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**Identification of genetic loci affecting the establishment and development of *Echinococcus multilocularis* larvae in mice**

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## ABSTRACT

Alveolar echinococcosis (AE) is a severe hepatic disorder caused by larval infection by the fox tapeworm *Echinococcus multilocularis*. The course of parasitic development and host reactions are known to vary significantly among host species, and even among different inbred strains of mice. As reported previously, after oral administration of parasite eggs, DBA/2 (D2) mice showed a higher rate of cyst establishment and more advanced protoscolex development in the liver than C57BL/6 (B6) mice. These findings strongly suggest that the outcome of AE is affected by host genetic factor(s). In the present study, the genetic basis of such strain-specific differences in susceptibility/resistance to AE in murine models was studied by whole-genome scanning for quantitative trait loci (QTLs) using a backcross of (B6 × D2)F<sub>1</sub> and D2 mice with varying susceptibility to *E. multilocularis* infection. For cyst establishment, genome linkage analysis identified one suggestive and one significant QTL on chromosomes (Chrs.) 9 and 6, respectively, whereas for protoscolex development, two suggestive and one highly significant QTLs were detected on Chrs. 6, 17 and 1, respectively. Our QTL analyses using murine AE models revealed that multiple genetic factors regulated host susceptibility/resistance to *E. multilocularis* infection. Moreover, our findings show that establishment of the parasite cysts in the liver is affected by QTLs that are distinct from those associated with the subsequent protoscolex development of the parasite, indicating that different host factors are involved in the

host–parasite interplay at each developmental stage of the larval parasite. Further identification of responsible genes located on the identified QTLs could lead to the development of effective disease prevention and control strategies, including an intensive screening and clinical follow-up of genetically high-risk groups for AE infection.

*Keywords: Echinococcus multilocularis, Alveolar echinococcosis, Zoonosis, QTL, Cyst, Protoscolex, Emcys1, Empsc1*

## 1. Introduction

Alveolar echinococcosis (AE) is a severe hepatic disorder caused by the metacestode stage of the tapeworm *Echinococcus multilocularis*. This zoonotic disease is endemic in many regions of the northern hemisphere and is one of the most significant life-threatening helminth diseases (Craig et al., 1996). The intermediate hosts, mainly rodents and occasionally humans, become infected by oral uptake of the eggs excreted in the feces of final host carnivores. The larval oncospheres hatch from eggs in the intestine, most probably penetrate the intestinal wall and disseminate primarily into the liver. The larvae establish in the liver and develop to harbor mature protoscoleces.

Many researchers have used inbred mice as experimental models of AE to study the host–parasite interplay. However, the course of parasitic development and host reactions are known to vary significantly among mouse strains (Yamashita et al., 1958; Webster and Cameron, 1961; Gottstein and Felleisen, 1995; Bauder et al., 1999; Hildreth and Granholm, 2003; Matsumoto et al., 2010). Previous study showed that the two inbred strains, DBA/2 (D2) and C57BL/6 (B6), differ markedly in their susceptibility to *E. multilocularis* infection (Matsumoto et al., 2010). When infective eggs were administered orally, D2 mice had a higher number of cysts established in the liver than did B6 mice, and moreover, mature protoscoleces were observed only in D2

mice at 16 weeks p.i.. Although this observation supported the belief that the susceptibility to *E. multilocularis* infection is genetically controlled, as has been previously suggested for animals and humans (Nakaya et al., 1997; Hildreth and Granholm, 2003; Vuitton, 2003), genetic factors responsible for the differences in susceptibility remain unknown.

Quantitative trait locus (QTL) mapping is a promising tool for the detection of genetic loci that contribute to differences in phenotypic variation. In this study, the traits and genotypes of offspring derived from two different strains of animals were investigated on a genome-wide scale to determine statistical linkages and to identify loci which contained genes affecting the traits. To date, the genetic background of susceptibility to nematode infection has been investigated using this approach in animals (Iraqi et al., 2003; Suzuki et al., 2006; Beraldi et al., 2007) as well as in humans (Williams-Blangero et al., 2008).

In this study, QTL analysis was conducted on offspring derived from D2 and B6 mice with varying susceptibility to *E. multilocularis* infection. Genetic linkage analysis identified one significant and one highly significant QTL for the establishment and development of *E. multilocularis* larvae in mice. Furthermore, our data show that establishment and development of parasites are controlled by distinct and multiple genetic factors.

## 2. Materials and methods

### 2.1. Mice

D2, B6 and (B6 × D2)F<sub>1</sub> (F<sub>1</sub>) mice were purchased from Japan SLC (Shizuoka, Japan). F<sub>1</sub> × D2 backcross (N<sub>2</sub>) mice were generated to conduct the genetic and phenotypic assessment experiments. All animal experiments were performed in accordance with the guidelines of Hokkaido Institute of Public Health, Japan and were approved by the ethics committee of the Institute.

### 2.2. Experimental infection

*Echinococcus multilocularis* eggs were obtained from the feces of two beagle dogs that had been orally inoculated with mature metacestode tissue containing protoscoleces (Hokkaido isolate). The eggs were then washed several times with physiological saline and kept at 4°C until use for experimental infections in mice. The experimental infections were performed in three independent and identical trials using these infective eggs, prepared 1–3 months prior to each experimental infection. The anesthetized mice, aged 4–5 weeks, were administered with either

200 or 2,000 eggs suspended in 0.5 ml of physiological saline orally. The animals were handled within a safety facility (Biosafety Level 3) at the Hokkaido Institute of Public Health, Japan.

### *2.3. Examination of cyst establishment and protoscolex development*

A total of 148 mice (D2 ( $n = 10$ ); B6 ( $n = 10$ ); F<sub>1</sub> ( $n = 10$ ); N<sub>2</sub> ( $n = 118$ )) infected with 200 eggs were sacrificed and necropsies were performed at 4 weeks p.i. for the assessment of cyst establishment. The livers were cut into approximately 0.5 mm-thick slices and the total numbers of cysts were counted. A total of 283 mice (D2 ( $n = 11$ ); B6 ( $n = 10$ ); F<sub>1</sub> ( $n = 12$ ); N<sub>2</sub> ( $n = 250$ )) infected with 2,000 eggs were sacrificed and necropsies were performed at 16 weeks p.i. for the assessment of protoscolex development. The livers were collected and the total weight of the organs and that of host liver tissue and parasitic cysts were measured. Several parts of the cysts (total weight 1–2 g) were minced, passed through a 125  $\mu\text{m}$  sieve, and then washed repeatedly with physiological saline. The numbers of mature protoscoleces were counted under a stereoscopic microscope as an index of the parasite development. The number of eggs used for the first experiment ( $n = 200$ ) was expected to produce a macroscopically countable number of cystic lesions in the host liver, while the number used in the second experiment ( $n = 2,000$ ) was expected to reduce the effect of the difference in the numbers of cysts established in individual mice.



