carnivores in the environment. The eggs hatch out into oncospheres in the intestine of intermediate hosts. The oncospheres migrate to the liver by penetrating the intestinal wall and develop to metacestode. Mature metacestodes produce huge numbers of protoscoleces in the intermediate hosts (Gottstein et al., 1995). AE is one of the most significant lethal zoonotic parasitic infection in the northern hemisphere. Currently, the threat to public health is increasing which showed the evidence by the rising prevalence rate of alveolar echinococcosis, as well as the invasion of urban areas by infected wild foxes. This threat is further increased due to the involvement of pet dogs, and probably cats, as emerging sources of infection. These increased threats to public health and associated economic risks (Kamiya et al., 2007). The occurrence of the causative cestode in Japan is restricted to the northern island of Hokkaido, although sporadic cases of human infections have been reported on other islands (Doi et al., 2003). Currently, this parasite is reported to be distributed throughout the island of Hokkaido. In addition, AE also occurs at lower rates in central and eastern Europe (Deplazes et al., 2006). According to the report of 2004, 482 patients in Hokkaido, Japan were confirmed to have been infected with AE, and the rate of occurrence during the endemic period was 48 cases per 100,000 residents every year (Oku, 2000). In

addition to its adverse effects on human health, an epidemic of this disease could adversely affect the local economy of Japan due to its potential impact on agricultural and tourist industries (Konno et al., 2003). Therefore, this disease demands immediate attention and decisive action for its effective and sustainable control.

Mice have been widely used as an experimental model to study the host-parasite interplay in the *E. multilocularis* infection, which may open the door for the establishment of better control strategy for AE. Matsumoto et al. (2010) demonstrated that two inbred strains, C57BL/6 (B6) and DBA/2 (D2) mice, differed markedly in their susceptibility to *E. multilocularis* infection. The oral administration of *E. multilocularis* eggs in D2 mice established a higher number of cysts in the liver as compared to B6 mice. Moreover, a significant number of protoscoleces were observed in the cysts of D2 mice, whereas protoscoleces were completely absent in B6 mice. The use of various recombinant antigens from *E. multilocularis* clearly revealed different antibody responses among mouse strains. D2 mice exhibited higher levels of IgG against recombinant antigens, whereas IgM levels remained low in response to any of recombinant proteins until 16 weeks post infection and D2 mice allowed the active growth of larval *E. multilocularis*. Specific IgG responses against these recombinant antigens were not effective in regulating the parasite growth but were just induced in response to active development of the parasite in the host tissues. On the other hand, specific antibody responses in the resistant mouse strains B6 and C57BL/10 were characterized by apparent IgM responses and relatively poor IgG responses against some of the recombinant antigens. However, AKR/N

mice, a relatively susceptible strain, also exhibited similar kinetics in the specific IgM levels to that of D2 mice, and thus these IgM responses do not seem to play a major role in regulating larval development of the parasite (Matsumoto et al., 2010). Additionally, the observation from other studies, have supported the results that the susceptibility to *E. multilocularis* infection is genetically controlled both in animals and humans (Hildreth and Granholm, 2003; Nakaya et al., 1997; Vuitton, 2003). However, the genetic factors responsible for difference in the susceptibility to *E. multilocularis* parasite still remains unknown. Several studies have reported that B6 and D2 mice have differences in susceptibility against various infectious diseases (Adrianus et al., 2009; David et al., 1995; Marquis et al., 2009; Simon et al., 2009). One study revealed a host genetic variation affects in increased production of proinflammatory mediators to infection with a highly pathogenic H5N1 influenza A virus in mice where D2 mice showed more inflammation than did B6 mice; TNF-α production was almost eightfold higher in virus-infected D2 mice than in B6 mice ($P < 0.01$), whereas IFN-α, IFN-β, and CCL2 production was approximately threefold higher; $P < 0.01$ for all three cytokines (Adrianus et al., 2009). Mouse models in case of leishmaniasis have uncovered more than 20 quantitative trait loci (QTLs) as being susceptibility genes, studies of which have made important contributions to the understanding of the host response to infection. The functional effects of individual QTLs differ widely, indicating a networked regulation of these effects. Several of these QTLs probably also influence susceptibility to other infections, indicating that their

characterization will contribute general understanding of susceptibility to infectious diseases (Lipoldova et al., 2006).

As mice and humans share 99% of their genes, they also share common diseases and the genomic resources are rapidly growing in the mouse, which facilitate complex trait analysis and extensive phenotypic differences exist between inbred strains of mice. The advances of the mouse genome and phenome provide the resources that are required to rapidly and cost-effectively identify QTLs and narrow QTL confidence intervals (Peters et al., 2007). Therefore, considering the marked difference between B6 and D2 mice in susceptibility to *E. multilocularis* infection, QTL analysis was conducted in backcrossed progenies from B6 and D2 mice (Nakao et al., 2011). QTL mapping is a promising tool for the detection of genetic loci that contribute to the determination of differences in phenotypic variation. QTL analysis has revealed a significant QTL, *Emcys1* for the establishment of *E. multilocularis* cysts in the mouse liver, suggesting that it controls the number of cysts in the liver (Nakao et al., 2011). Furthermore, the development of protoscoleces of cysts in the mouse liver was controlled by a distinct, highly significant QTL, *Empsc1*, indicating a role of different host factor interplaying with parasites at each developmental stage (Nakao et al., 2011). Lack of information on the interplay between the parasite and the intermediate host makes it difficult to focus on certain genes responsible for resistance/susceptibility to *E. multilocularis* infection. It is critically important to address the role of genetic factors to understand the course of infection as well as to get better treatment strategies. To verify that previously identified QTLs are indeed

responsible for susceptibility or resistance to *E. multilocularis* infection, making congenic mouse strains is one of the most reliable strategies. Congenic mouse strains are defined as those, in which genetic alteration or mutation is transferred into a standard inbred mouse strain (Markel et al., 1997). Several researchers have published valuable findings using congenic mice that could confirm the responsible genetic regions to address candidate genes in various diseases, including infectious and autoimmune diseases (Allen et al., 2006; Marquis et al., 2008; Rogner et al., 2003; Shimizu et al., 2007). The purpose of the current study is to verify that previously identified QTLs are indeed responsible for resistance/susceptibility to *E. multilocularis* infection by establishing congenic mouse strains from B6 and D2 mice, where the significant QTLs are introgressed from B6 to D2-genetic background and *vice versa* from D2 to B6-genetic background. Furthermore, subcongenic lines were generated to narrow down the critical region, including QTLs responsible for resistance/susceptibility to *E. multilocularis* infection.

