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Quantitative trait-locus analysis of ovarian cysts derived from rete ovarii in MRL/MpJ mice.

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Running title: Ovarian cysts in MRL/MpJ mice

Abstract

MRL/MpJ (MRL) is a model mouse for autoimmune diseases that shows dermatitis, vasculitis, arthritis, and glomerulonephritis. In addition to these immune-associated disorders, we found that aged MRL mice develop ovarian cysts originating from the rete ovarii, which is lined by ciliated or nonciliated epithelium and considered remnants of mesonephric tubules. Ovarian cysts, which are reported to have several sources, are associated with female infertility, but information regarding the genetic etiology of ovarian cysts originating from the rete ovarii is rare. In this study, to elucidate the genetic background of development of ovarian cysts, we performed quantitative trait-locus (QTL) analysis using 120 microsatellite markers, which cover the whole genome of murine chromosomes, and 213 backcross progenies between female MRL and male C57BL/6N mice. The quantitative trait measured was the circumferences of rete ovarii or ovarian cysts. As a result, suggestive linkages were detected on Chr 3, 4, 6, and 11, but significant linkages were located on Chr 14 by interval mapping. We thereby designated the 27.5-cM region of Chr 14 “MRL Rete Ovarian Cysts (*mroc*).” The peak regions of Chr 4 and 14 in particular showed a close additive interaction ($P < 0.00001$). From these results, we concluded that multiple loci on Chr 3, 4, 6, 11, and 14 interact to result in development of ovarian cysts in MRL mice.

Introduction

In humans and domestic animals, ovarian cysts are a major causes of female reproductive-system dysfunction, and are derived from several sources, including ovarian surface epithelium (OSE), follicles, corpus luteum and rete ovarii (MacLachlan 1987). Functional ovarian cysts, involving Graafian follicles or the corpus luteum, result from physiological variations in the ovulatory cycle and commonly regress spontaneously within 6 months (Holt et al. 1994). But nonfunctional ovarian cysts, with diverse histological origins, are not yet fully understood.

MRL/MpJ (MRL) mice serve as a representative model of autoimmune diseases, including dermatitis, vasculitis, arthritis, and glomerulonephritis (Singh 2005). In addition to these autoimmune-associated inflammatory characteristics, this strain shows interesting phenotypes—vigorous wound healing, metaphase-specific apoptosis in spermatogenesis, and heat-shock resistance in spermatocytes (Namiki et al. 2004). Furthermore, Kon et al. describe a new phenotype and spontaneous development of ovarian cysts in aged female MRL mice (Kon et al. 2007). The cysts were lined by various cell types such as cuboidal or columnar epithelium with or without cilia, and located in the ovarian hilus adjacent to the ovarian ligament. Use of lectin histochemistry and microscopy suggested that the cysts originated from the rete ovarii. These findings indicated that MRL mice could serve as a model for spontaneous ovarian cysts potentially providing valuable information pertaining dysfunction of female reproductive system.

As mentioned above, the origin of ovarian cysts in mammals is not solely the

rete ovarii. In women of childbearing age, most functional ovarian cysts are also derived from follicles, as evidenced by polycystic ovarian syndrome (PCOS) (Brassard et al. 2008). Interestingly, PCOS has been known to be closely associated with not only metabolic syndrome and sex-hormone imbalance but also with autoimmune diseases (Alborzi et al. 2009). Inevitably, multiple studies have used murine models to identify candidate genes or exacerbating factors in follicular cysts (Devin et al. 2007; Chapman et al. 2009). In addition, constitution of diagnostic criteria for clinical trials and the genetic approach via familial studies have been continued for PCOS patients, favored by affluent clinical reports (Gallinelli et al. 2003; Jakubowski 2005; Qin et al. 2006; Wu et al. 2007). In contrast, the genetic basis of nonfunctional ovarian cysts derived from epithelium has not been well established. Cysts can be asymptomatic or accompany nonspecific complaints like lower back pain, astriction, pollakiuria, abdominal bloating, fatigue, and menstrual irregularities. These symptoms are similar to premenstrual syndrome or climacteric disorder; therefore, early diagnosis is not easy to achieve. Cysts originating from the rete system are relatively rare in humans, but the possibility cannot be neglected since it might be linked to interruption of female reproductive function because of rupture or hemorrhage resulting from ovarian torsion (Jain 2002). Further, in the murine model, extremely dilated rete ovarii are cause compression of ovarian tissue, and lead to atrophy of the ovarian stroma, amyloidosis, or other degenerative changes not directly associated with cyst formation (Long 2002). Therefore, the paucity of reports on causative factors of cystic rete ovarii encouraged us to investigate this.

