

## Mapping of quantitative trait loci for ethanol preference in quasi-congenic strains

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### Abstract

Ethanol preference, a component of alcoholism, has been known for four decades to differ greatly between C57BL/6 and BALB/c inbred mouse strains. For mapping quantitative trait loci (QTLs) that affect ethanol preference, we used a set of B6.C Recombinant QTL Introgression (RQI) strains, which carry about 5% of the donor BALB/cJ (C) genome on a C57BL/6ByJ (B6) background. After characterizing males of the progenitor and RQI strains for variations in ethanol preference, we scanned their genome for polymorphisms at 244 dinucleotide-repeat marker loci known to differ between B6 and C. Because of the introgression of BALB/c-type QTLs onto the B6 background, some strains showed ethanol preference significantly lower or higher than that of the background strain, suggesting that genetic interaction between ethanol preference QTLs and the background can be operative. The genomic region showing the strongest influence on ethanol preference was on mouse chromosome 15, and corresponds to human chr.12 q11–q13. © 2000 Elsevier Science Inc. All rights reserved.

**Keywords:** Alcohol preference; QTL mapping; Recombinant QTL introgression; Mouse; Animal model

### 1. Introduction

One of the long-term goals of the human genome program is to expedite the identification of genes that are risk factors for complex disorders. Complex traits, which can be defined as the expression of interactions between multiple genetic and environmental components, have become the target of intensive genetic research because it is now evident that most of the prevalent and economically crippling diseases are complex diseases. Disorders such as alcoholism, diabetes, obesity, high blood pressure, schizophrenia, depression, and substance abuse are resistant to traditional genetic and functional analysis.

Quantitative Trait Locus (QTL) defines a section of a chromosome, which underlies a quantitative trait (Gelderman, 1975). Identification and confirmation of QTLs that predispose to alcoholism, a complex, composite disorder,

has been extremely difficult in humans (Long et al., 1998; Reich et al., 1998), because of a multitude of confounding factors. Among these are the involvement of several genes with individually small effect, genetic heterogeneity, coincident use of alcohol with other drugs of abuse, environmental variability, variable penetrance, genetic imprinting, heterogeneous genetic background, interaction between genes, and interaction between genes and environment. The use of animal models for specific aspects of alcoholism is an important alternative, and ethanol preference in rodents has been accepted as a valid model for alcohol-seeking behavior (Begleiter & Kissin, 1995).

Since the first demonstration of high ethanol preference in C57BL/6 mice, and low preference in BALB/c and DBA/2 mice (McClearn & Rodgers, 1959), these strain differences have been remarkably stable for four decades (Belknap et al., 1993). Experiments to map QTLs for ethanol preference were first carried out in the BXD set of recombinant inbred (RI) strains (Phillips et al., 1994; Rodriguez et al., 1994; Rodriguez et al., 1995). In these studies, five (Phillips et al., 1994) and ten (Rodriguez et al., 1995) chromosomes were reported to carry markers that showed

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significant ( $p < 0.05$ , uncorrected for multiple comparisons) correlation with the preference for 10% ethanol solution. Confirmation testing of these RI results was performed by short-term selective breeding for ethanol preference starting from a B6D2F2 population (Belknap et al., 1997) and by F<sub>2</sub> intercross studies (Phillips et al., 1998; Tarantino et al., 1998). In the selection study, Belknap et al. (1997) concluded that four provisional QTLs were implicated in both BXD RI studies and that their selection data provided supporting evidence for the existence of three of the four, with the most probable QTL on chr. 3. In the F<sub>2</sub> experiments Tarantino et al. (1998) found that the QTL replication rate was 28.6% supporting the argument for a multistage research design, while Phillips et al. (1998) reported verification of QTLs on chromosomes 9 and 2, and suggestive verification on chromosomes 3, 4, 7, and 15.

The LSxSS RI strains were also subjected to QTL analysis (Gehle & Erwin, 1998). Voluntary ethanol consumption was measured in a two-bottle free-choice paradigm, and chromosomes 1, 2, 4, 5, 9, 15, and X were reported to carry provisional QTLs. Recently, in another RI system (AXB/BXA), alcohol preference was studied in 27 strains (Gill et al., 1998). Putative sex-specific QTLs were mapped on chromosomes 2, 4, 5, 7, 9, 10, 11, 16, and 19.

A different approach, an intercross-backcross breeding design, was chosen by Melo et al. for mapping alcohol preference QTLs (Melo et al., 1996a). Using the progenitor strains of the BXD RI set, they localized sex-restricted QTLs on chromosome 2 and chromosome 11. Recently, two additional sex-specific QTLs were detected on chromosome 3 and chromosome 1 (Peirce et al., 1998).

For another measure of voluntary ethanol consumption (alcohol acceptance), marker-based genotypic selection (McClearn et al., 1997) confirmed evidence for the influence of a QTL on chromosome 15 (Rodriguez et al., 1995). The concordance across studies suggests that sex-specificity of QTLs is a significant factor in alcohol preference, and chromosomes 1, 2, 4, 7, 9, 11, and 15 may be identified as likely carriers of QTLs for alcohol preference.

Aiming to combine the advantages of i) inbred strains (precise strain means can be obtained for the complex phenotype because the number of isogenic animals tested can be increased as necessary), ii) congenic strains [strains that are nearly identical except for a short chromosomal segment are called congenic (Flaherty, 1981); the differential genes are on homogeneous genetic background, thus eliminating interactions], and iii) selection lines (the presence of QTLs affecting the expression of the complex trait is ensured by repeated phenotypic selection), we developed an alternative strategy for mapping complex traits. Quasi-congenic Recombinant QTL Introgression (RQI) strains were created by repeated backcross–intercross cycles with concomitant selection for the extreme values of a quantitative dopamine system-related phenotype, followed by at least 20 generations of strict brother–X–sister mating (Vadasz et al., 1987; Vadasz, 1990; Vadasz et al., 1994; Vadasz et al., 1996b;

Vadasz et al., 1998). In addition to mapping dopamine-system-related genes, this set of RQI strains may also be useful for the analysis of “passenger phenotypes”; i.e., for the analysis of complex traits whose QTLs (some or all of them) are passenger genes on introgressed chromosome segments. Here we use these RQI strains for the analysis of ethanol preference, which may be influenced by both pleiotropic effects of dopamine-system-related QTLs and passenger-gene effects. The rationale for this study is that the progenitor strains and RQI strains significantly differ in ethanol preference (Vadasz et al., 1996a) and that ethanol reinforcement appears to be mediated by release of dopamine (among several other factors) (Koob et al., 1998; Ingvar et al., 1998).