2. Materials and methods

2.1. Mice

Specific pathogen-free inbred mice, B6 and D2 were purchased from Japan SLC (Shizuoka, Japan) to generate congenic and sub-congenic lines. For generation of congenic and sub-congenic lines, the AAALAC International-accredited program and the Regulation for the Care and Use of Laboratory Animals, Hokkaido University was followed, and animal use protocol was approved by the President of Hokkaido University after the review by the Institutional Animal Care and Use Committee (#14-0154). Parental inbred, congenic, and sub-congenic mice were subjected to infection experiments with *E. multilocularis*. Infection experiments were performed in accordance with the regulation of Hokkaido Institute of Public Health and animal use protocol was approved by President of Hokkaido Institute of Public Health after the review by the Ethics Committee of the Institute.

2.1.1. Congenic lines

For *Emcys1*, two congenic lines were generated by the introgression of *Emcys1* in chromosome (Chr) 6 from B6 to D2-genetic background and *vice versa* from D2 to B6-genetic background. The (B6 x D2)F_1 mice were backcrossed to B6 or D2 parental strain [(B6 x D2)F_1 x B6 or (B6 x D2)F_1 x D2]. Congenic lines were generated by the speed congenic method (Markel et al., 1997). Briefly, in the case of B6-genetic background, backcrossing was repeated up to 5 generations and homozygous founders were established by brother-sister

mating. Similarly, for the D2-genetic background, backcrossing was repeated up to 7 generations and homozygous founders were established by brother-sister mating. For *Empsc1*, two congenic lines were generated according to the same method as for the *Emcys1*-congenic lines, where *Empsc1* in Chr 1 was introgressed from B6 to D2-genetic background and *vice versa* from D2 to B6-genetic background. For both genetic backgrounds (B6 and D2), backcrossing was repeated up to 6 generations and homozygous founders were established by brother-sister mating. Introgression of chromosomal regions was confirmed by genotyping of microsatellite markers shown in Figs. 1 and 5. Congenic lines were named according to the international nomenclature guidelines and abbreviated as in parenthesis; B6.D2-(*D6Mit188-D6Mit15*)/NSlcHkv (B6.D2-*Emcys1*), D2.B6-(*D6Mit188-D6Mit15*)/CrSlcHkv (D2.B6-*Emcys1*), B6.D2-(*D1Mit191-D1Mit291*)/NSlcHkv (B6.D2-*Empsc1*), and D2.B6-(*D1Mit191-D1Mit291*)/CrSlcHkv (D2.B6-*Empsc1*).

2.1.2. Sub-congenic lines

Two subcongenic lines were developed from B6.D2-*Empsc1*. B6.D2-*Empsc1* was mated with B6 parental strain to generate heterozygous F_1 and then F_1 generations were backcrossed to B6 to establish heterozygous subcongenic lines. After that, each line was intercrossed (brother-sister mating) to obtain homozygous subcongenic lines. Another subcongenic line was developed from D2.B6-*Empsc1* according to the same method as subcongenic line from B6.D2-*Empsc1*. D2.B6-*Empsc1* was mated with D2 parental strain to generate F_1 heterozygous

sub-congenic mice and homozygous sub-congenic line from D2.B6-*Empsc1* was established by brother-sister mating. Introgression of chromosomal regions was confirmed by genotyping of microsatellite markers as shown in Fig. 1. Subcongenic lines were named according to the international nomenclature guidelines and abbreviated as in parenthesis; B6.D2-(*D1Mit191-D1Mit290*)/NSlcHkv (B6.D2-*Empsc1.1*), B6.D2-(*D1Mit201-D1Mit291*)/NSlcHkv (B6.D2-*Empsc1.2*), and D2.B6-(*D1Mit191-D1Mit14*)/CrSlcHkv (D2.B6-*Empsc1.1*).

2.2 Microsatellite markers and genotyping

A total of 134 microsatellite markers (Table 1) were selected from the database in the Mouse Genome Informatics (MGI), The Jackson laboratory, ME, USA (*http://www.informatics.jax.org/*) to generate congenic and subcongenic lines. Twenty-eight microsatellite markers (Figs. 1 and 5) were used to confirm the introgressed regions for cyst establishment and protoscolex development in congenic and subcongenic mice. Briefly, a piece of ear-punched tissue was collected and genomic DNA was extracted by incubating samples at 54 °C for 3 h in 0.5 ml of lysis buffer [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA) and 1% sodium dodecyl sulfate (SDS)] with 5 µl of 10 mg/ml proteinase K. After genomic DNA extraction the touchdown PCR was performed with *Taq* DNA polymerase (Ampliqon A/S, Odense M, Denmark) as follows; denaturing at 95 °C for 1 min, followed by 10 cycles of denaturing at 95 °C for 30 sec, primer annealing at 65 °C for 30 sec (-2 °C in 2 cycles), and extension at 72 °C for 30 sec, and then,

35 cycles of denaturing at 95 °C for 30 sec, primer annealing at 55 °C for 30 sec, and extension at 72 °C for 30 sec, and final extension at 72 °C for 1 min, using a T100^{TM} thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA). The amplified samples were electrophoresed in 12% polyacrylamide gels in TBE buffer; pH-8.0 (89 mM Tris, 89 mM boric acid and 2 mM EDTA), and stained with ethidium bromide for the visualization under a UV light. The genotype was identified by the size of the PCR product.

2.3 Infection experiments and phenotype assessment

The infection experiments were performed according to Nakao et al., (2011). Briefly, *E. multilocularis* eggs were collected from feces of the infected dogs. Experimental infection was conducted in parental inbred strains (B6 and D2), congenic, and subcongenic mice by oral administration of *E. multilocularis* eggs. For the assessment of cyst establishment, mice were infected with 200 eggs, sacrificed, and necropsied at 4 weeks after infection. Mouse liver was cut into small slices with approximately 0.5-mm thickness and the total number of cysts was counted in each liver. For the assessment of protoscolex development in the cyst, mice were infected with 2,000 eggs, sacrificed, and necropsied at 16 weeks after infection, because larger number of eggs and longer period were needed to assess protoscolex development in the cyst. Livers were collected, parasitic cysts were dissected from the liver, and total weight of the cysts was measured. A part of the cysts (1-2 g) was minced, passed through a 125-μm sieve, and washed repeatedly with saline. The number of mature protoscoleces was counted under a

stereoscopic microscope and expressed as number/gram of cysts. Moreover, protoscoleces were counted in histopathological sections of liver to confirm the protoscolex development in cysts.