In this study, we first attempted to analyze the genetic background of

nonfollicular ovarian cysts originating from the rete ovarii in an autoimmune disease model—MRL mice. The purpose of this research was to define candidate quantitative trait loci (QTL) associated with cyst formation in ovaries and lay the foundations for more detailed research to determine trigger or modifier genes in cystic changes of the female reproductive system. Furthermore, we will discuss the current views on the relationships between female infertility and autoimmune diseases.

Material and methods

Animals

Mouse strains used in this study were MRL and C57BL/6N (B6). MRL and B6 mice were bred to generate 213 ((MRL $\times$ B6) $\times$ MRL) backcross progenies for QTL analysis. To determine the incidence of ovarian cysts by strain, MRL (n = 12), B6 (n = 5), F1 (n = 9), and F2 (n = 76) progenies of MRL and B6 crosses were raised. Animals were maintained in a controlled ambience at 21–24°C, with relative humidity of 40–60%, and subjected to a 12-h light–dark cycle in the animal facility of the Graduate School of Veterinary Medicine, Hokkaido University. Mice were treated according to the Guideline for the Care and Use of Laboratory Animals, Hokkaido University, Graduate School of Veterinary Medicine.

Histology and phenotyping

Ovaries of MRL and B6 mice were harvested at 6–18 months of age, and F1, F2, and backcross progenies were harvested at 8 months of age. Ovaries of each animal were fixed in Bouin's solution overnight, processed through graded alcohol,

and embedded in paraffin with a routine procedure. Sections of 3- μ m thickness were cut every 30- μ m using both ovaries from each animal. Sections were stained with hematoxylin and eosin (H&E) using standard procedures.

Only sections containing cysts of maximal size were chosen for phenotype evaluation. These were photographed using a digital camera, and the diameter, circumference, and area of each ovarian cyst were measured by Image J (NIH, <http://www.nih.gov/>). In one individual without noticeable ovarian cysts, tubular rete ovarii, adjacent to the ovarian hilus and ovarian ligament, were count.

Genotyping

The genome-wide scan was performed using 213 backcross progenies. Genomic DNA was extracted from spleens tissues of these backcross progenies using a standard protocol and was subjected to a genome-wide scan at 10–20 cM-resolution (average = 12.3 cM) using 120 polymorphic microsatellite markers (Table 1). PCR primers of the markers were identified in the Mouse Genomic Database of the Jackson Laboratory (www.informatics.jax.org) and Mouse Microsatellite Data Base of Japan (MMDBJ).

PCR was carried out using a Promega PCR thermal cycler (iCycler, Madison, WI, USA) with the cycling sequence of 94°C for 2 min (one cycle), followed by 38–40 cycles consisting of comprising denaturation 94°C for 40 sec, primer annealing at 58°C for 30 sec, and extension at 72°C for 30 sec. The PCR dinucleotid triphosphate mixture and the polymerase enzyme (Taq DNA polymerase) were purchased from Sigma (St. Louis, MO, USA). Amplified samples were electrophoresed in 2 6% NuSieve 3:1 agarose gel (Cambrex BioScience Rockland, Inc., Rockland, ME, USA) or Agarose 3:1 (aMRESCO, Inc., Solon, Ohio,

USA), stained with ethidium bromide, and photographed under an ultraviolet lamp.

Linkage analysis

To identify the ovarian cyst loci, genotyping data and the trait score were analyzed by MapManager QTX (Manly et al. 2001), whereby permutation tests were done in 1-cM steps for 10,000 permutations to determine the suggestive, significant, or very significant levels of statistics.

Results

Histology and size distribution of cysts derived from rete ovarii

As shown in Fig. 1, characteristics of lining cells in the lumen of rete ovarii or cystic lesions in backcross progeny varied for each individual. Some rete tubules had tightened lumens with similar cuboidal epithelia in the B6 progenies. Whereas the slightly dilated intraovarian rete ovarii were prone to having flattened epithelia and balloon-like features. Large cysts were usually composed of various epithelial structures. Some individuals had both dilated and nondilated rete ovarii, while others had extremely expanded cysts that compressed the ovarian stroma.