## 2. Methods

### 2.1. Animals

RQI strains were constructed as described (Vadasz et al., 1987; Vadasz, 1990; Vadasz et al., 1994; Vadasz et al., 1996b; Vadasz et al., 1998). Briefly, QTLs that are responsible for the continuous variation of mesencephalic tyrosine hydroxylase activity (TH/MES), were introgressed onto the C57BL/6By (B6) strain background from BALB/cJ (C) and CXBI (I) donor strains with high and low TH/MES, respectively. B6 served as the background strain, because its TH/MES was intermediate between those of the donor strains, and it had already been used as a background strain for numerous congenic lines. The QTL transfer was carried out in two directions by four or five backcross–intercross cycles with concomitant selection for the extreme high and low expressions of TH/MES in replicates, resulting in four QTL introgression lines. In the B6.C and B6.I introgression lines, the top and bottom one-third of the population was selected, respectively (Vadasz et al., 1994). The QTL introgression phase was followed by brother X sister mating for at least 20 generations.

### 2.2. Nomenclature

$\alpha$  (alpha) and  $\beta$  (beta) replicates were derived from the (B6XC)F<sub>2</sub>, and  $\alpha$  and  $\beta$  replicates were derived from the (B6XI)F<sub>2</sub> segregating selection generations. The F<sub>2</sub> generation was followed by the first backcross (b<sub>1i0</sub>), the first intercross (b<sub>1i1</sub>), then by the second backcross (b<sub>2i1</sub>), etc. The last selection generation was b<sub>5i7</sub>, which reflected five backcross–intercross cycles. The name of an RQI strain begins with the abbreviation for the background strain followed by a period and the abbreviation for the donor strain; e.g., B6.C. This is followed by the total number of backcrosses and intercrosses experienced and by the designation of the replicate line of origin; e.g., B6.Cb<sub>5i7</sub>- $\alpha$ . From the b<sub>5i7</sub> generation B6.Cb<sub>5i7</sub>- $\alpha$ , B6.Cb<sub>5i7</sub>- $\beta$ , B6.Ib<sub>5i7</sub>- $\alpha$ , and B6.Ib<sub>5i7</sub>- $\beta$  sets of RQI strains were derived. In addition to the b<sub>5i7</sub> series, another replicate series with four backcross–intercross cycles, designated B6.Cb<sub>4i5</sub>- $\alpha$  and B6.Cb<sub>4i5</sub>- $\beta$ , were derived

from a cross between the  $b_{3i5}$  generation and the B6 background strain. Numbers from 1 through 14 were assigned to each new strain; e.g., B6.Cb $_{4i5}$ - $\alpha$ 10/Vad.

In the present study, we genotyped 12 B6.C RQI strains of the  $b_{4i5}$  series, 1 B6.I strain of the  $b_{5i7}$  series, and the 3 progenitor strains (B6, C, and I). The B6.Ib $_{5i7}$ - $\alpha$ 16at/Vad strain was included for the preliminary assessment of the effect of five backcross–intercross cycles on the genomic contribution of a donor strain, in contrast to four backcross–intercross cycles represented by the  $b_{4i5}$  series. The two series differ from each other in the average expected proportion of the homozygous background genes because with each backcross half of the nonselected, nonlinked donor genome was lost during the development of the RQI strains (Green, 1981; Vadasz et al., 1994). Without selection, after four and five backcrosses the average proportion of the homozygous background genomes are 93.75% and 96.875%, respectively. If inbreeding follows the backcrosses, half of the heterozygous donor loci will be fixed as background type genes. If backcrossing with concomitant phenotypic selection is exercised, the proportions of the background genome is expected to be smaller, but this effect depends on many factors, such as the number of QTLs involved, effect size, interactions, etc., which are difficult to predict. All RQI strains had at least 20 generations of brother-X-sister mating.

The capacity of the testing system allowed us to carry out ethanol preference tests in batches of 40–50 males. Females were not tested. Depending on their availability, animals from all the strains were included in a batch. The  $n$  was large for the progenitor strains because they were included in each batch. The experiments began in October 1996 and ended in December 1997. Animals were kept on a 12/12 light/dark schedule (lights on at 6 A.M., lights off at 6 P.M.) with free access to food (Purina #5008). After weaning, 3 males and 3 females (littermates) were housed in a cage. For the alcohol experiment 9–11-week-old mice were used and had been in the study room for at least one week prior to the 3% trial. Body weight was determined on the first and the last day of the experiment to assess the effect of experimental stress on the subjects. No loss of body weight was found.

### 2.3. Measurement of ethanol and water consumption

For the present study, we used a “two-bottle choice” paradigm with escalating ethanol concentration (Le et al., 1994) and with limited social isolation to measure ethanol preference. The test consisted of four 3-day trials, in which mice were allowed to choose between ethanol solution and tap water. To acclimate the animals to the taste of ethanol, the ethanol solution was offered in escalating concentrations: a 3% solution for trial 1 (day 1–3) was increased to 6% in trial 2 (day 3–6), and further increased to 12% for trials 3 (day 6–9) and 4 (day 9–12). This arrangement provided duplicate measures of ethanol preference at 12% concentration (v/v). To diminish potential social isolation-induced

neurochemical and behavioral changes (Essman & Valzelli, 1984; Valzelli, 1969; Valzelli, 1973), in trial 2 a littermate female was housed together with the tested male (day 3–6). In all other trials (1, 3, and 4), males were housed singly. Preference was expressed as the proportion of ethanol solution consumption to total liquid (ethanol solution + water) consumption.

Because offering ethanol solutions of 3% and 6% served the purpose of acclimation of animals to 12% solution, we did not wish to carry out experiments in duplicate for 3% and 6% ethanol consumption, or to distinguish between male and female consumption at 6% ethanol concentration. Our data analysis focused on preference for 12% ethanol solution, measured in duplicate (i.e., two subsequent 3-day trial periods), the values used for analysis were 3-day access values. The liquids were offered in custom-made drinking tubes composed of centrifuge tubes fitted with single-hole rubber stoppers into which stainless steel sippers were inserted. Stainless-steel washers were glued to the bottom part of the rubber stoppers, which faced the cage-covers, to prevent chewing and playing with the drinking tubes. We also used stainless-steel springs to fasten the tubes firmly to the top of the cage covers. Two empty cages with alcohol and tap water drinking tubes were put on the racks to obtain control weights of leakage and evaporation (Phillips et al., 1994). For each trial, the mean control values were subtracted from the alcohol and water consumption data. Food was located on the cover at the front of the cage, with one drinking tube always nearer to food than the other. The position of the water and alcohol drinking tubes on the cage cover was alternated in each 3-day preference trial to avoid a position effect. The weights of the drinking tubes were measured before and after a 3-day trial by an A&D electronic analytical balance connected to an IBM AT computer. Data were entered automatically using A&D COLLECT software and QUATTRO spreadsheets.