2.4. Histopathology

Liver tissues were fixed in 10% formalin in phosphate-buffered saline (pH 7.4). The specimen was dehydrated in consecutive stages with increasing concentrations of alcohol and cleaned by xylene. Finally, liver samples were processed for paraffin embedding and cut into 2-μm-thick sections. Tissue sections were stained with haematoxylin and eosin for microscopic examinations with an All-in-One Fluorescence Microscope BZ-X710 (Keyence, Osaka, Japan) to confirm protoscolex development in the liver section. The number of protoscolex was counted in10 cysts selected randomly in each mouse liver and the mean value was calculated in each mouse.

2.5. Statistical analysis

All data were subjected to non-parametric test, Mann-Whitney *U*-test to compare between two groups and one-way ANOVA with Bonferroni post-hoc analysis for more than two groups. Statistical analyses were performed using a GraphPad Prism 5 software version 5 for windows (GraphPad software Inc., San Diego, CA, USA). $P < 0.05$ was considered to be significant.

3. Results

3.1 Confirmation of Empsc1 using congenic mice

In congenic mice, B6.D2-*Empsc1* and D2.B6-*Empsc1*, introgressed chromosomal region (*D1Mit7* to *D1Mit511*) was confirmed by genotyping of microsatellite markers (Fig. 1). This region covered highly significant region of the QTL peak detected in the previous QTL analysis (Nakao et al., 2011). Other chromosomes except for the Chr 1 were confirmed to be recipient-genetic background by genotyping microsatellite markers located in other chromosomes as shown in Table 1. To confirm the *Empsc1* for protoscolex development, B6.D2-*Empsc1* and D2.B6-*Empsc1* mice were subjected to the examination at 16 weeks after oral administration of 2,000 eggs of *E. multilocularis*. Infection experiments were performed separately in different point of time depending on the establishment of *Empsc1* congenic lines of B6 and D2 genetic background. Firstly, B6.D2-*Empsc1* mice as well as B6 and D2 mice (parental strains) were challenged and the number of mature protoscoleces in the cysts was counted and calculated for all of them. Protoscolex development in B6.D2-*Empsc1* was comparable to that in D2 and significantly greater than that in B6 mice, which was completely absent (Fig. 2). On the other hand, D2.B6-*Empsc1* and D2 mice (parental strains) were challenged with *E. multilocularis* and protoscolex development in D2.B6-*Empsc1* mice was significantly less than that in D2 control mice (Fig. 3). These results indicate that the *Empsc1*

is definitely located between *D1Mit7* and *D1Mit511* and contributes to the difference in protoscolex development between B6 and D2 mice.

3.2 Narrowing the critical genetic region

To narrow down the length of critical region, three subcongenic lines were developed from congenic mice, B6.D2-*Empsc1* and D2.B6-*Empsc1*. Genotyping of microsatellite markers revealed that two lines of B6.D2-subcongenic mice, B6.D2-*Empsc1.1* and B6.D2-*Empsc1.2*, were introgressed D2-derived chromosomal region between *D1Mit7* and *D1Mit399* (38.6 cM and 70.3 cM) and *D1Mit496* and *D1Mit511* (63.1 cM and 97.3 cM), respectively (Fig. 4). On the other hand, D2.B6-sub-congenic line, D2.B6-*Empsc1.1,* was confirmed to be introgressed B6-derived chromosomal region between *D1Mit7* and *D1Mit290* (38.6 cM and 68.1 cM) in the D2-genetic background (Fig. 4).

These three lines of sub-congenic mice were challenged with *E. multilocularis* and protoscoleces in the cysts were counted under a light microscope. B6.D2-*Empsc1.2* and D2.B6-*Empsc1.1* mice showed significant development of protoscoleces as did D2 mice, whereas B6.D2-*Empsc1.1* and B6 mice did not (Fig. 5). Thus, these results conclude that the *Empsc1* is located in the critical region between *D1Mit290* and *D1Mit511* (68.1 cM and 97.3 cM) and is secured in the introgressed region of B6.D2-*Empsc1.2* (Fig. 4).

3.3 Confirmation of protoscolex in sub-congenic mice with histopathological analysis

Histopathological analysis using liver sections confirmed that protoscoleces were developed in the B6.D2-*Empsc1.2* as well as D2 mice (Fig. 6A). In contrast, B6.D2-*Empsc1.1* mice did

not develop any protoscolex as seen in B6 mice (Fig. 6A). Protoscolex number in B6.D2-*Empsc1.2* was significantly higher than that of the parental strain B6 and B6.D2-*Empsc1.1* (Fig. 6B). These results are consistent with results from the actual counting under a light microscope.

3.4 Congenic lines for cyst establishment

Congenic lines, B6.D2.-*Emcys1* and D2.B6-*Emcys1*, for cyst establishment phenotype were confirmed introgressed region by genotyping microsatellite markers (Fig. 7). Both congenic lines were replaced chromosomal region between *D6Mit188* and *D6Mit15* with donor haplotype, in which *Emcys1* locus could be included based on the QTL analysis data in the previous paper (Nakao et al., 2011). Other chromosomes except for Chr 6 were confirmed to be recipient-genetic background by genotyping of microsatellite markers locating in other chromosomes as shown in Table 1. These congenic mice were then challenged with *E. multilocularis* and investigated cyst establishment (Fig. 8). The phenotype of cyst establishment in B6.D2.-*Emcys1* and D2.B6-*Emcys1* congenic mice were the same as that of B6 and D2 parental strains, respectively, indicating that the replacement of *Emcys1* locus with the donor genotype did not alter the recipient phenotype. The reason for the ineffectiveness of the *Emcys1* introgression is unknown; however, the result from this study indicates that the number of cysts is not controlled the *Emcys1* alone.

Table 1: Lists of all microsatellites in all chromosomes used for the generation of congenic and sub-congenic lines.