For phenotyping indices, areas, diameters and circumferences of ovarian cysts were measured. Amongst these values, circumference well represented the quantitative trait of ovarian cysts because paraffin-embedded ovarian cysts had irregular features. The specificity and sensitivity of circumference as a quantitative trait were confirmed by preliminary experiments using MapManager QTX, analyzing 119 backcross progenies.

Circumference size distributions for individual ovarian cysts of each progeny

is presented as dots on the graph (Fig. 2a). The distribution of the backcross progeny was fairly scattered, while those of the MRL and B6 progenies tended to fall on one of the two extremes. The mean score of the backcross progeny (2,279 μm in circumference), composed of MRL homozygotes and heterozygotes, was significantly higher than those of the F1 (1,088 μm in circumference) and F2 (1,106 μm in circumference) progenies. The F2 progenies were composed of each homozygote of MRL and B6, and the heterozygotes were the same as the F1 progenies.

In Fig. 2b, individual traits of backcross progeny ($n = 213$) are arranged by size of the rete ovarii or its cyst. The trait-value graph shows a gentle curve, which suggests that ovarian cysts in MRL mice are under the control of polygenic inheritance.

QTL analysis mapping of ovarian cysts

Final results of interval mapping were considered suggestive, significant, or highly significant linkages when the threshold likelihood-ratio statistics (LRS) were 6.5, 11.9, and 18.1 (Lander et al. 1995), respectively. As shown in Fig. 3, suggestive linkages ($6.5 < \text{LRS} < 11.9$) appeared on Chr 3, 4, 6, and 11. In addition, the highest linkage over the significant level ($\text{LRS} > 11.9$) manifested on Chr 14. The maximum LRS score were 7.8 on Chr 3, 11.1 on Chr 4, 10.8 on Chr 6, 7.3 on Chr 11, and 13.5 on Chr14.

Linkage details are shown in Fig. 4. The marker positions at *D3Mit182-244*, *D4Mit248*, *D6Mit316*, and *D11Mit212* show suggestive linkages to ovarian cyst. The locus at the *D14Mit37* marker position, showing the highest linkage ($\text{LRS} = 13.5$), was designated “MRL Rete Ovarian Cyst (*mroc*).” As a result of a manual χ^2

test, the loci from Chr 6 and 11 were revealed to be heterozygous between the MRL and B6 mice, while those of Chr 3, 4, and 14 were meaningfully homozygous for MRL alleles (data not shown).

Significant interactions were searched in the 55.2-cM region of Chr 4 and the 27.5-cM region of Chr 14 by MapManager QTX ($P < 0.00001$). To gain further insight into the interactions between Chr 4 and 14, we chose 30 populations of high traits over 5,000 μm in circumference in the backcross progeny and manually performed a χ^2 test (Table 2). The results explain that individuals with high trait values tended to contain homozygous loci on Chr 4 and 14 in MRL mice. On the other hand, there was no suggestive linkage in the group of 30 resistant individuals ($\chi^2 = 1.745$, $P = 0.62698$).

Discussion

The rete ovarii is the counterpart of the rete testis and develops as a result of differentiation of mesonephric cells that have migrated to the developing gonad of the embryo. While the rete system plays an integral role in ovarian development, the rete ovarii in the adult is generally considered a nonfunctioning vestige (Wartenberg 1982). However, some authors have hypothesized a secretory role for the rete ovarii, based on observations of 1) a holocrine secretion of eosinophilic material, 2) PAS-positive secretion, or 3) the columnar epithelium and wide lumina of the rete tubules in several species (Wenzel and Odend'hal 1985). In mice, the rete ovarii becomes dilated with age and, in some, the epithelium becomes hyperplastic and/or hypertrophic (Long 2002).