### 2.4. Analysis of blood alcohol level

We also wished to establish the relationship between ethanol preference and blood alcohol level (BAL). This relationship cannot be determined by taking blood at the end of a 3-day-long ethanol preference test because ethanol levels are too low (Phillips et al., 1994). Therefore, at the end of trial 4, the animals were deprived of water at 1600. On the next morning at 1000, they were subjected to a 2-h preference test (12% alcohol vs. tap water), followed by decapitation and collection of trunk blood in heparinized test tubes. Blood ethanol determinations were performed with Abbott Laboratories (Abbott Park, IL 60064) Radiative Energy Attenuation (REA) Ethanol Reagents. Analyses were performed on an Abbott Laboratories TDx/FLx analytical instrument. In the radiative energy attenuation method, alcohol concentration is determined by the combined catalytic reaction of alcohol dehydrogenase and diaphorase to generate a fluorescent dye. Levels of ethanol in mouse

blood were calculated from a standard curve generated with ethanol calibrators of concentration 0, 25, 50, 100, 200, and 300 mg/dL. The total sample sizes for alcohol preference tests ( $n = 457$ ) and BAL ( $n = 348$ ) are different because not every batch of alcohol preference tests was followed by the 2-h preference test and blood taking, and we did not have enough BAL data for one of the strains (B6.Ib<sub>5</sub>i<sub>7</sub>- $\alpha$ 16at/Vad).

### 2.5. Genotyping

DNA was isolated from spleen. From each strain one male, derived from a continuous line of brother X sister mating, was used. To genotype the DNA samples, radiolabeled PCR reactions were performed using 244 microsatellite markers (Research Genetics, Huntsville, AL), and the products were visualized on acrylamide gels. One primer was end-labelled with <sup>32</sup>P- $\gamma$ -ATP (6000 Ci mmol<sup>-1</sup>) using T4 kinase as described (Vadasz et al., 1996b). Genomic DNA (5  $\mu$ L; 4 ng/ $\mu$ L) was amplified in a 10  $\mu$ L PCR reaction using AmpliTaq DNA polymerase (Perkin Elmer). The PCR primer concentration was 230 nM for each of the two primers. Forty  $\mu$ L of light mineral oil (Sigma) was layered over the reaction mixture. Reactions were performed in flexible 96-well plates and amplified on a Hybaid OmniGene Thermal Cycler (Labnet, Woodbridge, NJ) using the following thermocycling protocol: initial denaturation at 94 °C for 3 min, followed by 25 cycles of 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 1.5 min, followed by a final extension period at 72 °C for 7 min. PCR products were mixed with 10  $\mu$ L of loading buffer consisting of xylene cyanol and bromophenol blue dyes in 100% formamide, denatured for 5 min on a 100 °C heating block, and electrophoresed on 6% polyacrylamide gels for 2.5 h at 85 W. Gels were wrapped in plastic wrap and exposed directly to film for 4–24 h at –80 °C. Size determinations of alleles were made by comparison of allele bands against a radiolabeled DNA ladder (BioVentures) present on each gel. Sixteen inbred strains (12 RQI strains of the b<sub>4</sub>i<sub>5</sub> series, 1 RQI strain of the b<sub>5</sub>i<sub>7</sub> series, and 3 progenitor strains) were genotyped for 244 microsatellite markers.

### 2.6. Estimation of introgressed segment length

The lengths of chromosome segments of donor origin were estimated as follows: When two or more adjacent donor loci were observed in a chromosome region, the interval between the two outermost donor-type loci was calculated, and half of the intervals between the outermost donor-type loci and the flanking background-type loci were calculated on each side, then added to the interval between the outermost donor-type loci.

### 2.7. Statistical analysis of phenotypic data

After data from sick animals or from mice that died during the experiment were excluded (3 animals), 472 mice (distributed across 18 batches) remained. Duplicate mea-

asures of 12% ethanol preference were considered inconsistent if they differed by more than twice of the standard deviation of ethanol preference (Melo et al., 1996). The standard deviation was  $\pm 0.3$  for both AP<sub>3</sub> and AP<sub>4</sub> in the total population. Animals were excluded if the difference between AP<sub>3</sub> and AP<sub>4</sub> was larger than 0.6. Based on this criterion 15 animals were excluded ( $n = 457$ , after exclusions). 1–3 animals per strain were excluded (including high-, intermediate-, and low-preference strains); this data trimming did not affect significantly the mean values. For statistical analysis the SPSS software package (release 7.5.2) was used.

### 2.8. Genetic analysis

Mapping and other genetic analyses were carried out with QGene, a Macintosh computer program written to aid in marker-based genetic analysis (Nelson, 1997) and with SPSS (7.5.2). Map positions are from the 1998 Chromosome Committee Report (<http://www.informatics.jax.org/>). QTLs for 12% ethanol preference in the third and fourth trials (AP<sub>3</sub> and AP<sub>4</sub>) were mapped by regression analysis, the dependent variable being the strain mean value of the phenotype and the independent variable the marker score. Significance levels were corrected for multiple comparisons by Bonferroni's method. Because 171 markers were B6 background type and 73 were polymorphic in the RQI strains, corrections were made for 73 comparisons. Permutation was used as another way of providing Type-I error thresholds for multiple analyses involving linked, nonindependent markers (Churchill & Doerge, 1994).

The animal care and use for these studies met the standards and recommendations of the IACUC of The Nathan S. Kline Institute for Psychiatric Research.

## 3. Results

In the final dataset, significant differences were found between AP<sub>3</sub> and AP<sub>4</sub> by paired samples T test in the B6.Cb<sub>4</sub>i<sub>5</sub>- $\beta$ 14/Vad and B6.Cb<sub>4</sub>i<sub>5</sub>- $\beta$ 10/Vad strains ( $p < 0.05$ ), while in the other strains AP<sub>3</sub> and AP<sub>4</sub> were not significantly different ( $p > 0.1$ ). After correction for multiple comparisons, AP<sub>3</sub> and AP<sub>4</sub> were not significantly different in any one of the strains ( $p > 0.05$ ).

Total fluid volume consumptions in trial 3 (TF<sub>3</sub>) and trial 4 (TF<sub>4</sub>) were evaluated for strain differences by one-way ANOVA. For both variables significant strain dependent variation was detected ( $p < 0.001$ ). Tukey's HSD multiple comparison test of both TF<sub>3</sub> and TF<sub>4</sub> data showed that B6.Cb<sub>4</sub>i<sub>5</sub>- $\beta$ 10/Vad ( $p < 0.01$ ), B6.Cb<sub>4</sub>i<sub>5</sub>- $\beta$ 4A/Vad ( $p < 0.01$ ), and CXBI ( $p < 0.001$ ) had higher total liquid volume intake than the background B6. Of these strains, in comparison to B6, only CXBI showed significantly lower alcohol preference ratios (AP<sub>3</sub> and AP<sub>4</sub>,  $p < 0.001$ , Tukey's HSD).