D1Mit118	*D1Mit150*	*D4Mit235*	*D6Mit188*	*D8Mit261*	*D10Mit14*	*D14Mit102*	*D17Mit49*
D1Mit170	*D1Mit291*	*D4Mit172*	*D6Mit1005*	*D8Mit31*	*D11Mit226*	*D14Mit225*	*D17Mit89*
D1Mit324	*D1Mit511*	*D4Mit139*	*D6Mit39*	*D8Mit234*	*D11Mit21*	*D14Mit165*	*D17Mit142*
D1Mit7	*D2Mit296*	*D4Mit152*	*D6Mit104*	*D8Mit242*	*D11Mit4*	*D14Mit266*	*D17Mit221*
D1Mit191	*D2Mit91*	*D4Mit308*	*D6Mit150*	*D8Mit200*	*D11Mit212*	*D15Mit12*	*D18Mit132*
D1Mit30	*D2Mit185*	*D4Mit204*	*D6Mit254*	*D8Mit156*	*D11Mit199*	*D15Mit5*	*D18Mit17*
D1Mit445	*D2Mit62*	*D4Mit54*	*D6Mit194*	*D9Mit90*	*D11Mit48*	*D15Mit156*	*D18Mit124*
D1Mit496	*D2Mit286*	*D4Mit42*	*D6Mit59*	*D9Mit91*	*D12Mit172*	*D15Mit159*	*D18Mit184*
D1Mit201	*D2Mit229*	*D5Mit180*	*D6Mit15*	*D9Mit302*	*D12Mit5*	*D15Mit161*	*D18Mit7*
D1Mit55	*D2Mit200*	*D5Mit108*	*D6Mit340*	*D9Mit133*	*D12Mit101*	*D16Mit182*	*D19Mit69*
D1Mit34	*D3Mit164*	*D5Mit258*	*D7Mit114*	*D9Mit355*	*D12Mit20*	*D16Mit59*	*D19Mit80*
D1Mit451	*D3Mit182*	*D5Mit208*	*D7Mit82*	*D9Mit200*	*D13Mit17*	*D16Mit140*	*D19Mit13*
D1Mit14	*D3Mit22*	*D5Mit188*	*D7Mit318*	*D9Mit18*	*D13Mit9*	*D16Mit152*	*D19Mit1*
D1Mit290	*D3Mit78*	*D5Mit370*	*D7Mit66*	*D10Mit248*	*D13Mit148*	*D16Mit106*	*DXMit166*
D1Mit399	*D3Mit14*	*D5Mit222*	*D7Mit333*	*D10Mit3*	*D13Mit262*	*D17Mit113*	*DXMit130*
D1Mit145	*D3Mit291*	*D6Mit159*	*D8Mit4*	*D10Mit61*	*D14Mit10*	*D17Mit198*	*DXMit186*
D1Mit355	*D3Mit129*	*D6Mit74*	*D8Mit100*	*D10Mit186*	*D14Mit120*		

Table 2: Lists of possible candidate genes between *D1Mit290* and *D1Mit511* loci.

Gene	Position in Chr. 1 of mouse genome	Genetic position (cM)
Tumor necrosis factor (ligand) superfamily, member 4	161395409-161418410	69.8
Fasl (Fas ligand, TNF super family)	161780689-161788495	70.0
Sele (selectin, endothelial cell)	164048234-164057677	71.3
Sell (selectin, lymphocyte)	164061982-164084181	71.4
Selp (selectin, platelet)	164115264-164150026	71.4
CD247	165788681-165877277	73.1
Fc receptor, IgG, low affinity III (Fcgr3)	171051174-171064935	78.8
Fc receptor, IgE, high affinity I, gamma polypeptide (Fcer1g)	171229572-171234365	79.2
Dedd	171329145-171342331	79.3
CD244 natural killer cell receptor 2B4 (Cd244)	171559193-171609746	79.5
CD48 antigen (Cd48)	171682009-171705258	79.5
C-reactive protein, pentraxin-related (crp)	172698055-172833031	80.1
TNF receptor-associated factor 5 (Traf5)	191997205-192059162	97.1

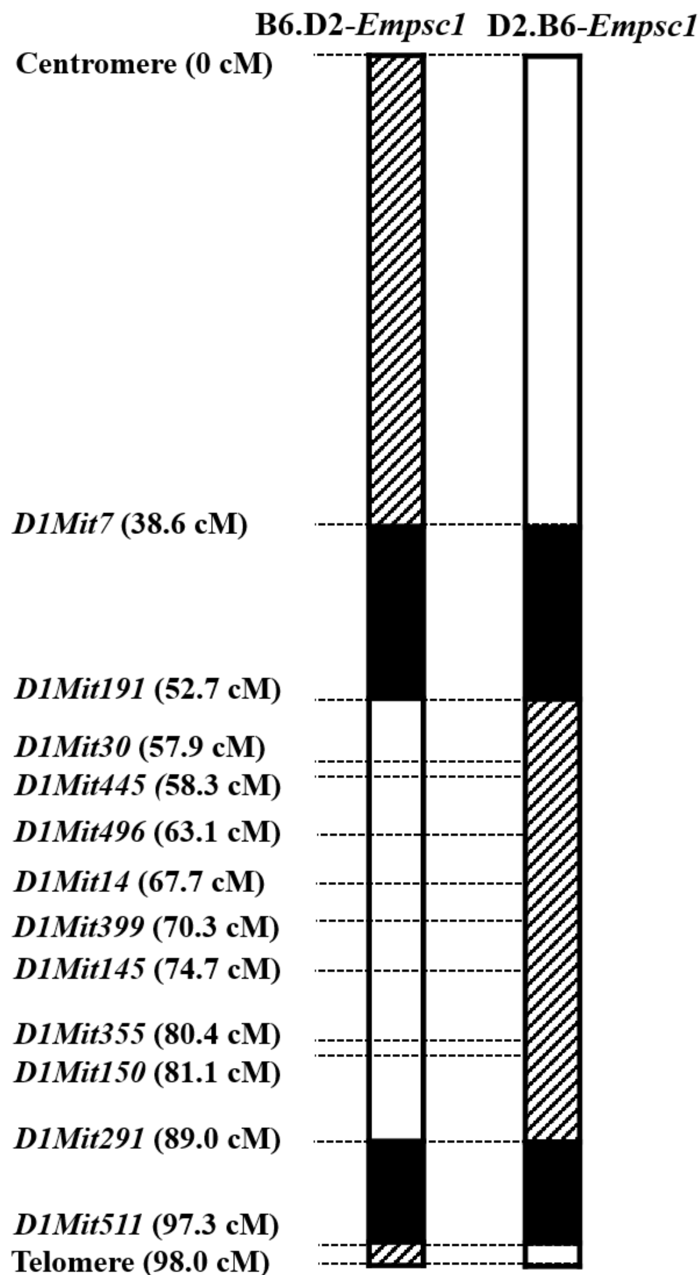

Fig. 1. Schematic diagram of Chr1 in congenic mice for *Empsc1* in B6 and D2 genetic background. Shaded, open, and black columns indicate B6-derived, D2-derived, and unknown chromosomal portions, respectively.