#### *2.4. Genotype analysis*

To identify and map QTLs, a total of 121 informative microsatellite markers—113 for cyst establishment and 117 for protoscolex development—were chosen from a database maintained by Mouse Genome Informatics (MGI), The Jackson Laboratory, USA (<http://www.informatics.jax.org/>), as listed in Table 1. After the initial QTL scan, extra markers were included in the vicinity of the provisional QTLs to refine their position. A total of 232 N<sub>2</sub> mice, 118 for cyst establishment and 114 for protoscolex development, were genotyped using each microsatellite marker. After genomic DNA extraction and PCR amplification, amplified PCR products were electrophoresed in 10% polyacrylamide or 3% agarose gels, stained with ethidium bromide and visualized under UV light. The genotype at each marker locus was determined by the size of the PCR product.

#### *2.5. Data analysis*

Two phenotypes—cyst establishment and protoscolex development in mice—were analyzed separately and the following phenotypic parameters were used for the analyses. For the analysis of cyst establishment, the number of cysts in the liver was converted to a percent value against a mean number of cysts in susceptible D2 mice to avoid the bias resulting from egg infectivity between two independent experimental infections. For the analysis of protoscolex

development, the number of mature protoscoleces in the liver was indicated in terms of number/gram of cystic lesion, which was subsequently log-transformed for QTL analysis. Zero values were assigned a value of 1 to allow log transformation. Linkage analyses of two phenotypes to chromosomal loci were performed using the MapManager QTXb20 software (Manly et al., 2001). Recombination frequencies (%) were converted into genetic distances (centiMorgan; cM) using the Kosambi map function, in which linkage data are provided as likelihood ratio statistic (LRS) scores. Genome-wide significance thresholds were calculated in terms of LRS by carrying out permutation tests for 1,000 permutations based on the established guidelines (Lander and Kruglyak, 1995). The thresholds in the backcrossed progenies were determined by the QTL software program for finding suggestive, significant and highly significant linkages. Two-way interactions (epistasis) were estimated with a QTL scan and statistical significance for these gene to gene interaction tests were based on  $P < 10^{-5}$  using 1,000 permutations of the observed data. Statistical analysis was performed using the Stat-View program (SAS Institute, Cary, NC, USA).  $P < 0.05$  was considered to be significant. A Scheffé's F test was conducted for multiple comparisons between the mean values of genotypes at the detected QTLs.

### 3. Results

#### 3.1. Establishment of *E. multilocularis* cysts

At 4 weeks after oral administration of 200 eggs, the total numbers of cysts in the liver were counted. In two independent experiments, the average numbers of cysts in D2 mice were different (average  $\pm$  S.D.):  $79.0 \pm 30.4$  and  $60.6 \pm 13.5$  for the first and second experiments, respectively. Therefore, the numbers of cysts observed in B6, F<sub>1</sub> and N<sub>2</sub> mice were converted to percent values against a mean number of cysts in susceptible D2 mice in each experiment. The distribution of percent values are shown in Fig. 1A. All animals had cestodes in the livers. The mean values for B6 mice (40.2%) were significantly lower than those of D2 mice ( $P < 0.01$ , Scheffé's F test), while those of N<sub>2</sub> progenies varied from 1.7 to 182.5% with an average of 72.0%.

#### 3.2. Identification of QTLs and their effects on cyst establishment

The genome-wide linkage analysis of cyst establishment was performed using 118 N<sub>2</sub> mice, where one suggestive and one significant QTL were detected on chromosomes (Chrs.) 9 and 6 (*Emcys1*), respectively (Figs. 2A and 3A). The significant QTL had a peak LRS score of 15.0 and accounted for 12% of the total variance (Table 2). No significant epistatic interactions were observed between all of the tested markers (data not shown). Segregation of N<sub>2</sub> mice according to their genotypes at *Emcys1* (*D6Mit150*) showed that individuals homozygous for the

D2 allele (DD) had a statistically higher number of cysts than did the heterozygous mice (DB) ( $P < 0.01$ , Scheffé's F test), and that there were no significant differences between D2 and DD, and between B6, F<sub>1</sub> and DB (Fig. 4A). A list of *Emcys1* candidates on Chr. 6 is shown in Table 3.

### 3.3. Development of *E. multilocularis* protoscoleces

At 16 weeks after oral administration of 2,000 eggs, the total numbers of mature protoscoleces in the liver were calculated. The distributions of numbers of mature protoscoleces per gram of cystic tissue are shown in Fig. 1B. Although all animals had *Echinococcus* cysts in their enlarged livers, the number of mature protoscoleces differed greatly among individual mice even within the same mouse group. In D2 mice,  $1.5 \times 10^4$ – $8.5 \times 10^5$  protoscoleces were recovered per gram of cystic lesion, whereas in all B6 mice and some N<sub>2</sub> progenies, protoscolex formation was completely absent (Fig. 1B).

### 3.4. Identification of QTLs and their effects on protoscolex development

The genome-wide linkage analysis of protoscolex development using 114 N<sub>2</sub> mice identified two suggestive and one highly significant QTLs on Chrs. 6, 17 and 1 (*Empsc1*), respectively (Figs. 2B and 3B). The highly significant QTL had a peak LRS score of 75.4 and accounted for 48% of total variants (Table 2). The two suggestive QTLs with LRS scores of 7.1 and 10.3 explained only 6 and 9% of the variants, respectively. Although weak epistatic interaction was observed between *Empsc1* and suggestive QTLs, the scores did not reach the

significant level (data not shown). N<sub>2</sub> carrying D2 mice homozygous at *Empsc1* (*D1Mit14*) had statistically higher numbers of mature protoscoleces than did DB heterozygous mice ( $P < 0.001$ , Scheffé 's F test), and there were no significant differences between D2 and DD and between F<sub>1</sub> and DB (Fig. 4B). A list of *Empsc1* candidates on Chr. 1 is shown in Table 3.

#### 4. Discussion

As shown in a previous investigation, susceptibility/resistance to *E. multilocularis* infection was different among mouse strains including D2 and B6 mice (Matsumoto et al., 2010). D2 mice were more susceptible to infection than were B6 mice. D2 mice showed a higher number of cysts established in the liver after administration of infective eggs than those of B6, and at 16 weeks p.i., maturation of protoscoleces occurred only in D2, but not in B6 mice. The objective of this study was to perform a genome-wide linkage analysis of a cross between D2 and B6 mice to localize genes associated with the increased susceptibility of D2 mice against *E. multilocularis* infection.