To test the reliability of the alcohol preference measures, progenitor strains were included in each batch (three males

per strain per batch). One-way ANOVA of the preference data (AP\_3 and AP\_4) indicated no significant variation across batches for the C and I strains, while the variations were significant for B6 ( $p < 0.01$ ). However, after correcting for multiple comparisons no significant differences were found between batches in the overlapping subsets ( $p > 0.05$ , Tukey HSD). Post-hoc power analysis indicated that there was sufficient power to detect differences in the C and I strains (power  $> 0.8$ ), while the power was smaller (0.6) for B6. The batch averages of AP\_34 (using the mean of the duplicate trials AP\_3 and AP\_4) were calculated for 18 batches. In the B6 strain, the 95% confidence interval for AP\_34 was 0.35 (lower bound) and 0.54 (upper bound) with a range of 0.81.

Confirming our previous report (Vadasz et al., 1996a), we found that the C57BL/6By background strain expressed higher ethanol preference than the donor BALB/cJ mice (Table 1). However, preference scores for 12% ethanol solution in both the C57BL/6By and BALB/cJ strains were lower than the reported values for C57BL/6J and BALB/cJ, respectively, at 10% ethanol solution (McClearn & Rodgers, 1959).

One-way ANOVA was carried out using the mean of the duplicate ethanol preference measures for 12% ethanol solution for each individual in the 16 inbred strains (3 progenitor strains, 12 B6.Cb4i5 RQI strains, and 1 B6.I b5i7 RQI strain). Significant between-strain variability was detected ( $p < 0.0001$ ). Broad-sense heritability was estimated as  $h^2 = (MS_b - MS_w) / (MS_b + (k-1)MS_w)$ , where  $MS_b$  is between-groups Mean Square,  $MS_w$  is within-group Mean Square, and  $k$  is the harmonic mean of the group sizes. The broad-sense heritability of ethanol preference for 12% ethanol solution (using the mean of the duplicate trials) was 0.47.

To assess the deviations from the genetic background strain, we compared all the strains to B6 using one-way ANOVA and LSD post-hoc test. The results indicated that BALB/cJ ( $p < 0.0001$ ), CXBI ( $p < 0.0001$ ), B6.Cb4i5- $\alpha$ 10/Vad ( $p < 0.001$ ), B6.Cb4i5- $\alpha$ 11/Vad ( $p < 0.001$ ) and, B6.Cb4i5- $\alpha$ 13/Vad ( $p < 0.01$ ) expressed significantly lower ethanol preference (AP\_3 and AP\_4) than B6. Interestingly, two RQI strains [B6.Cb4i5- $\beta$ 13C/Vad ( $p < 0.0001$ ) and B6.Cb4i5- $\beta$ 14/Vad ( $p < 0.01$ )], carrying introgressed chromosome segments from the low ethanol preference BALB/cJ donor strain, expressed significantly higher ethanol preference than the background B6.

Alcohol preference in the 2-h test (AP\_BAL) and BAL showed significant between-strain correlation ( $r = 0.88$ ;  $p < 0.001$ ,  $N = 15$ , Fig. 1). Analyzing the entire population, we found a significant correlation (0.74;  $p < 0.001$ ,  $N = 348$ ) between AP\_BAL and BAL.

The blood alcohol level after the 2-h test was significantly correlated with AP\_BAL ( $r = 0.87$ ,  $p < 0.001$ ), AP\_3 ( $r = 0.74$ ,  $p < 0.001$ ) and AP\_4 ( $r = 0.79$ ,  $p < 0.001$ ).

The average amount of introgressed donor genome is shown in Table 2. In the b4i5 series we found 4.4% (average) BALB/c donor loci (maximum 7.3%, minimum 1.2%). In the CXBI strain the proportion of BALB/cJ markers was much higher (42.5%) than that in the RQI strains. The average level of heterozygosity was 0.8%, with 0% being the lowest (5 RQI strains) and 5.4% the highest in B6.Cb4i5- $\alpha$ 6/Vad. All markers were of background type on chromosomes 6, 11, and X.

In the b4i5 RQI strains, the total numbers of segments carrying homozygous and heterozygous donor loci were 75

Table 1

Alcohol preference ratio (weight of 3%, 6%, or 12% ethanol solution consumed divided by total fluid weight consumed) in genotyped quasi-congenic RQI strains and in their progenitor strains

Strain	AP_1 (3%, M)			AP_2 (6%, M+F)			AP_3 (12%, M)			AP_4 (12%, M)		
	Mean	N	SD	Mean	N	SD	Mean	N	SD	Mean	N	SD
C57BL/6ByJ	0.53	53	0.38	0.67	49	0.25	0.42	52	0.28	0.47	52	0.30
BALB/cJ	0.14	43	0.23	0.17	42	0.21	0.02*	42	0.05	0.02*	42	0.05
CXBI	0.46	45	0.38	0.37	40	0.30	0.08*	44	0.10	0.06*	44	0.08
B6.Cb4i5- $\alpha$ 10/Vad	0.39	27	0.44	0.41	23	0.31	0.21*	27	0.21	0.26*	27	0.28
B6.Cb4i5- $\alpha$ 11/Vad	0.31	35	0.38	0.57	28	0.33	0.25*	33	0.25	0.30*	33	0.25
B6.Cb4i5- $\alpha$ 12/Vad	0.48	30	0.41	0.51	26	0.33	0.42	27	0.24	0.47	27	0.27
B6.Cb4i5- $\alpha$ 13/Vad	0.53	22	0.35	0.54	19	0.29	0.22*	22	0.18	0.30*	22	0.30
B6.Cb4i5- $\alpha$ 4/Vad	0.48	25	0.36	0.57	20	0.27	0.38	24	0.27	0.41	24	0.27
B6.Cb4i5- $\alpha$ 6/Vad	0.54	26	0.38	0.58	23	0.27	0.51	26	0.32	0.41	26	0.27
B6.Cb4i5- $\alpha$ 8/Vad	0.48	29	0.43	0.53	21	0.29	0.41	25	0.28	0.42	25	0.27
B6.Cb4i5- $\beta$ 10/Vad	0.47	26	0.40	0.59	20	0.28	0.26*	26	0.21	0.37	26	0.26
B6.Cb4i5- $\beta$ 13C/Vad	0.64	29	0.38	0.64	26	0.34	0.72*	27	0.29	0.70*	27	0.28
B6.Cb4i5- $\beta$ 14/Vad	0.47	23	0.42	0.84	23	0.20	0.59*	23	0.25	0.74*	23	0.23
B6.Cb4i5- $\beta$ 4A/Vad	0.57	21	0.44	0.63	18	0.29	0.37	20	0.26	0.35	20	0.22
B6.Cb4i5- $\beta$ 9/Vad	0.53	22	0.37	0.59	19	0.32	0.40	22	0.26	0.49	22	0.28
B6.Cb4i7- $\alpha$ 16at/Vad	0.47	16	0.41	0.60	17	0.31	0.35	17	0.32	0.38	17	0.29

M = male; F = female; AP\_1, AP\_2, AP\_3, and AP\_4 = alcohol preference in trial 1, trial 2, trial 3, and trial 4, respectively.