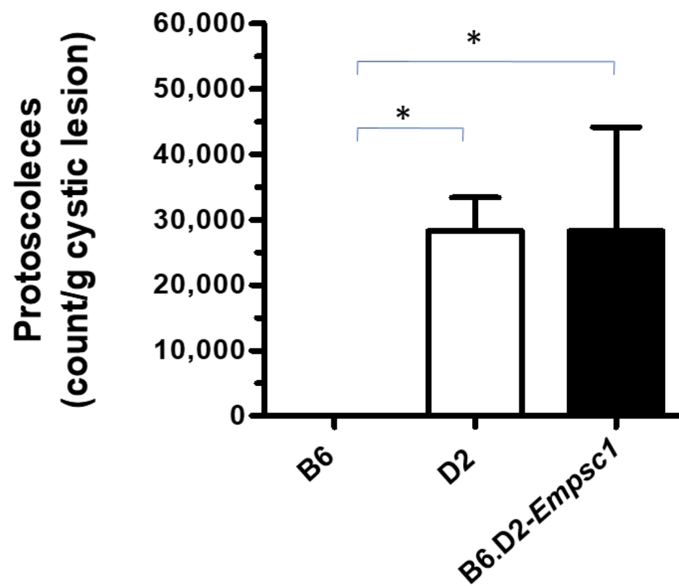

Fig. 2. Protoscolex development in the liver of B6, D2, and congenic mice for *Empsc1* in B6 genetic background. The number of protoscolex in cystic lesions of liver was counted and expressed as number of protoscolex per 1 gram of cystic lesion. Data represent the mean ± SEM for B6 (n=4), D2 (n=4), and B6.D2-*Empsc1* (n=5). * indicate P <0.05.

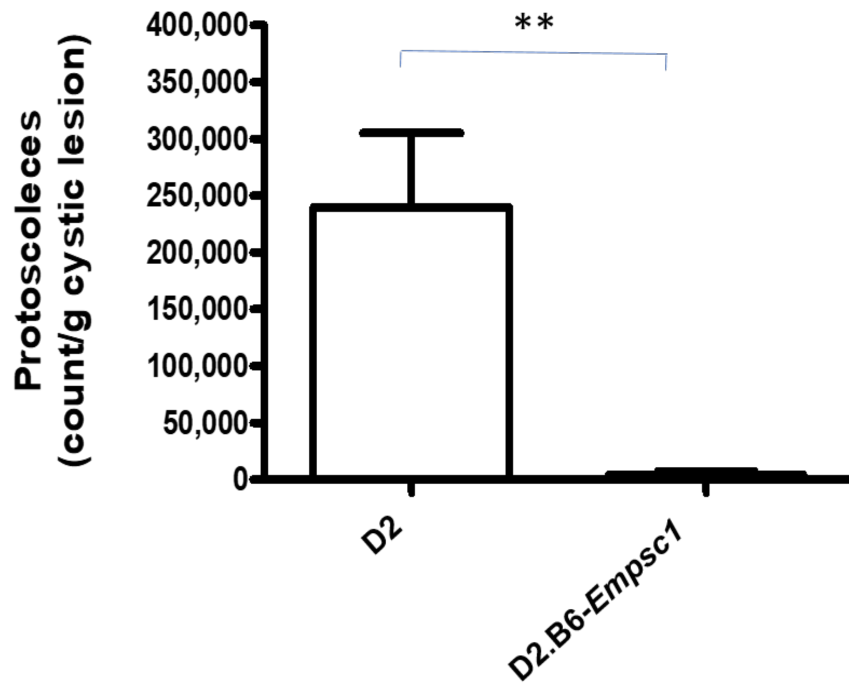

Fig. 3. Protoscolex development in the liver of D2, and congenic mice for *Empsc1* in D2 genetic background. The number of protoscolex in cystic lesions of liver was counted and expressed as number of protoscolex per 1 gram of cystic lesion. Data represent the mean ± SEM for D2 (n=9) and D2.B6-*Empsc1* (n=3). ** indicate P <0.01.