We found significant linkage between cyst establishment and genotypes at *Emcys1* on Chr. 6 with a peak LRS score of 15.0, accounting for 12% of the variation (Table 2). The segregation analysis supported a strong influence of *Emcys1* on cyst establishment (Fig. 4A). However, the continuous distribution of N<sub>2</sub> phenotypes implies that multiple genes control

susceptibility to *E. multilocularis* infection. Although statistical modeling of the current data did not detect any QTL interacting with *Emcys1*, the suggestive QTL on Chr. 9, and even others slightly below the suggestive level (LRS = 6.7) such as those on Chrs. 3, 13 and 17 (Fig. 2A), may have an influence on susceptibility. At this time, it is difficult to identify a gene responsible for cyst establishment on *Emcys1*, a region between *D6Mit188* and *D6Mit15*, as there are nearly 1,000 genes located within this chromosomal region. Even with the aid of the web-based program Positional Medline ([PosMed; http://omicspace.riken.jp/PosMed](http://omicspace.riken.jp/PosMed)) (Yoshida et al., 2009), approximately 200 immune-related genes that may be involved in host–parasite interplay, were identified, some of which are listed in Table 3.

The safety requirements for handling infective *E. multilocularis* eggs hinder the research on cyst establishment after oral infection with eggs and thus only limited information is available for further discussion on candidate genes. Gottstein et al. (2010) used microarray analysis to compare the expression levels of hepatic genes in mice between pre- and 31-days p.i. of eggs. The study identified only 38 genes whose expression levels were significantly changed. None of those were located on *Emcys1*, implying that the host factors, apart from those expressed in the liver, may have more influence on the cyst establishment and that the earlier stages of infection such as hatching, activation, penetration, migration and predilection of oncospheres might be determinants of susceptibility to the infection. As recently reviewed by Vuitton and Gottstein

(2010), the cell-mediated immune responses, especially acute inflammatory Th1 response, are known to play an important role in the early stage of *E. multilocularis* infection. Furthermore, early inflammation induced by complement activation was shown to be important in controlling the establishment of the metacestode of *Echinococcus granulosus*, a closely related parasite (Breijo et al., 2008). The *Emcys1* locus includes genes related with the Th1 response, such as *Cd4*, *Lag3* and *Cd69*, and those associated with complement components, such as *C1s*, *C1r*, *C1rl* and *C3ar1* (Table 3).

In the Chinese village of Nanwan, a highly endemic area for AE, only certain family members were likely to be more susceptible to AE than others, despite sharing similar life patterns (Yang et al., 2006), implying that host genetic factors contribute to the susceptibility to *E. multilocularis* infection in humans. Some genes showing quantitative genetic variation in mice have also been revealed to affect the phenotypic variance in humans (Korstanje et al., 2004; Hillebrandt et al., 2005; Peters et al., 2007). Therefore, the data obtained in this study could provide an experimental basis for further identification of human genetic factors that are associated with the response after ingestion of *E. multilocularis* eggs. Such information is expected to facilitate the development of effective disease prevention and control programs, for example, an intensive screening of genetically high-risk groups for AE infection.

The identification of genetic factors affecting the protoscolex development of *E. multilocularis* will lead to a better understanding of host and parasite interplay in the intermediate hosts. In our analysis, highly significant linkage was observed between protoscolex development and genotypes at *Empsc1* on Chr. 1 (a peak LRS score of 75.4) (Fig. 3B). A significant influence of *Empsc1* on variants was demonstrated by a high value of contribution (48%) (Table 2) as well as significant differences between DD homozygous and DB heterozygous in segregation analysis (Fig. 4B), whereas the detection of other suggestive QTLs suggests that the protoscolex development is under multigenic control, as in the case of cyst establishment.

The physical barrier between growing parasites and host tissue is the laminated layer, an acellular and carbohydrate-rich outer membrane of cysts, which protect parasites from direct contact with host immune cells. Meanwhile, several host-derived molecules were previously reported to exist in the cyst fluid or on the wall (Kassis and Tanner, 1977; Ali-Khan and Siboo, 1981), indicating that certain host-derived factors are transported through the laminated layer and are involved in parasite development in the hosts. In recent years, great progress has been made in elucidating the mechanisms of parasite development in the hosts by identifying the evolutionarily conserved signaling systems in *E. multilocularis*, which can interact with host-derived molecules (Konrad et al., 2003; Spiliotis et al., 2003, 2005, 2006; Zavala-Góngora et al.,



2003, 2006, 2008; Gelmedin et al., 2008, 2010; Brehm, 2010; Förster et al., 2011). Specifically, interactions between parasite receptors and host-derived molecules, such as epidermal growth factor and insulin have been demonstrated experimentally (Spiliotis et al., 2006; Gelmedin et al., 2008; Brehm, 2010), strongly suggesting that parasite development is triggered by host signaling systems. Of 450 genes located on the *Empsc1* locus, more than 100 were previously associated with various kinds of signaling systems in mammals, as exemplified in Table 3. Thus, a better understanding of evolutionarily conserved *E. multilocularis* signaling systems may lead to further identification of host factors important for parasite development from the existing list of candidates.

In humans, major histocompatibility complex (MHC) genotypes were associated with the clinical severity of AE (Eiermann et al., 1998; Godot et al., 2000; Zhang et al., 2003). In mice, the genes coding for MHC molecules are located on Chr. 17 and thus are not included in the *Empsc1* locus determined in this study. In our experimental infections of mice, there was no statistically significant correlation between the number of mature protoscoleces and whole liver weight at 16 weeks p.i. measured as an index of metacystode growth in size (data not shown). These findings suggest that the metacystode growth of *E. multilocularis* is regulated, at least in part, by host factors different from those affecting protoscolex development. As generally recognized, mature protoscoleces are critical for the parasite to maintain its lifecycle, whereas

the growth of the metacestode mass is intimately associated with the clinical outcome of AE. Hence, further investigations to identify QTLs responsible for metacestode growth in murine models may experimentally demonstrate the association between MHC genotypes and disease severity that was suggested in AE cases.