\*indicates significant differences from B6 ( $p < 0.05$ ).

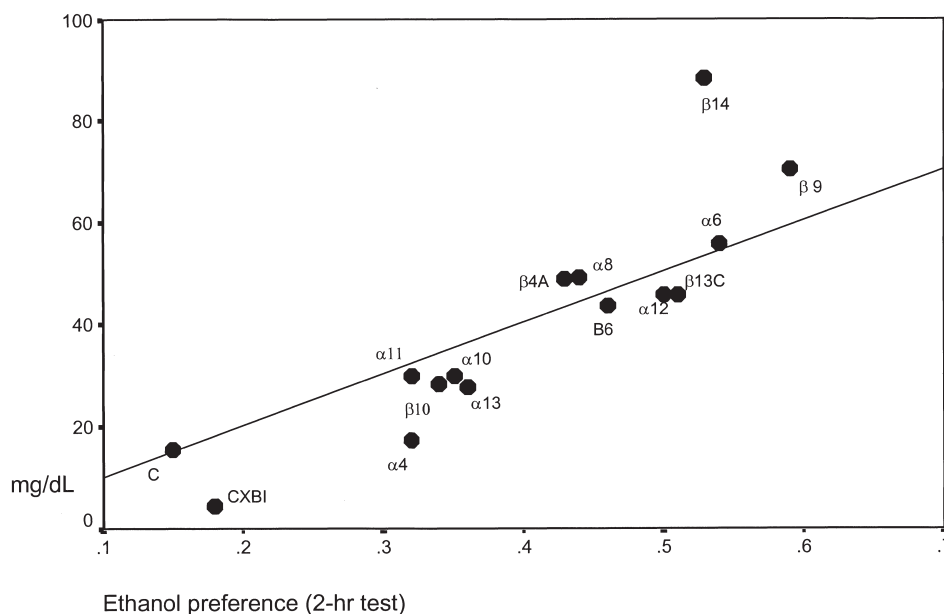


Fig. 1. Scatterplot of RQI and progenitor strains. Ethanol preference in a short-term (2-h) test showed significant between-strain correlation ( $r = 0.88$ ;  $p < 0.0001$ ,  $n = 15$ ) with blood ethanol level (mg/dL) at the end of the test.

and 9, respectively. The results indicate that in each RQI strain only a limited number of chromosomes (3–6 per RQI strain) were carriers, while the rest of the chromosomes were of background type. Typically, we found 1–2 segments on each carrier chromosome.

The estimated mean donor chromosome lengths of homozygous and heterozygous loci were 11.11 and 12.33 cM, respectively (Fig. 2). The smallest and largest estimated segment sizes were 1 and 45 cM, respectively. Because the current average marker density is about 6.6 cM, a small proportion of the introgressed donor genome might have gone undetected.

Table 2  
Proportion of homozygous donor-type loci and heterozygous loci in RQI strains

Strain	Homozygous donor loci (%)	Heterozygous loci (%)
C57BL/6ByJ	0.00	0.00
BALB/cJ	100.00	0.00
CXBI	42.50	1.66
B6.Cb4i5- $\alpha$ 10/Vad	3.67	0.42
B6.Cb4i5- $\alpha$ 11/Vad	4.08	0.42
B6.Cb4i5- $\alpha$ 12/Vad	2.87	0.42
B6.Cb4i5- $\alpha$ 13/Vad	6.12	0.85
B6.Cb4i5- $\alpha$ 4/Vad	6.94	1.28
B6.Cb4i5- $\alpha$ 6/Vad	4.08	5.44
B6.Cb4i5- $\alpha$ 8/Vad	7.35	0.43
B6.Cb4i5- $\beta$ 10/Vad	3.67	0.00
B6.Cb4i5- $\beta$ 13C/Vad	4.47	0.00
B6.Cb4i5- $\beta$ 14/Vad	5.69	0.00
B6.Cb4i5- $\beta$ 4A/Vad	1.22	0.00
B6.Cb4i5- $\beta$ 9/Vad	2.49	0.00
B6.Cb4i7- $\alpha$ 16at/Vad	3.69	1.68
Mean (RQI strains only)	4.39	0.77

To designate QTL-carrying regions, regression analysis was performed relying on the quantitative value of strain phenotypic mean and on the marker genotype, using QGene (Nelson, 1997) (Fig. 3). Our primary interest was to map QTLs that affect 12% ethanol preference; therefore, this test was done in duplicate (AP\_3 and AP\_4). The two donor strains were omitted from the regression analysis. A threshold for genome-wide significance of a single marker was set as  $p < 0.05$ .

For AP\_3, a block of three chr. 15 markers, on an estimated 6.5 cM-long region (49.7–56.2 cM, Chromosome Committee map position) were the most significant ( $p < 0.002$ ,  $R^2 = 0.58$ ,  $F = 16.79$ ). Although 244 markers were tested as polymorphic for the 16 strains, after excluding the donor strains (C and I), the number of markers polymorphic between B6 and the RQI strains, was only 73. After correcting for 73 comparisons, the genome-wide significance was  $p > 0.05$ . *D17Mit129* was also significant ( $p < 0.005$ ,  $R^2 = 0.52$ ,  $F = 13.15$ ), but after correction for multiple comparisons the marker association with alcohol preference was not significant. The 95th percentile of the  $F$  values yielded by permutation for the above chr. 15 markers was  $F = 4.19$ , for *D17Mit129* was  $F = 7.14$ , while the experimentwise 95th percentile was  $F = 24.44$ , indicating that the permutation test supports the results of the regression test and that of the corrections.