Although the etiology of dilation of rete ovarii in the murine model still remains unclear, some researchers hypothesize that rete ovarii appears to be a

common source of ovarian cysts and ovarian epithelial neoplasms in mice. Ovaries of CD-1 mice subjected to incessant ovulation for long periods of time showed an increased number of surface invaginations, OSE stratification, cortical inclusion cyst formation, and dilation of the rete ovarii tubules, similar to preneoplastic changes in the human ovary (Fleming et al. 2007; Tan and Fleming 2004; Tan et al. 2005). This agrees with the theory that deep OSE invaginations and insufficient tissue arrangement after incessant ovulation for long periods of time might induce cyst formation (Tan et al. 2005). Ovarian epithelial tumors in humans are primarily because of OSE invaginations (Dubeau 2008), representing a naïve cell type capable of differentiating into many morphologically-different epithelia (Auersperg et al. 2001). Inevitably, ovulation and normal follicular development are accompanied by interactive wounding, healing, and remodeling of the ovarian surface and stroma. In this process, disruption of intercellular junctions within OSE or stromal tissue remodeling might lead to inappropriate proliferation of the rete ovarii (Burdette et al. 2007).

The incidence and circumferences of ovarian cysts in backcross progeny were higher and larger, respectively, than those of F1 and F2 progenies. The F2 progenies were homozygous for MRL and B6, and the heterozygotes were the same as the F1 progenies. On the other hand, backcross progenies were composed by homozygotes of MRL and the heterozygotes. Based on these facts, we considered that development of ovarian cysts in the backcross progeny is dependent on MRL host's genetic background recessively, and the B6 locus might offset the factors of the homozygous MRL locus. Although the possibility that environmental effects and the combination of parental strains may affect the

results during the long, 8-month period of the study cannot be ruled out, the result of our QTL analysis showed that ovarian cysts in MRL mice were inherited in a polygenic manner.

Several candidate quantitative trait loci appeared at the *D3Mit182-D3Mit244* marker on Chr 3 (map position 23–31 cM), *D4Mit248* on Chr 4 (55.2 cM), *D6Mit316* on Chr 6 (28 cM), *D11Mit212* on Chr 11 (50 cM), and *D14Mit37* on Chr 14 (27.5 cM). Unexpectedly, the susceptibility loci on Chr 6 and 11 manifested in heterozygotes between MRL and B6 mice. That is, these two loci from MRL mice operated as inhibitory factors rather than trigger loci in backcross progenies. This could be because of allelic polymorphisms derived from the genomes of the LG/J, AKR/J, C3H/HeDi, and C57BL/6J strains of mice that comprise the MRL substrains (Andrews et al. 1978). This suggests that particular combinations of genes with allelic polymorphism by backcross breeding might become evident under these experimental conditions (Nishihara et al. 1999). The peak region of Chr 6, in particular, overlaps the results of another QTL report regarding ovarian teratomas in LT/Sv mice (Lee et al. 1997).

According to the Mouse Genomic Informatics (MGI) database, Chr 14 harbors 83 genes adjacent to '*mroc*' (Chr 14: 20.0–38.0 cM). Especially, as shown in Fig. 5, in this region of Chr 14, several genes, which are associated with upregulation of cell proliferation, cell differentiation, cell death, and cell–cell signaling, attracted our interest. Furthermore, our interest was strengthened by the findings that, in aged mice, the epithelium of the cystic rete ovarii is hyperplastic and/or hypertrophic, indicated by PCNA immunoreactivity (Tan and Fleming 2004) or BrdU incorporation (Fleming et al. 2007). Above all, the

chromosomal locations of clusterin (*clu*) (30.5 cM), fibroblast growth factor 9 (*Fgf9*) (20 cM), fibroblast growth factor 17 (*Fgf17*) (38 cM), and GATA-binding protein 4 (*Gata4*) (28 cM) agree with our experimental findings.

Originally isolated from ram rete testis fluid (Blaschuk et al. 1983; Grima et al. 1990), clusterin is a ubiquitous glycoprotein, which that has been proposed to have many physiological functions, including cell aggregation, complement regulation, protein secretion, membrane recycling, and apoptosis (Rosenberg et al. 1995). In addition, its association with pathological processes including glomerulonephritis, cystic renal diseases, renal tubular injury, and cancer suggest that clusterin is involved in immune response and tissue-remodeling (Rosenberg and Silkensen 1995). Members of the FGF family play important roles in development, proliferation, differentiation, cellular migration, tissue repair, injury response, angiogenesis, and cancer metastasis (Ornitz and Itoh 2001). FGF9 is involved in regulation of mesenchymal proliferation and mesonephric migration into the gonad (Colvin et al. 2001). Even though very little is known about the pattern of expression of FGF17 in the reproductive system, some researchers have suggested a physiological role for FGF17 in the control of granulosa cell differentiation in bovines (Machado et al. 2009). Members of the GATA family of transcription factors are emerging as essential players in reproductive cell-lineage determination during development and as mediators of adult reproductive function (LaVoie 2003).