The database available at the MGI website ([http://www.informatics.jax.org/strains\\_SNPs.shtml](http://www.informatics.jax.org/strains_SNPs.shtml)) provides information on the strain-specific genomic features of mice. In the present investigation, however, only limited numbers of genomic differences were found between D2 and B6 strains on the identified QTLs (nine and 18 for *Emcys1* and *Empsc1*, respectively), all of which were located outside the coding region (data not shown). Since the database will be extended regularly, it might become useful to further narrow down the candidate genes in the future.

Using congenic techniques, we are currently attempting to generate mice strains that harbor QTLs from one selection line on the opposite line to investigate whether each allele has a different effect on the phenotype. At the same time, several approaches are currently being employed to identify candidate genes located on *Emcys1* and *Empsc1*. Some of these approaches include comparisons of gene expression levels of D2 and B6 mice in response to *E. multilocularis* infection using microarray and next-generation RNA sequencing technologies. This analytical combination that includes QTL mapping and gene expression profiles has proven

useful in the selection of candidate genes (Wayne and McIntyre, 2002; Rennie et al., 2008; Ahn et al., 2010; Stark et al., 2010).

A lack of existing comprehensive information on the interplay between parasite and intermediate host makes it difficult to focus on certain genes responsible for resistance/susceptibility to *E. multilocularis* infection. However, our study provides several important conclusions. First, using QTL analysis, we were able to localize chromosomal sites where the allelic differences in genes present in D2 and B6 mice strongly affect mouse susceptibility to *E. multilocularis* infection. Second, multiple QTLs were detected on different Chrs., indicating that susceptibility to infection is complex and is determined by multiple host genes. Additionally, larval establishment and development in mouse livers are controlled by distinct QTLs, indicating that there are different host factors interplaying with parasites at each developmental stage. Further identification of responsible genes located on the identified QTLs could lead to the development of effective disease prevention and control strategies, for example, an intensive screening and clinical follow-up of genetically high-risk groups for AE infection.

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**Table 1. List of microsatellite markers used for whole genome scanning in this study.**

Markers	cM	CYS	PSC	Markers	cM	CYS	PSC	Markers	cM	CYS	PSC
<i>D1Mit118</i>	1.65	+	+	<i>D6Mit159</i>	12.36	+	+	<i>D13Mit17</i>	7.73	+	+
<i>D1Mit324</i>	29.13	+	+	<i>D6Mit74</i>	23.70	+	+	<i>D13Mit60</i>	14.44	+	+
<i>D1Mit415</i>	43.94	+	+	<i>D6Mit188</i>	32.53	+	+	<i>D13Mit63</i>	21.00	+	+
<i>D1Mit191</i>	52.66	+	+	<i>D6Mit104</i>	51.53	+		<i>D13Mit9</i>	42.19	+	+
<i>D1Mit30</i>	57.91	+	+	<i>D6Mit150</i>	53.75	+	+	<i>D13Mit148</i>	59.69	+	+
<i>D1Mit445</i>	58.27		+	<i>D6Mit254</i>	59.32	+		<i>D13Mit262</i>	63.93	+	+
<i>D1Mit14</i>	67.71	+	+	<i>D6Mit374</i>	64.60	+					
<i>D1Mit145</i>	74.68		+	<i>D6Mit59</i>	69.36	+		<i>D14Mit10</i>	6.41	+	+
<i>D1Mit355</i>	80.36		+	<i>D6Mit15</i>	77.70	+	+	<i>D14Mit120</i>	20.88	+	+
<i>D1Mit150</i>	81.08		+					<i>D14Mit102</i>	34.36	+	+
<i>D1Mit291</i>	88.97	+	+	<i>D7Mit114</i>	15.42	+	+	<i>D14Mit225</i>	39.46	+	+
<i>D1Mit511</i>	93.70		+	<i>D7Mit82</i>	32.76	+	+	<i>D14Mit165</i>	56.16	+	+
				<i>D7Mit318</i>	42.27	+	+	<i>D14Mit266</i>	64.86	+	+
<i>D2Mit293</i>	17.24	+	+	<i>D7Mit66</i>	64.30	+	+				
<i>D2Mit296</i>	21.81	+	+	<i>D7Mit333</i>	82.25	+	+	<i>D15Mit12</i>	1.80	+	+
<i>D2Mit91</i>	39.24	+	+					<i>D15Mit5</i>	16.74	+	+
<i>D2Mit185</i>	55.23	+	+	<i>D8Mit4</i>	18.89	+	+	<i>D15Mit156</i>	32.19	+	+
<i>D2Mit62</i>	59.34	+	+	<i>D8Mit100</i>	29.70	+	+	<i>D15Mit159</i>	41.96	+	+
<i>D2Mit286</i>	76.74	+	+	<i>D8Mit234</i>	39.33	+	+	<i>D15Mit161</i>	52.78	+	+
<i>D2Mit229</i>	88.99	+	+	<i>D8Mit242</i>	50.07	+	+				
<i>D2Mit200</i>	102.29	+	+	<i>D8Mit200</i>	61.37	+	+	<i>D16Mit182</i>	2.57	+	+
								<i>D16Mit59</i>	26.86	+	+
<i>D3Mit164</i>	2.01	+	+	<i>D9Mit90</i>	17.80	+	+	<i>D16Mit140</i>	40.30	+	+
<i>D3Mit182</i>	21.73	+	+	<i>D9Mit91</i>	20.74	+	+	<i>D16Mit152</i>	48.23	+	+
<i>D3Mit28</i>	39.27	+	+	<i>D9Mit302</i>	36.36	+	+	<i>D16Mit106</i>	57.68	+	+
<i>D3Mit14</i>	61.32	+	+	<i>D9Mit133</i>	45.80	+	+				
<i>D3Mit129</i>	80.49	+	+	<i>D9Mit355</i>	51.41	+	+	<i>D17Mit113</i>	8.14	+	+
				<i>D9Mit18</i>	71.49	+	+	<i>D17Mit198</i>	14.59		+
<i>D4Mit235</i>	3.57	+	+					<i>D17Mit139</i>	27.40		+
<i>D4Mit237a</i>	22.38	+	+	<i>D10Mit248</i>	5.21	+	+	<i>D17Mit89</i>	32.82	+	+
<i>D4Mit139</i>	29.65	+	+	<i>D10Mit61</i>	34.80	+	+	<i>D17Mit218</i>	43.76	+	+
<i>D4Mit152</i>	39.46	+	+	<i>D10Mit186</i>	38.56	+	+	<i>D17Mit187</i>	50.17	+	+
<i>D4Mit303</i>	45.55	+	+	<i>D10Mit14</i>	66.75	+	+	<i>D17Mit221</i>	59.77	+	+
<i>D4Mit308</i>	57.66	+	+	<i>D10Mit297</i>	72.31	+	+				
<i>D4Mit54</i>	70.02	+	+					<i>D18Mit132</i>	11.92	+	+
<i>D4Mit42</i>	82.64	+	+	<i>D11Mit226</i>	5.64	+	+	<i>D18Mit17</i>	21.09	+	+
				<i>D11Mit21</i>	25.94	+	+	<i>D18Mit124</i>	32.15	+	+
<i>D5Mit180</i>	11.93	+	+	<i>D11Mit140</i>	32.13	+	+	<i>D18Mit184</i>	39.70	+	+
<i>D5Mit108</i>	23.91	+	+	<i>D11Mit4</i>	41.87	+	+	<i>D18Mit7</i>	51.92	+	+
<i>D5Mit258</i>	33.95	+	+	<i>D11Mit212</i>	54.34	+	+				
<i>D5Mit208</i>	48.51	+	+	<i>D11Mit199</i>	65.48	+	+	<i>D19Mit69</i>	8.93	+	+
<i>D5Mit188</i>	57.51	+	+	<i>D11Mit48</i>	82.96	+	+	<i>D19Mit80</i>	18.24	+	+
<i>D5Mit370</i>	65.23	+	+					<i>D19Mit33</i>	51.76	+	+
<i>D5Mit222</i>	81.53	+	+	<i>D12Mit219</i>	9.69	+	+				
				<i>D12Mit172</i>	21.09	+	+	<i>DXMit166</i>	28.26	+	+
				<i>D12Mit5</i>	37.16	+	+	<i>DXMit130</i>	55.45	+	+
				<i>D12Mit101</i>	51.55	+	+	<i>DXMit186</i>	76.75	+	+