For AP\_4 the same three chr. 15 markers (*D15Mit159*, *D15Mit34*, and *D15Mit242*) emerged as significant ( $p < 0.0002$ ,  $R^2 = 0.74$ ,  $F = 35.97$ ). After correction for multiple comparisons the genome-wide significance was  $p < 0.01$ . The next most significant markers were *D1Mit135* and *D1Mit311* ( $p < 0.02$ ,  $F = 8.78$ ), which were not significant after correction for multiple comparisons. The 95th percentile of the  $F$  values yielded by permutation for the above

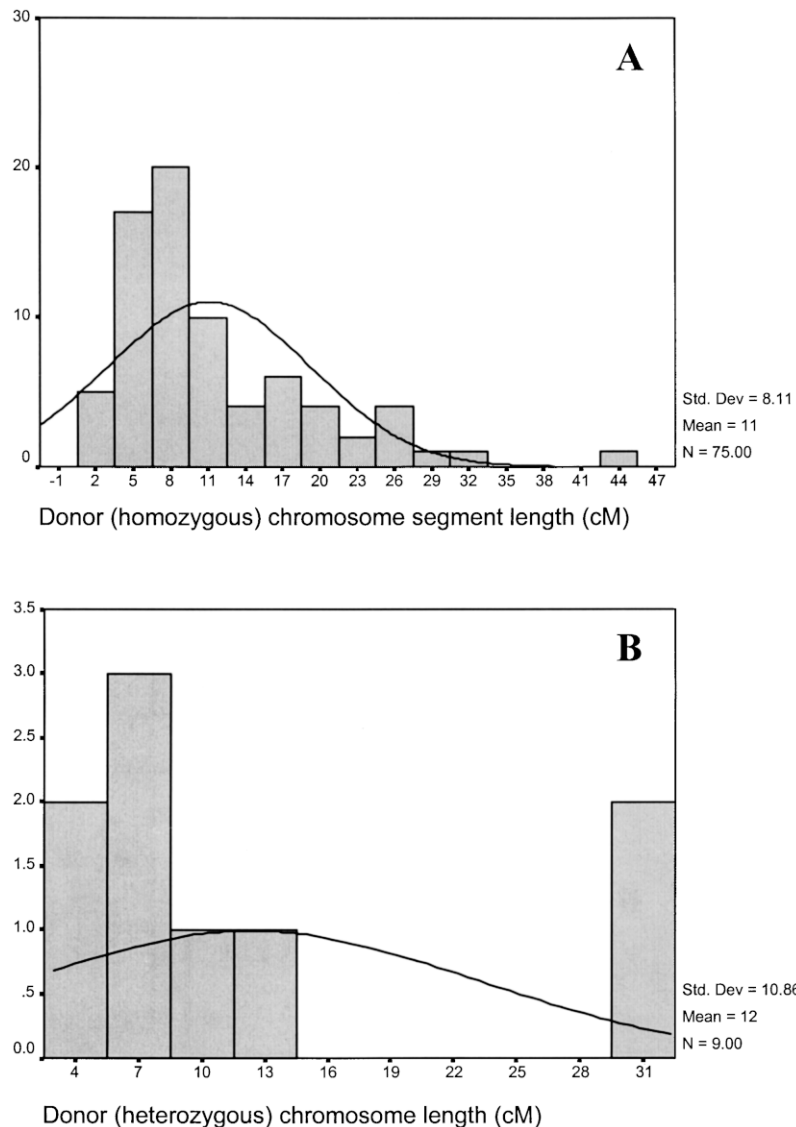


Fig. 2. Chromosome segment length distribution in RQI strains carrying homozygous (A) or heterozygous (B) donor loci.

three chr. 15 markers was  $F = 3.61$  (compare to  $F = 35.97$ ), for *D1Mit135* and *D1Mit311* was  $F = 3.84$  (compare to  $F = 8.78$ ), for *D17Mit129* was  $F = 7.14$  (compare to  $F = 13.03$ ), for *D18Mit144* was  $F = 3.86$  (compare to  $F = 4.19$ ), while the experiment-wise 95th percentile, yielded by permutation, was  $F = 35.97$ . Thus, the permutation test offered some evidence for the assignment of genome-wide significance of the above chr. 15 markers, while the other markers reached only point-wise significance.

Distribution of BALB/c-type markers in quasi-congenic strains that significantly differ in ethanol preference from the B6 background strain is shown in Table 3.

The RQI strains were developed by introgressing QTLs for a brain dopamine system-related trait (mesencephalic tyrosine hydroxylase activity, TH/MES). Because dopamine has been implicated in substance abuse (Koob & Bloom, 1988; Koob et al., 1998), we performed a correlation analysis using our previously published TH/MES data (Vadasz et

al., 1998) and the ethanol preference data (present report). Strain means for TH/MES were not significantly correlated with AP\_3 ( $r = -0.001$ ,  $p > 0.05$ ) and AP\_4 ( $r = 0.078$ ,  $p > 0.05$ ).

## 4. Discussion

### 4.1. Ethanol preference in progenitor and RQI strains

Results of the analyses of inter-batch variability suggest that AP\_3 and AP\_4 are valid and reliable measures of alcohol preference. Male mice of the C57BL/6By strain, a sub-strain of C57BL/6, did not show the anticipated high level of ethanol preference characteristic of C57BL/6 animals (0.7–0.8) (McClern & Rodgers, 1959). BALB/cJ mice, however, as anticipated, showed very low preference for 12% ethanol. These differences may be attributed to the higher ethanol concentration used in our study, and to the

Table 3

Number of donor segments and donor-type markers in four RQI strains that significantly differ in ethanol preference from the B6 background strain

Strain	Chr.	Segm.	Marker
B6.Cb4i5- $\alpha$ 10/Vad (low ethanol preference)	2	2	D2Mit365-D2Mit372; D2Mit224
	6	2	D6Mit86; D6Mit228-D6Mit19
	7	1	D7Mit100-D7Mit105-D7Mit373
B6.Cb4i5- $\alpha$ 11/Vad (low ethanol preference)	2	1	D2Mit372
	5	1	D5Mit240
	9	1	D9Mit4-D9Mit54-D9Mit196
	13	2	D13Mit275-D13Mit88; D13Mit53-D13Mit371
	17	1	D17Mit127
B6.Cb4i5- $\beta$ 13C/Vad (high ethanol preference)	1	1	D1Mit135-D1Mit311
	7	1	D7Mit124-D7Mit100-D7Mit105-D7Mit371
	15	1	D15Mit159-D15Mit34-D15Mit242
	17	1	D17Mit129
	18	1	D18Mit144
	19	4	D19Mit46; D19Mit65; D19Mit37; D19Mit34
B6.Cb4i5- $\beta$ 14/Vad (high ethanol preference)	1	1	D1Mit135-D1Mit311
	7	1	D7Mit124-D7Mit100-D7Mit105-D7Mit371-D7Mit47
	8	1	D8Mit100-D8Mit249
	10	1	D10Mit233
	12	1	D12Mit263
	15	1	D15Mit159-D15Mit34-D15Mit242

genetic differences between the sublimes C57BL/6J and C57BL/6By. Our other donor strain, CXBI, also showed a low level of ethanol preference, however, for this strain, the relatively high total liquid volume consumed could also be considered as a contributing factor. To our best knowledge, this is the first report using C57BL/6By and CXBI in alcohol preference experiments. Because CXBI is one of the CXB RI strains, and the progenitor strains for this set were C57BL/6By and BALB/cBy, the expected proportion of the BALB/c donor loci is 50%, which corresponds well to the observed 42.5%. The CXB set is a potentially interesting tool for mapping or confirmation, although its usefulness is limited by the small number of RI strains ( $n = 13$ ). Also, this finding suggests that future QTL mapping with the B6.I quasi-congenic RQI strains is feasible because we may expect significant strain-dependent variation in alcohol preference.