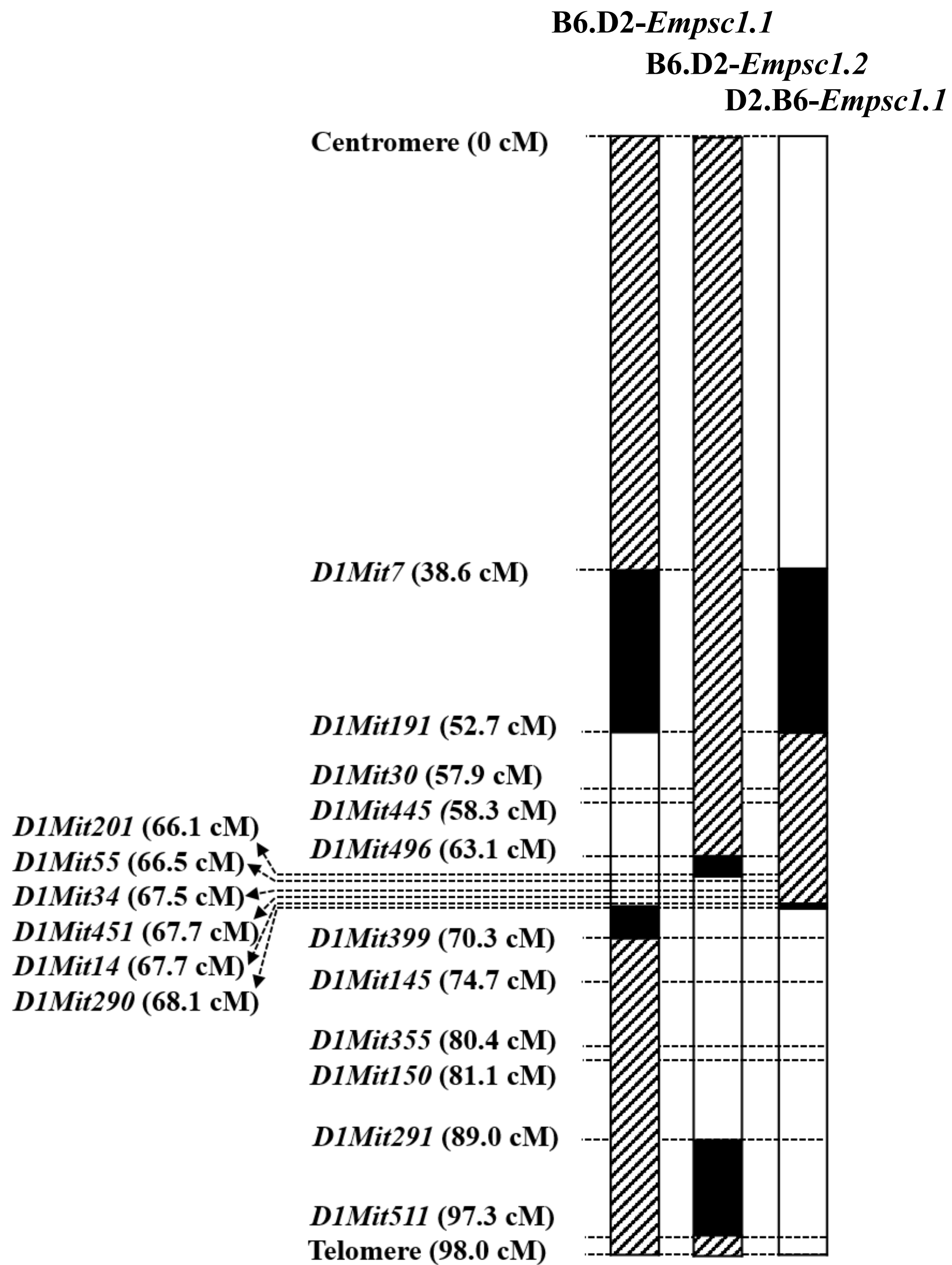

Fig. 4. Schematic diagram of Chr1 in subcongenic mice for *Empsc1* (B6.D2-*Empsc1.1* and B6.D2-*Empsc1.2*) in B6 genetic background and subcongenic mice for *Empsc1* (D2.B6-*Empsc1.1*) in D2 genetic background. Shaded, open, and black columns indicate B6-derived, D2-derived, and unknown chromosomal portions, respectively.

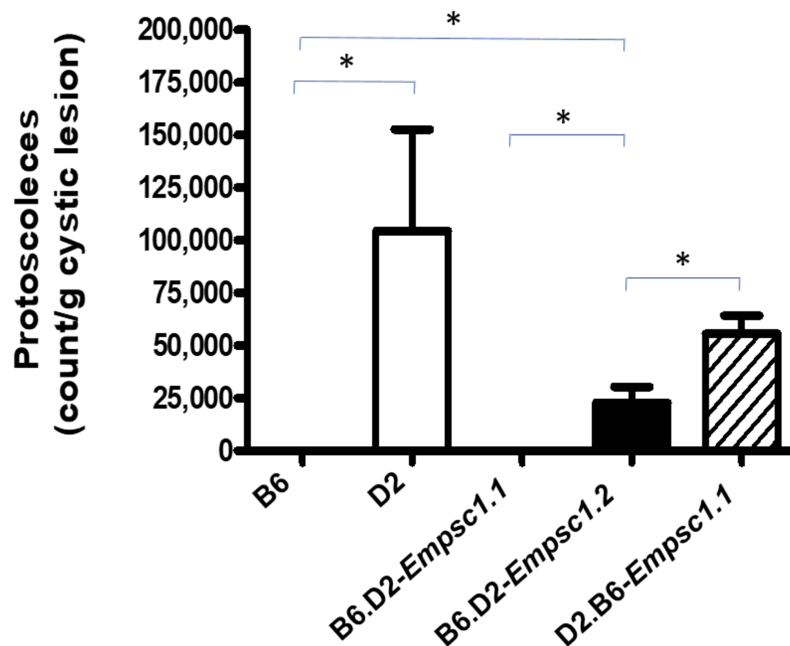

Fig. 5. Protoscolex development in the liver of B6, D2, and subcongenic mice for *Empsc1*. The number of protoscolex in cystic lesions of liver was counted and expressed as number of protoscolex per 1 gram of cystic lesion. Data represent the mean ± SEM for B6 (n=3), D2 (n=3) and subcongenic mice for *Empsc1*, B6.D2-*Empsc1.1* (n=3), B6.D2-*Empsc1.2* (n=3), and D2.B6-*Empsc1.1* (n=5). * indicates $P < 0.05$.