As a result of the χ^2 test, peak regions of Chr 4 and 14 apparently manifested high additive interactions. This result has an interesting interpretation that several loci involved in susceptibility to autoimmune diseases

could be involved in the development of ovarian cysts in this autoimmune-disease model. Around 55 cM of Chr 4 contains many autoimmune-associated QTLs in MRL mice, such as autoimmune glomerulonephritis in MRL 2 (*Agnm2*) in 53 cM (Miyazaki et al. 2005), autoimmune renal vasculitis 2 (*Arvm2*) in 58 cM (Qu et al. 2000), autoimmune sialadenitis in MRL mice 1 (*Asm2*) in 51 cM (Nishihara et al. 1999), lupus in MRL and B6 F2 cross, QTL 1 (*Lmb1*) in 57.5 cM (Vidal et al. 1998) and sialoadenitis susceptibility (*Sials*) in 48.5 cM (Nose et al. 2000). Moreover, in addition to the MRL strain, other murine strains also show autoimmune-like features such as autoimmune biliary disease 1 (*Abd1*) in 49 cM (Irie et al. 2006), anti-dsDNA antibody production in NZM 1 (*Adaz1*) in 49.6 cM (Waters et al. 2001), anti-erythrocyte antibodies 3,4 (*Arigg3,4*) in 47.8, 59.9 cM (Lee et al. 2004), splenomegaly modifier (*Spm1*) in 49 cM (Ochiai et al. 2000), erosive arthritis susceptibility 2 (*Erars2*) in 53.6 cM (Mountz et al. 2005), and systemic lupus erythematosus suppressor 2 (*Sles2*) in 58 cM (Morel et al. 1999). Likewise, numerous QTLs, associated with autoimmune diseases and autoantibody production, belong to specific regions on Chr 4, which overlap our results describing ovarian cysts in MRL mice. These results likely indicate a possible connection between ovarian cysts and uncertain autoimmune diseases in MRL mice.

Rete testis, the counterpart of rete ovarii, could also be affected by cystic disorders in humans and animals (Kutzler et al. 2006; Smith et al. 2008). Congenital cystic rete testis (CRT) is commonly observed in conjunction with renal developmental abnormalities (Eberli et al. 2002; Robson et al. 1998). Acquired CRT can result from extratesticular disorders such as mechanical

obstruction secondary to chronic renal insufficiency or chronic epididymitis (Nistal et al. 1996). By analogy, chronic inflammations associated with autoimmune traits might result in cystic transformations of the female rete system. In some cases of autoimmune diseases, adjacent organs or tissues indirectly targeted by the autoimmune reaction are typically spared from the autoimmune attack (Betterle et al. 2004). Therefore, it seems to be quite reasonable to educate those women, who suffer from autoimmune diseases, to pay close attention to their menstrual cycles and to participate in periodical gynecological monitoring for their future pregnancy planning.

A close association between female infertility and autoimmunity has been reported in conditions such as premature ovarian failure (POF), endometriosis, and PCOS. POF is early-onset menopause because of autoantibody attack of the ovaries (Bakalov et al. 2005). Endometriosis is described as uncontrolled endometrial proliferation whereby endometrial tissue undergoes ectopic growth in the pelvic lymph nodes, uterine serosa, omentum, and ovaries (Hever et al. 2007), and it has been postulated to coincide with autoimmune disorders (Sinaii et al. 2002). PCOS is characterized by formation of multiple, non-ovulated follicular cysts accompanied by hyperandrogenism, amenorrhea, hirsutism, excess weight because of insulin resistance, and autoantibodies against ovarian components (Gallinelli et al. 2003; Palacio et al. 2006). It has also been reported that patients with autoimmune disorders such as rheumatic disease have a tendency to experience impaired fertility (Skomsvoll et al. 2000). Likewise, autoimmune disorders and symptoms of ovarian dysfunction should be considered as part of, rather than inclusive of, an individual's health problems.