cM, centiMorgan; CYS, markers used for Quantitative trait loci (QTLs) analysis of cyst establishment; PSC, markers used for QTL analysis of protoscolex development.

**Table 2. Quantitative trait loci (QTLs) with high likelihood ratio statistic (LRS) scores (> 4.0) detected by marker regression analysis.**

Chr.	Locus	LRS	Contribution (%)	<i>P</i> value	CI	Additive effect
Cyst establishment						
3	<i>D3Mit164</i>	4.8	4	0.02847	113	-14.07
6	<i>D6Mit104</i>	13.1	10	0.00030	43	-22.72
6	<i>D6Mit150</i>	15.0	12	0.00011	38	-24.32
6	<i>D6Mit254</i>	14.7	12	0.00013	38	-24.05
6	<i>D6Mit374</i>	11.0	9	0.00092	51	-20.95
6	<i>D6Mit59</i>	7.2	6	0.00724	76	-17.14
6	<i>D6Mit15</i>	4.3	4	0.03725	124	-13.37
9	<i>D9Mit91</i>	6.7	5	0.00989	82	16.44
13	<i>D13Mit9</i>	5.7	5	0.01724	96	-15.21
13	<i>D13Mit148</i>	5.3	4	0.02152	103	-14.73
13	<i>D13Mit262</i>	5.2	4	0.02211	103	-14.63
17	<i>D17Mit187</i>	4.5	4	0.03457	121	13.55
17	<i>D17Mit221</i>	4.0	3	0.04627	136	12.78
Protoscolex development						
1	<i>D1Mit415</i>	7.2	6	0.00734	76	-0.75
1	<i>D1Mit191</i>	17.9	15	0.00002	32	-1.15
1	<i>D1Mit30</i>	31.4	24	0.00000	19	-1.48
1	<i>D1Mit445</i>	31.4	24	0.00000	19	-1.48
1	<i>D1Mit14</i>	75.4	48	0.00000	10	-2.12
1	<i>D1Mit145</i>	73.5	48	0.00000	10	-2.09
1	<i>D1Mit355</i>	42.5	31	0.00000	15	-1.70
1	<i>D1Mit150</i>	40.4	30	0.00000	16	-1.68
1	<i>D1Mit291</i>	29.5	23	0.00000	20	-1.46
1	<i>D1Mit511</i>	18.5	15	0.00002	31	-1.18
2	<i>D2Mit296</i>	4.2	4	0.03958	127	-0.58
3	<i>D3Mit182</i>	4.4	4	0.03551	122	0.59
5	<i>D5Mit222</i>	5.4	5	0.02063	101	-0.67
6	<i>D6Mit188</i>	7.1	6	0.00765	77	0.74
14	<i>D14Mit165</i>	6.2	5	0.01274	88	-0.71
17	<i>D17Mit198</i>	4.5	4	0.03307	119	0.60
17	<i>D17Mit139</i>	7.3	6	0.00679	75	0.76
17	<i>D17Mit89</i>	5.6	5	0.01809	97	0.66
17	<i>D17Mit218</i>	9.2	8	0.00247	60	0.85
17	<i>D17Mit187</i>	10.3	9	0.00130	54	0.89
19	<i>D19Mit69</i>	6.0	5	0.01397	90	-0.69
X	<i>DXMit186</i>	5.9	5	0.01557	93	0.70