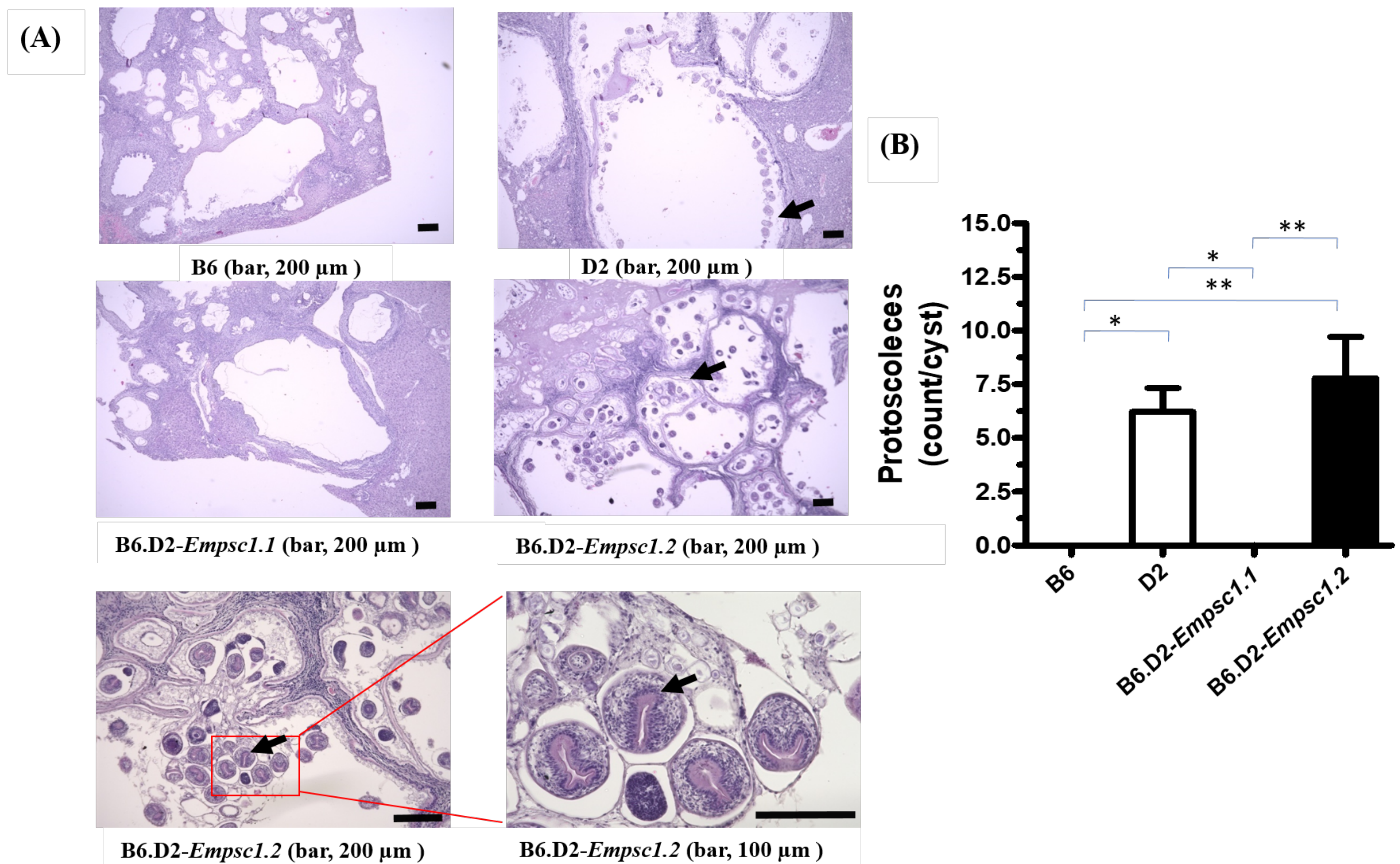

Fig. 6. (A) Photographs of liver sections in B6, D2, and sub-congenic mice for *Empsc1*. Arrows indicate protoscolecces in the cyst. (B) Protoscolex counts in liver sections in B6 (n=6), D2 (n=6), B6.D2-*Empsc1.1* (n=6), and B6.D2-*Empsc1.2* (n=8). Data represent mean ± SEM. ** and * indicate P <0.01 and P <0.05, respectively.

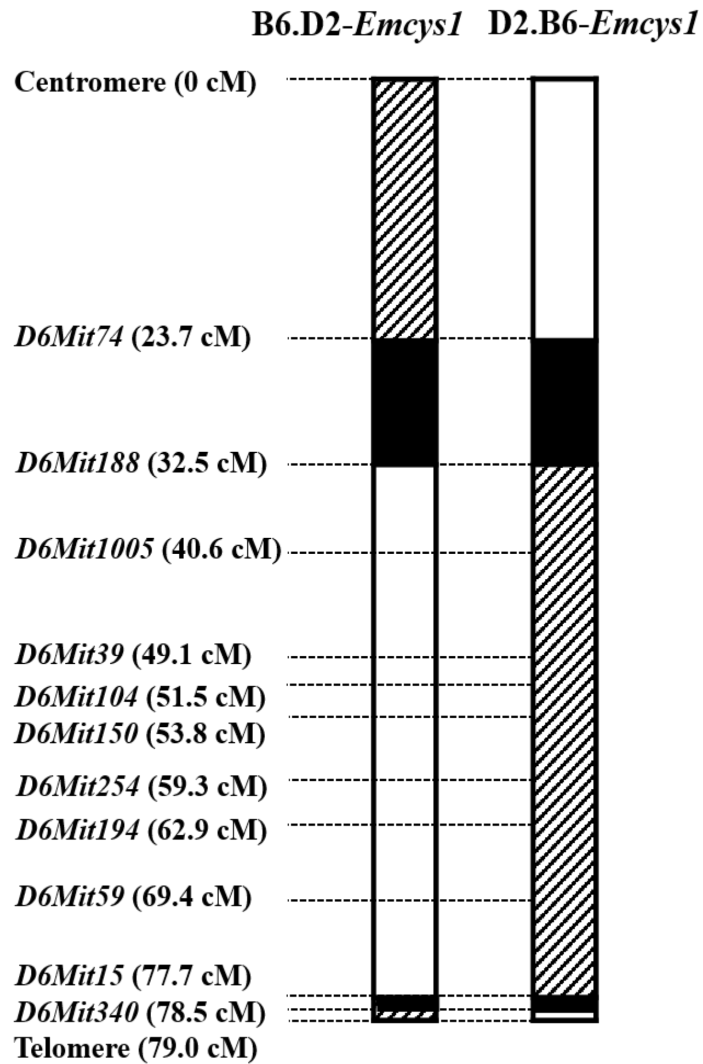

Fig. 7. Schematic diagram of Chr 6 in congenic mice for *Emcys1*; B6.D2- *Emcys1* and D2.B6-*Emcys1*. Shaded, open, and black columns indicate B6-derived, D2-derived, and unknown chromosomal portions, respectively.

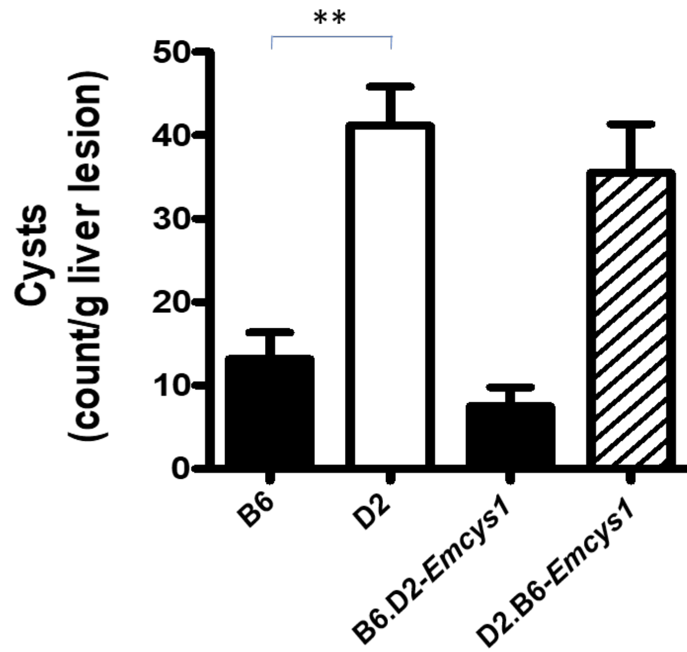

Fig. 8. Cyst establishment in liver of B6 (n=6), D2 (n=6), and congenic mice for *Emcys1*; B6.D2- *Emcys1* (n=5) and D2.B6- *Emcys1* (n=6). The data represent mean ± SEM. Data are representative of two independent experiments. ** indicates $P < 0.01$.