In summary, we performed genome-wide screenings for spontaneous ovarian cysts in MRL mice. We found that specific loci on Chr 4 (55.2 cM) and 14 (27.5 cM) were crucial for triggering ovarian cystic changes. We conclude that development of ovarian cysts in MRL mice is a multifactorial disorder having autoimmune characteristics. Although detection of candidate genes remains our task, use of this unique phenotype in a typical autoimmune model could be an alternative means to unveil many other etiopathological factors common between autoimmune diseases and epithelium-derived ovarian cysts. To determine the candidate genes, further studies, such as generating and analysis of congenic mice harboring the *mroc* region, are contemplated.

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Figure legends

Fig. 1. Gross and microscopic features of ovarian cysts from rete ovarii. a. Gross appearance of ovaries of a 19-month-old MRL mouse. Bilateral ovaries are significantly enlarged and filled with blood or fluid (arrows; bar = 1 cm). b. Histological variations of rete ovarii involved in 8-month-old backcross progenies. b. Some rete tubules are observed as fairly tightened (arrows; bars = 200 μ m). c. Normal rete ovarii with cuboidal epithelium (arrow). d–e. Dilated rete ovarii with flattened epithelium (asterisks). Cluster of cell debris (arrow head) were observed in the lumen of rete ovarii located within or outside of the ovary. f. Remarkably expanded rete ovarii account for half of the volume of the ovary (asterisk) and are accompanied by relatively normal rete ovarii (arrow).

Fig. 2. a. Size distribution of rete ovarii in MRL, B6, F1, F2, or backcross progenies. Mean values are indicated by horizontal lines. b. Distribution of circumferences rete ovarii in backcross progeny.

Fig. 3. Result of interval-mapping scans by MapManager QTX. From the bottom, the dotted lines represent the suggestive (LRS score = 6.5), significant (LRS score = 11.9), or very significant (LRS score = 18.1) levels determined by the permutation test. Linkages above the suggestive level were detected on Chr 3, 6, 4, 11, and 14.

Fig. 4. Details of suggestive and significant linkages in QTL analysis of ovarian cysts. Loci on chromosomes 6 and 11 manifested heterozygotes in MRL and B6 mice, while the others were suggestive of homozygous loci in MRL mice. Maximum LRS (13.5) was scored around the *D14Mit37* marker position (27.5 cM in Chr 14), designated as “MRL Rete Ovarian Cyst (*mroc*).”

Fig. 5. Schematic view of significant and suggestive loci. Several genes related to up-regulation of cell proliferation, differentiation, programmed cell death, and cell–cell signaling belong to *mro* loci on chromosome 14. A 55.2-cM region of Chr 4 contains numerous previously described autoimmune-associated loci.