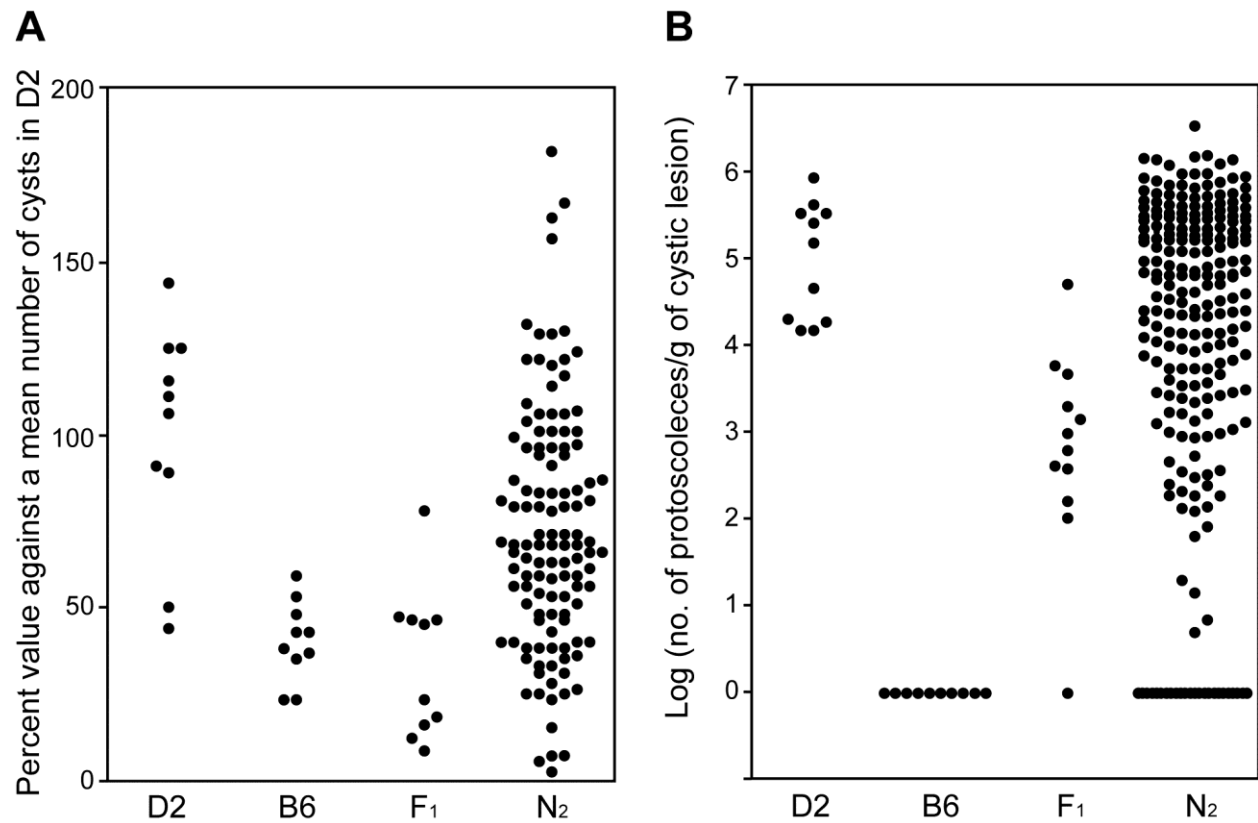
Chr., chromosome; CI, confidence interval.

**Table 3. List of candidate genes for *Emcys1* and *Empsc1* of *Mus musculus*.**

Gene symbol	Gene function	cM
<i>Emcys1</i> candidates on Chr. 6		
<i>D6Mit188</i>	Flanking marker	32.53
<i>Cd207</i>	CD 207 antigen	35.94
<i>Trh</i>	Thyrotropin releasing hormone	41.03
<i>Il5ra</i>	Interleukin 5 receptor, alpha	49.19
<i>Vhllh</i>	Von Hippel-Lindau syndrome homolog	52.81
<i>Ghrl</i>	Ghrelin	52.84
<i>Hrh1</i>	Histamine receptor H 1	53.05
<i>D6Mit150</i>		53.75
<i>8-Mar</i>	Membrane-associated ring finger (C3HC4) 8	53.78
<i>Cxcl12</i>	Chemokine (C-X-C motif) ligand 12	54.81
<i>C3ar1</i>	Complement component 3a receptor 1	57.93
<i>Cd163</i>	CD163 antigen	59.13
<i>C1rl</i>	Complement component 1, r subcomponent-like	59.16
<i>C1r</i>	Complement component 1, r subcomponent	59.16
<i>C1s</i>	Complement component 1, s subcomponent	59.17
<i>Cd4</i>	CD4 antigen	59.17
<i>Lag3</i>	Lymphocyte-activation gene 3	59.17
<i>Tnfrsf1a</i>	Tumor necrosis factor receptor superfamily, member 1a	59.32
<i>D6Mit254</i>		59.32
<i>Fgf23</i>	Fibroblast growth factor 23	61.92
<i>Klrblc</i>	Killer cell lectin-like receptor subfamily B member 1C	63.09
<i>Cd69</i>	CD69 antigen	63.21
<i>Clec7a</i>	C-type lectin domain family 7, member a	63.34
<i>D6Mit15</i>	Flanking marker	77.70
<i>Empsc1</i> candidates on Chr. 1		
<i>D1Mit445</i>	Flanking marker	58.27
<i>Ptprc</i>	Protein tyrosine phosphatase, receptor type, C	60.73
<i>Rgs1, 2, 13</i>	Regulator of G-protein signaling 1, 2, 13	62.56
<i>Rgs18</i>	Regulator of G-protein signaling 18	62.99
<i>Pla2g4a</i>	Phospholipase A2, group IVA (cytosolic, calcium-dependent)	63.51
<i>Ptgs2</i>	Prostaglandin-endoperoxide synthase 2	63.84
<i>Rgs8</i>	Regulator of G-protein signaling 8	65.41
<i>Rgs1, 2</i>	Regulator of G-protein signaling like 1, 2	65.43
<i>Rgs16</i>	Regulator of G-protein signaling 16	65.43
<i>Abl2</i>	V-abl Abelson murine leukemia viral oncogene 2 (arg, Abelson-related gene)	67.71
<i>D1Mit14</i>		67.71
<i>Fasl</i>	Fas ligand (TNF superfamily, member 6)	69.95
<i>Sele</i>	Selectin, endothelial cell	71.35
<i>Sell</i>	Selectin, lymphocyte	71.37
<i>Selp</i>	Selectin, platelet	71.42
<i>Tiprl</i>	TIP41, TOR signalling pathway regulator-like ( <i>S. cerevisiae</i> )	72.55
<i>Rgs4, 5</i>	Regulator of G-protein signaling 4, 5	76.84
<i>Atf6</i>	Activating transcription factor 6	76.96
<i>Fcgr2b</i>	Fc receptor, IgG, low affinity Iib	78.02
<i>Fcgr3</i>	Fc receptor, IgG, low affinity III	78.80
<i>Fcer1g</i>	Fc receptor, IgE, high affinity I, gamma polypeptide	79.23
<i>Slamf1</i>	Signaling lymphocytic activation molecule family member 1	79.54
<i>Cd48</i>	CD48 antigen	79.54
<i>Ncastrin</i>	Nicastrin	79.54
<i>Crp</i>	C-reactive protein, petaxin related	80.13
<i>Fcer1a</i>	Fc receptor, IgE, high affinity I, alpha polypeptide	80.33
<i>D1Mit355</i>	Flanking marker	80.36

cM, centiMorgan; Chr., chromosome.