The broad-sense heritability of ethanol preference for 12% ethanol solution was 0.47. This measure of heritability does not reflect dominance effects, but includes inter-locus interaction effects, and, as with other measures of heritability, it is specific to this set of strains. Recently Rodriguez et al. (1994) reported that estimates of narrow-sense heritability for acceptance and preference of 10% ethanol in BXD recombinant inbred strains were 0.19 and 0.20, respectively, while Gill et al., found that the narrow-sense heritability for preference of 10% alcohol solution was 0.33 in both males and females (Gill et al., 1998).

Interestingly, two RQI strains [B6.Cb<sub>4i5</sub>- $\beta$ 13C/Vad ( $p < 0.0001$ ) and B6.Cb<sub>4i5</sub>- $\beta$ 14/Vad ( $p < 0.01$ )], carrying introgressed chromosome segments from the low-ethanol preference BALB/cJ donor strain, expressed significantly higher ethanol preference than the background B6. This result suggests genetic interaction between ethanol preference QTL(s) and the background.

#### 4.2. Between-strain ("genetic") correlations

In QTL analysis, multivariate techniques will play an increasingly important role. To this end, as shown by Blizard and Bailey for RI strains (Blizard & Bailey, 1979), between-strain correlation can be used as an estimate for genetic correlation because correlations can be based on precise phenotypic mean values, and the approach lends itself easily to multivariate genetic analysis (Vadasz et al., 1982).

Originally the RQI strains were developed by selection for a dopamine-system related phenotype, TH/MES. We found no significant between-strain correlation between TH/MES and alcohol preference. However, this does not indicate that the dopamine system and alcohol preference are genetically independent. TH/MES is a complex trait, and it is only one aspect of the dopamine system, and in terms of dopamine neurotransmitter function, the consequences of genetic variation of TH/MES are not completely understood.

#### 4.3. Proportion of introgressed donor genome and residual heterozygosity

The estimated mean donor chromosome lengths of homozygous and heterozygous loci were 11.11 and 12.33 cM, respectively (Fig. 2). The smallest and largest estimated segment sizes were 1 and 45 cM, respectively. Because the current marker density is about 6.6 cM, a small proportion of the introgressed donor genome might have gone undetected. Accordingly, the marker density needs to be increased to achieve a spacing that ensures covering most of the donor genome. Distribution of BALB/c-type markers in RQI is shown in Table 3.

The level of heterozygosity in an inbred strain is a factor that may interfere with the reliability of QTL mapping using

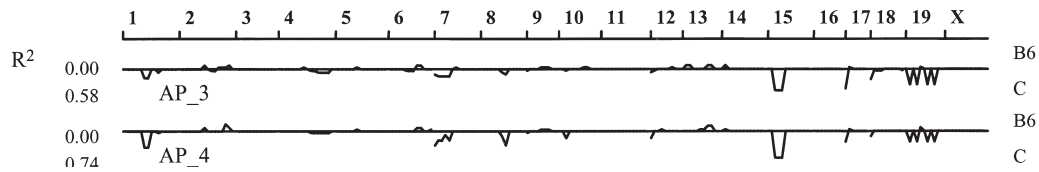


Fig. 3. Regression of strain means on marker genotype. Results of the regression analyses are supplied as a plot of  $R^2$  from the regression performed with the QGene computer program for preference of 12% alcohol solution in trial 3 (AP\_3) and trial 4 (AP\_4). The  $R^2$  score scale is along the y axis. The highest  $R^2$  scores for AP\_3 and AP\_4 (0.58 and 0.74, respectively) were obtained with Chr. 15 markers. Above the plots the positions of the 20 chromosomes (representing 244 microsatellite markers) are shown. Downward peaks indicate the parent (BALB/cJ) whose marker allele at the corresponding locus is associated with the higher mean for the phenotype.

sets of RI or RQI strains. In 1997, at the time of taking the tissue samples for genotyping, B6.Cb<sub>4</sub>i<sub>5</sub>- $\alpha$ 6/Vad was still segregating at various microsatellite loci after 30 generations of brother X sister matings (Table 3). In a new inbred strain a low level of heterozygosity can be expected because of residual heterozygosity. In addition, the contribution of new mutations may even allow selection for quantitative traits in inbred strains with modest divergence (Knightly, 1998). The relatively high level of heterozygosity in B6.Cb<sub>4</sub>i<sub>5</sub>- $\alpha$ 6/Vad may also be attributed to the selective advantage of the heterozygous condition at certain loci. In a recent study (Vadasz et al., 1998) we found that B6.Cb<sub>4</sub>i<sub>5</sub>- $\alpha$ 6/Vad expressed the highest level of TH/MES among the RQI strains. If high TH/MES is genetically correlated with maladaptive physiological changes (e.g., disturbance in the central dopamine system, stress sensitivity, reproduction), heterozygotes with lower TH/MES may have selective advantage. The detected residual polymorphism limits the experiment's probability of detecting linkages. Future availability of sufficiently large SNP sets may alleviate the problem of microsatellite mutation.

#### 4.4. QTL mapping

For genotyping, the current study used the B6 background, the C and I donor strains, and 13 RQI strains. All 16 strains were typed at microsatellite loci spaced in the genome on the average at 6.6 cM apart.

Potential QTL-carrying chromosome segments can be roughly outlined by RQI-vs.-background contrast, which is not a mapping method, just an orienting tool, and it is obvious that many of the differential markers are not ethanol-preference-related. This simple comparison takes advantage of the genomic similarity and the significant phenotypic dissimilarity between a selected RQI and its background strain.

The X chromosome in these RQI strains carried no donor markers, as expected. Chromosomes 6 and 11 were also of background type at the current marker density. Possibly some smaller donor segments were not exposed by this genome scan, or these chromosomes are void of donor segments. This outcome underscores the importance to a QTL search of generating sufficient strains that every region in the genome is represented with donor material in at least a few strains.

The analysis was restricted to B6 and RQI strains because of their similar genetic background. The reason for the restriction is that a QTL effect on a phenotype has a better chance for being detected if the rest of the genome is kept constant (invariant) as much as possible, to eliminate genetic interaction with other loci whose genotype would vary from strain to strain in a heterogeneous genetic background. The genome scan with 244 markers suggests that on average each RQI strain of the b<sub>4</sub>i<sub>5</sub> series has about 95% genomic similarity to the B6 background strain. In comparison, two RI strains, on the average, share 50% of their genome. Thus, it is plausible that the probability of interactions is higher in RI strains than in RQI strains, or in recombinant congenic (RC) strains (Fijneman & Demant, 1995).