Table 1. List of the microsatellite markers used for genotyping

Marker	cM	Marker	cM	Marker	cM	Marker	cM
<i>D1Mit64</i>	5.0	<i>D5Mit353</i>	24.0	<i>D9Mit91</i>	17.0	<i>D14Mit228</i>	46.0
<i>D1Mit211</i>	15.0	<i>D5Mit254</i>	34.0	<i>D9Mit302</i>	35.0	<i>D14Mit226</i>	60.0
<i>D1Mit123</i>	21.0	<i>D5Mit201</i>	42.0	<i>D9Mit181</i>	48.0		
<i>D1Mit303</i>	34.8	<i>D5Mit24</i>	60.0	<i>D9Mit76</i>	49.0	<i>D15Mit111</i>	17.8
<i>D1Mit134</i>	49.0	<i>D5Mit101</i>	81.0	<i>D9Mit18</i>	71.0	<i>D15Mit156</i>	39.0
<i>D1Mit191</i>	63.0					<i>D15Mit71</i>	46.7
<i>D1Mit107</i>	85.0	<i>D6Mit166</i>	0.6	<i>D10Mit166</i>	5.0	<i>D15Mit245</i>	58.9
<i>D1Mit456</i>	95.8	<i>D6Mit74</i>	20.5	<i>D10Mit42</i>	44.0		
<i>D1Mit403</i>	99.5	<i>D6Mit33</i>	25.5	<i>D10Mit134</i>	58.0	<i>D16Mit131</i>	4.3
		<i>D6Mit316</i>	28.0	<i>D10Mit271</i>	70.0	<i>D16Mit59</i>	27.7
<i>D2Mit369</i>	28.7	<i>D6Mit243</i>	30.4			<i>D16Mit140</i>	42.8
<i>D2Mit249</i>	47.5	<i>D6Mit188</i>	32.5	<i>D11Mit62</i>	1.6	<i>D16Mit70</i>	57.0
<i>D2Mit107</i>	63.4	<i>D6Mit323</i>	36.5	<i>D11Mit230</i>	16.0	<i>D16Mit106</i>	71.5
<i>D2Mit340</i>	71.2	<i>D6Mit105</i>	45.5	<i>D11Mit5</i>	37.0		
<i>D2Mit452</i>	80.0	<i>D6Mit10</i>	48.7	<i>D11Mit320</i>	43.0	<i>D17Mit198</i>	16.0
<i>D2Mit456</i>	86.8	<i>D6Mit194</i>	61.5	<i>D11Mit212</i>	50.0	<i>D17Mit135</i>	17.0
<i>D2Mit148</i>	92.4	<i>D6Mit374</i>	74.0	<i>D11Mit288</i>	55.0	<i>D17Mit119</i>	38.5
				<i>D11Mit199</i>	62.0	<i>D17Mit221</i>	56.7
<i>D3Mit130</i>	3.9	<i>D7Mit178</i>	0.5	<i>D11Mit48</i>	77.0		
<i>D3Mit182</i>	23.3	<i>D7Mit82</i>	25.0			<i>D18Mit177</i>	20.0
<i>D3Mit244</i>	38.3	<i>D7Mit321</i>	48.5	<i>D12Mit185</i>	11.0	<i>D18Mit53</i>	27.0
<i>D3Mit103</i>	51.0	<i>D7Mit105</i>	63.5	<i>D12Mit136</i>	13.0	<i>D18Mit151</i>	37.0
<i>D3Mit288</i>	58.8	<i>D7Mit189</i>	72.4	<i>D12Mit158</i>	38.0	<i>D18Mit186</i>	45.0
<i>D3Mit320</i>	71.0			<i>D12Mit19</i>	58.0	<i>D18Mit4</i>	57.0
<i>D3Mit129</i>	84.9	<i>D8Mit224</i>	17.0				
		<i>D8Mit226</i>	22.0	<i>D13Mit17</i>	8.0	<i>D19Mit68</i>	6.0
<i>D4Mit235</i>	1.9	<i>D8Mit205</i>	30.0	<i>D13Mit13</i>	35.0	<i>D19Mit80</i>	22.0
<i>D4Mit178</i>	26.9	<i>D8Mit8</i>	32.0	<i>D13Mit260</i>	65.0	<i>D19Mit91</i>	47.0
<i>D4Mit301</i>	31.5	<i>D8Mit343</i>	37.0			<i>D19Mit33</i>	53.0
<i>D4Mit145</i>	44.5	<i>D8Mit50</i>	41.0	<i>D14Mit10</i>	3.0		
<i>D4Mit12</i>	49.5	<i>D8Mit200</i>	58.0	<i>D14Mit133</i>	10.0	<i>DXMit166</i>	15.5
<i>D4Mit248</i>	55.2	<i>D8Mit56</i>	73.0	<i>D14Mit141</i>	15.0	<i>DXMit25</i>	27.8
<i>D4Mit251</i>	59.8			<i>D14Mit142</i>	19.5	<i>DXMit16</i>	37.0
<i>D4Mit42</i>	69.9			<i>D14Mit37</i>	27.5	<i>DXMit130</i>	55.0
<i>D4Mit127</i>	77.5			<i>D14Mit193</i>	40.0	<i>DXMit186</i>	69.0

Table 2. Interaction between microsatellite markers

Genotype at susceptibility loci		n	χ test	<i>p</i> value
<i>D4Mit248</i> (55.2cM)	<i>D4Mit37</i> (27.5cM)			
M*	M	20	28.133	0.00000341
M	H**	2		
H	M	4		
H	M	4		
M*: MRL homozygous H**: Heterozygous		Total: 30	Degree of freedom: 3	