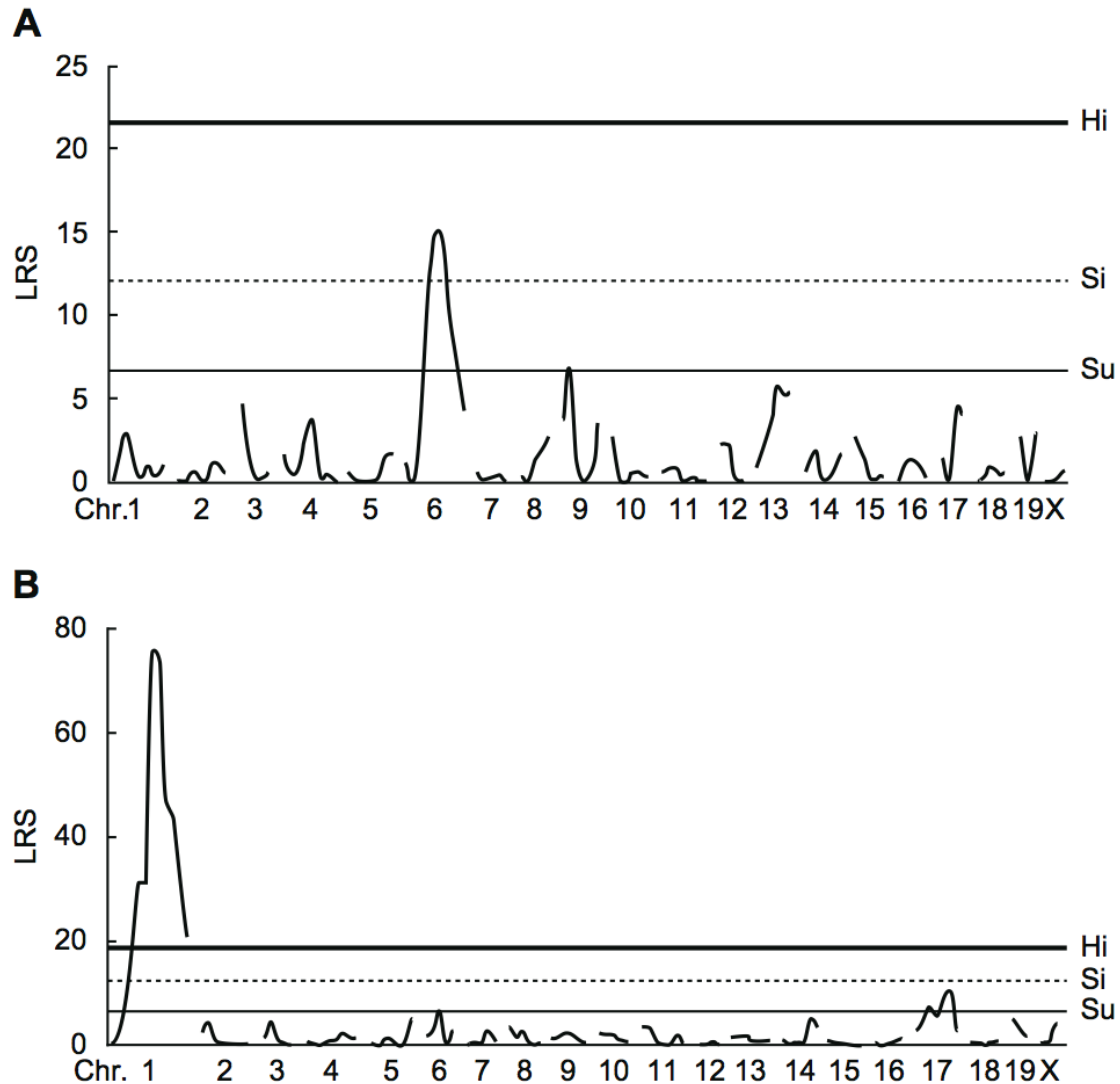
**Fig. 1. Distribution of cyst establishment (A) and protoscolex development (B) of *Echinococcus multilocularis* in the livers of D2, B6, F<sub>1</sub> and N<sub>2</sub> mice.**



(A) The number of cysts in the liver was counted 4 weeks p.i. with 200 eggs for the assessment of cyst establishment. The numbers were converted to percent values against a mean number of cysts in susceptible D2 mice. (B) The number of protoscolexes was counted 16 weeks p.i. with 2,000 eggs for the assessment of protoscolex development. The numbers were indicated as number/gram of cystic lesion and subsequently log-transformed. Zero values were assigned a value of 1 to allow log transformation.