4. Discussion

A previous investigation showed that susceptibility or resistance to *E. multilocularis* infection was genetically controlled and D2 mice were more susceptible to infection than B6 mice (Matsumoto et al., 2010). Furthermore, another study identified a significant QTL, *Emcys1* in Chr 6 and a highly significant QTL, *Empsc1* in Chr 1 as responsible for cyst establishment and protoscolex development, respectively (Nakao et al., 2011).

In this study, congenic lines, B6.D2-*Empsc1* and D2.B6-*Empsc1*, were established from B6 and D2 parental strains and it was confirmed that the *Empsc1* is responsible for the difference in the protoscolex development. As 834 protein-coding genes are present in the introgressed chromosomal region between *D1Mit7* and *D1Mit511*, narrowing down the introgressed region could lead to the identification of candidate genes responsible for protoscolex development. Therefore, subcongenic lines, B6.D2-*Empsc1.1* and B6.D2-*Empsc1.2*, were generated from B6.D2-*Empsc1* and D2.B6-*Empsc1.1* line was generated from D2.B6-*Empsc1* (Fig. 4). These subcongenic mice were challenged with *E. multilocularis* and protoscolex development was observed in B6.D2-*Empsc1.2* mice, but not in B6.D2-*Empsc1.1*. On the other hand, D2.B6-*Empsc1.1* mice showed protoscolex development as seen in D2 mice. From these results, a critical region narrowed down between *D1Mit290* and *D1Mit511*, in which a gene responsible for protoscolex development must be located. Based on MGI database, there are still 331 protein-coding genes locating in the critical region, among which several candidate genes such as those relating to the immune response or inflammation are listed in Table 2. It has been

explained that the cell mediated immune responses particularly acute inflammatory Th1 response may play an important role in early stage of *E. multilocularis* infection (Vuitton et al., 2010). However, at this point it is difficult to address responsible gene(s) until functional verification of gene(s) that control the protoscolex development is carried out. Yang et al. (2006) reported that host genetic factors could contribute to the susceptibility of humans to *E. multilocularis*. Moreover, some genes showed quantitative genetic variation in mice as well as in humans (Hellebrandtet et al., 2005; Korstanje et al. 2004). Many researchers have proposed that the interplay between parasites and hosts has been developed by an evolutionarily conserved signaling system, specifically the interaction between parasitic receptors and host-derived molecules (Brehm, 2010; Gelmedin et al., 2008). The obtained results from this study could facilitate the identification of candidate gene(s) involving this signaling pathway for protoscolex development in both mouse and human, leading to the implementation of the most effective control strategy for AE. Other researchers have successfully addressed candidate genes responsible for some genetic phenotypes with the linkage analysis using congenic and sub-congenic lines (Kanagaratham et al., 2014; Pelletier et al., 2016) and gene expression profiling in subcongenic mouse lines (Ahn et al., 2010; Rennie et al., 2008; Sander et al., 2007; Stark et al., 2010). Therefore, generation of backcrosses from B6.D2-*Empsc1.2* have been started to identify responsible gene(s) with the genetic linkage analysis.

In the case of cyst establishment phenotype, two congenic lines, B6.D2-*Emcys1* and D2.B6-*Emcys1*, were generated from parental strains B6 and D2, respectively (Fig. 7). The results

from the current study could not confirm that the genetic region between *D6Mit188* and *D6Mit15* was responsible for the cyst establishment in mouse liver (Fig. 8). This result suggests that other genetic factors may be involved in the cyst establishment of *E. multilocularis* in the mouse liver.

In conclusion, a QTL *Empsc1* for protoscolex development of *E. multilocularis* was confirmed by the generation of congenic lines. Moreover, the critical genetic region was narrowed down to the length of 34.2 Mb between *D1Mit290* and *D1Mit511* and secured candidate gene(s) in the introgressed region of B6.D2-*Empsc1.2*. These findings will lead to the identification of the candidate gene(s) for protoscolex development of *E. multilocularis*. Identification of candidate gene(s) will enrich the scientific knowledge of this zoonotic disease echinococcosis and would lead to the development of new and effective drugs against echinococcosis.

5. Summary

The resistance/susceptibility to *Echinococcus multilocularis* infection in mice are genetically controlled. However, genetic factors responsible for these differences remain unknown. Previous study in genetic linkage analysis has revealed that there is a significant quantitative trait locus (QTL) for the establishment of cyst (*Emcys1*), and a highly significant QTL for development of protoscolex of *E. multilocularis* larvae (*Empsc1*), on mouse chromosomes 6 and 1, respectively. The current study aimed to confirm these QTLs and narrow down the critical genetic region that controls resistance/susceptibility to *E. multilocularis* infection by establishing congenic and subcongenic lines from C57BL/6 (B6) and DBA/2 (D2) mice. For protoscolex development phenotype, two congenic lines, B6.D2-*Empsc1* and D2.B6-*Empsc1* were developed, where responsible QTL, *Empsc1* was introgressed from D2 into B6 background and *vice versa*. For cyst establishment phenotype, two congenic lines, B6.D2-*Emcys1* and D2.B6-*Emcys1* were developed, where responsible QTL, *Emcys1* was introgressed from D2 into B6 background and *vice versa.* Because there was no significant difference in cyst establishment between B6.D2-*Emcys1* and D2.B6-*Emcys1* mice after challenge with *E. multilocularis*, it is suggested that the *Emcys1* does not solely control the cyst establishment in mouse liver. However, infection experiment with B6.D2-*Empsc1* and D2.B6-*Empsc1* mice showed a significant difference in protoscolex development in the cyst. It confirms that the *Empsc1* controls phenotype of the protoscolex development in the cyst. Subsequently, two subcongenic lines, B6.D2-*Empsc1.1* and B6.D2-*Empsc1.2*, from B6.D2-

Empsc1 and one subcongenic line, D2.B6-*Empsc1.1* from D2.B6-*Empsc1* were developed to narrow down the critical region responsible for protoscolex development. From the results of infection experiments with *E. multilocularis* in these subcongenic mice, it is concluded that a gene responsible for protoscolex development is located between *D1Mit290* (68.1 cM) and *D1Mit511* (97.3 cM).

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