Are the observed marker associations genetically meaningful? The association of preference with three markers lying on a less than 8-cM stretch of chromosome 15 is based on the co-occurrence, in just two of the 13 RQI strains used in the study, of higher preference score with the BALB/cJ marker genotype at those marker loci. Based on the overall frequency of donor loci in the dataset, any given locus had a 0.044 probability of having the donor genotype in a given strain, and thus in any two given strains an 0.0012 probability of sharing the donor genotype not carried at that locus by the remaining 11 lines. Of all 73 polymorphic loci, the probability that solely by chance at least one locus would have the donor genotype in, and in only, the two high-preference lines is not negligible at 0.08, and it would then not be surprising that closely linked loci should carry the same genotype.

Chromosome 15 markers have already been implicated in earlier mapping studies. Phillips et al. (Phillips et al., 1994) found association between *D15Mit3* (37 cM) and consumption of 10% ethanol in saccharine in females ( $r = -0.65$ ,  $p < 0.01$ ). Rodriguez et al. (1995) found significant correlation between *D15Mit12* (6.4 cM) and alcohol preference ( $r = -0.48$ ;  $p < 0.05$ ), while *D15Bir1* and *D15Mit3* were not significant ( $p > 0.05$ ). A reanalysis of the RI data with a larger marker set (>1500) and expressing the results in terms of an alcohol preference ratio confirmed the association with chromosome 15, but indicated a different region (*Hoxc*, 48 cM, (Tarantino et al., 1998)). The same authors in their alcohol preference confirmation study, using an F2

generation derived from C57BL/6 and DBA/2, did not detect chromosome 15 effects on alcohol preference (Tarantino et al., 1998). Belknap et al. (1997), based on selective breeding experiments, reported a presumed chromosome 15 QTL represented by *D15Mit33* (48.6 cM,  $p = 0.002$ ; LOD 2.4), which did not reach genome-wide significance as defined in their study. They also genotyped *D15Mit34*, which was not significant ( $p > 0.1$ ). Recently, B6D2F<sub>2</sub> mice were utilized in a verification testing strategy, in which suggestive verification was obtained for QTLs on chromosome 15 (*D15Mit33*, 48.6 cM,  $p < 0.01$ ) (Phillips et al., 1998).

Significant associations were found on chromosome 15 for alcohol acceptance, another operational measure of alcohol consumption (Rodriguez et al., 1995). In BXD RI strains, the highest correlations were detected in the central region of the chromosome (between 30 and 40 cM), and a sharp but smaller peak was shown at approximately 48 cM (McClearn et al., 1997; Rodriguez et al., 1995). The putative QTL on chromosome 15 was further examined by McClearn et al. (1997). F<sub>2</sub> interval mapping results for males indicated peak LOD scores (LOD  $> 3.0$ ) at approximately 17 cM, and additional significant LOD scores in the region of 30 cM. Haplotype analysis of genotypically selected offspring supported the hypothesis of a chromosome 15 QTL between 26.1 and 37.6 cM. In an earlier study on alcohol acceptance, Crabbe et al. did not report provisional QTL location on chromosome 15 (Crabbe et al., 1983); however, reanalysis of the original data with a greater number of markers provided suggestive evidence for a correlation between alcohol acceptance and *D15Ncvs21* at 53 cM. Gehle and Erwin (1998) studied the LS x SS RI strains, which exhibit phenotypic extremes in alcohol consumption comparable to the C57BL/6 and DBA/2 mice. Confirming Phillips' results, mapping identified a putative QTL for consumption of 10% ethanol associated with *D15Mit3* in females ( $p < 0.02$ ), but not in males.

Our results support the hypothesis of a QTL on chromosome 15 for ethanol preference. The provisional QTL location (49–57 cM) is compatible with associations found between ethanol preference and *D15Mit33* [48.6 cM, (Phillips et al., 1998)], with the location of a smaller peak of correlation coefficients at about 48 cM in the BXD RI analysis of alcohol acceptance [Fig. 1, Panel b of McClearn et al. (1997)], and with the results of the reanalysis (McClearn et al., 1997) of an alcohol acceptance study (Crabbe et al., 1983) implicating *D15Ncvs21* (53 cM). Developmental studies on a chromosome 15 QTL, which appeared sex-specific, suggested that the effect on alcohol acceptance, observable at about 100 days of age, diminished or disappeared at 300 days of age (McClearn et al., 1998).

QTL analysis did not provide putative chromosome locations with genome-wide significance for BALB/c alleles, which decrease alcohol preference, although we have solid evidence that some of the quasi-congenic RQI strains exhibit significantly lower ethanol preference than that of the B6 background strain. Lack of such results indicates the

need for a denser marker characterization of the RQI strains and for the inclusion of a larger number of RQI strains in the mapping studies.

QTL mapping of alcohol-related behaviors is also in progress in humans and rats. A recent report by the Collaborative Study of the Genetics of Alcoholism (COGA) centers (Reich et al., 1998) provided suggestions of loci on human chromosomes 1, 7, and 2. Database search indicated that none of the involved genomic regions are homologous to segments on mouse chr. 15 (<http://www.ncbi.nlm.nih.gov>, and <http://www.informatics.jax.org>). Mapping experiments with alcohol-preferring (P) and alcohol-nonpreferring (NP) rats identified a region on chr. 4, near to the locus for neuropeptide Y (Carr et al., 1998). Rat chr. 4 does not carry regions that are homologous to segments on mouse chr. 15 (<http://www.informatics.jax.org>).

In the 49–57 cM region of mouse chr. 15 there are genes whose function might be relevant to ethanol preference or acceptance. Platelet-derived growth factor, contactin, kinesin, transcription factor (POU domain, class 6), and aquaporin 5 may influence alcohol-related behaviors through their effects on neurodevelopment, neurotransmitter function, and liquid balance.

In summary, we found significant association between chromosome 15 markers and ethanol preference, suggesting the possibility of a QTL (or QTLs) in the 49–57 cM region for this phenotype. All results point to caution in assigning more than suggestive significance to putative QTL associations in this small strain set. Although other markers did not reach the  $p < 0.05$  level of genome-wide significance, the mapping results suggest that chrs. 1, 2, 4, 8, 9, 13, 17, 18, and 19 deserve further attention. As with other similar QTL mapping studies, the designation of QTLs should be regarded as provisional and in need of confirmation. Experiments with RQI strains of the b<sub>5</sub>i<sub>7</sub> series, which comprises nearly 100 strains, is in progress. In future studies, towards the isolation of genes for alcohol preference, the characterized quasi-congenic strains can be used. This approach requires the saturation of the target regions with markers to map the introgressed donor segments at high resolution, the generation of backcross or F<sub>2</sub> populations between the background strain and specific RQI strains, the identification of recombinants, and testing of the recombinants for alcohol preference in sufficient numbers. Following this strategy, selective backcrossing of recombinants can narrow down the target region to a  $< 1$ cM interval allowing the identification of candidate genes and positional cloning of QTLs.

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