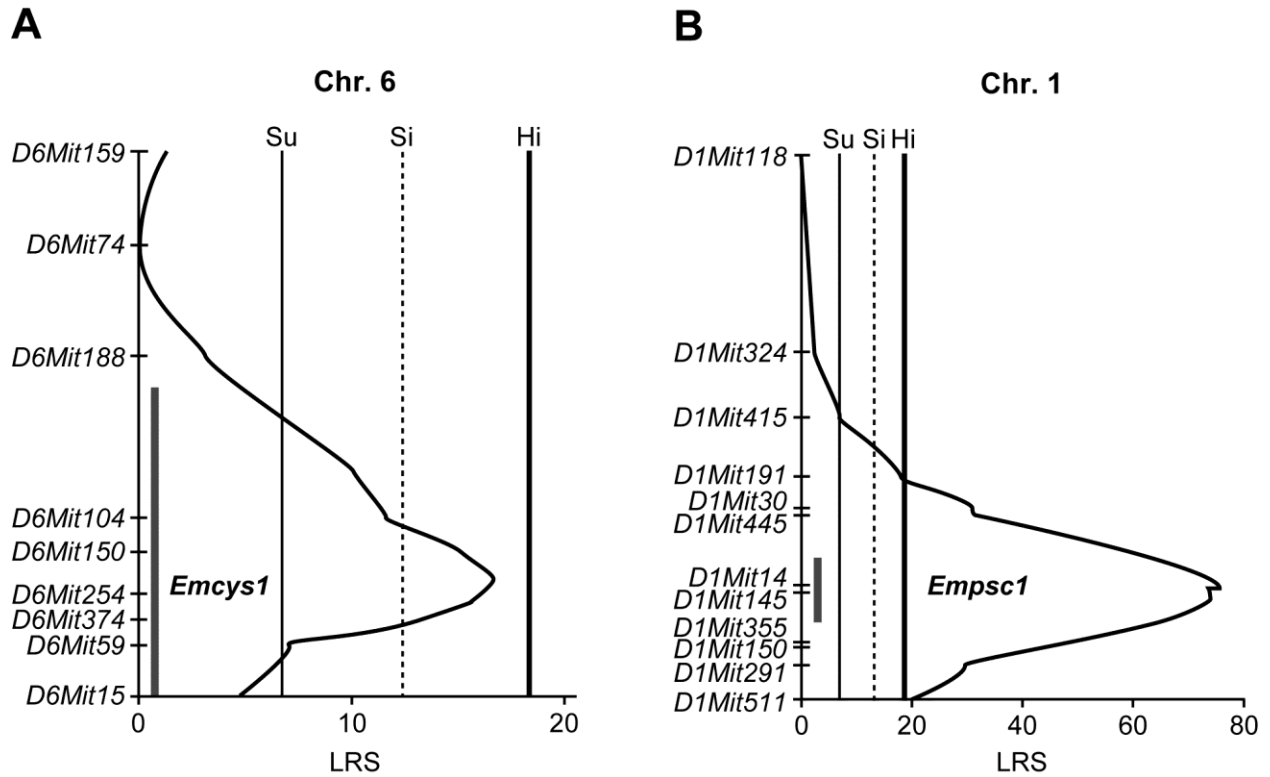


**Fig. 2. Whole genome scans for chromosome sites associated with cyst establishment (A) and protoscolex development (B) of *Echinococcus multilocularis* in the liver of N<sub>2</sub> mice.**



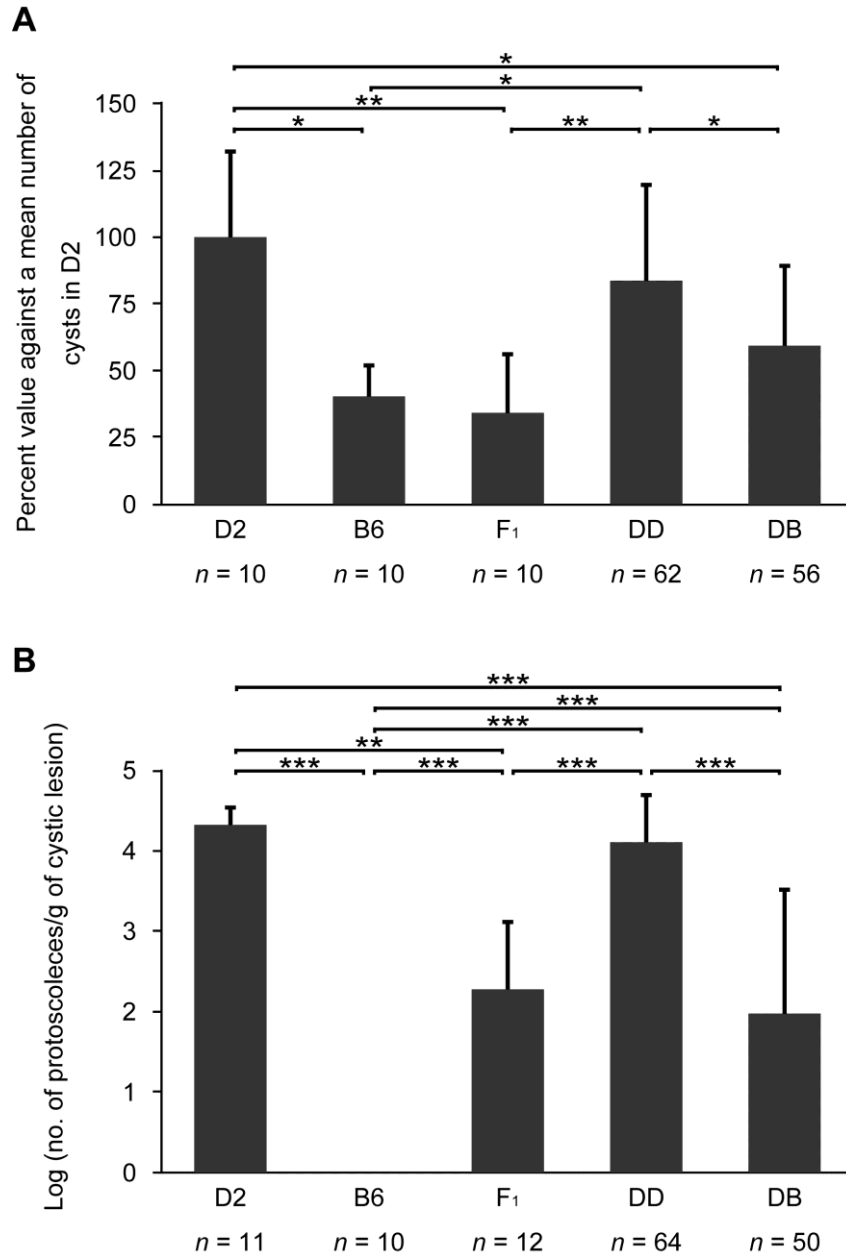
Analyses of linkage of cyst establishment (A) and protoscolex development (B) in the liver of N<sub>2</sub> mice to chromosomal loci were performed using the MapManager QTXb20 software. Recombination frequencies (%) were converted into genetic distance (centiMorgan; cM) using the Kosambi map function, in which linkage data are provided as likelihood ratio statistic (LRS) scores. Genome-wide significance thresholds were calculated in terms of LRS by carrying out permutation tests for 1,000 permutations. The thresholds for suggestive (Su), significant (Si) and highly significant (Hi) linkages are indicated in thin, dotted and thick lines, respectively.

**Fig. 3. Quantitative trait loci (QTLs) affecting cyst establishment (A) and protoscolex development (B) of *Echinococcus multilocularis* in the liver.**



The QTLs on chromosomes (Chrs.) 6 (*Emcys1*) and 1 (*Empsc1*) showed significant and highly significant linkage to (A) cyst establishment and (B) protoscolex development, respectively. The gray bars on the graph indicate approximate 95% confidence intervals. The thin, dotted and thick lines represent suggestive (Su), significant (Si) and highly significant (Hi) thresholds calculated by 1,000 times permutation tests, respectively. The microsatellite markers used for determining genotypes of N<sub>2</sub> mice are presented along the Y-axis. LRS, likelihood ratio statistic.

**Fig. 4. Effect of allelic combination at *D6Mit150* (A) and *D1Mit14* (B) on  $N_2$  mice.**



$N_2$  mice were segregated according to their respective genotypes at (A) *D6Mit150*, a marker representing the *Emcys1* locus and (B) *D1Mit14*, a marker representing the *Empsc1* locus. The mean value of each group was shown with the bar representing the S.D.. DD and DB indicate individuals homozygous for the D2 allele and heterozygous for D2 and B6 alleles, respectively. The number of animals in each group is indicated at the bottom. Statistical analysis for multiple comparisons between the mean values of each group was conducted using Scheffé's F test (\* $P < 0.05$ ; \*\* $P < 0.001$ ; \*\*\* $P < 0.0001$ ).