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Identification of QTLs Influencing Alcohol Preference in the High Alcohol Preferring (HAP) and Low Alcohol Preferring (LAP) Mouse Lines

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The High- and Low-Alcohol Preferring (HAP1/LAP1 and HAP2/LAP2) mouse lines were developed by selective breeding for differences in alcohol preference. They represent the only extant selectively bred mouse lines developed for this alcohol phenotype. Therefore, they provide a unique resource for QTL detection and mapping. Importantly, neither of the replicate lines is inbred and therefore, novel study designs can be employed to detect loci contributing to alcohol preference. Two independent studies, with very different approaches, were conducted in the HAP and LAP replicate lines. In *Study 1*, microsatellite markers were genotyped in the replicate HAP1/LAP1 and HAP2/LAP2 mice in QTL regions nominated by other mouse RI and F2 studies in order to detect divergence of allele frequencies in the two oppositely selected lines. Significant differences in allele frequencies were observed in the HAP1/LAP1 mice with markers on chromosome 9 ($p < 0.01$). In the HAP2/LAP2 mice, significant differences in allele frequencies were identified on chromosomes 2 and 9 ($p < 0.01$). In *Study 2*, a genome-wide screen was performed in a sample of 432 HAP1×LAP1 F2 animals and a QTL on chromosome 9 (LOD = 5.04) was found which met criteria for genome wide significance ($p < 0.001$). Gender specific analyses supported a greater effect of the QTL among female mice (LOD = 5.19; $p < 0.0008$) than male mice (LOD = 1.19). This study provides additional evidence and confirmation that specific regions on chromosomes 9 and perhaps 2 are important for alcohol preference.

KEY WORDS: Alcohol preference; HAP and LAP mice; QTL; selective breeding.

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BACKGROUND

The contribution of genetic factors to alcohol use and the etiology of human alcoholism is well documented. There is also a strong genetic component in the response to alcohol in animals. This verity has given investigators the tools to develop animal models with phenotypic traits similar to that seen in human alcoholism. These animal models may provide important genetic clues to improve the efficiency with which genes underlying human alcohol-seeking behavior can be identified.

To identify loci that influence alcohol preference, investigators have most commonly used populations

derived from the C57BL/6J (B6) and DBA/2J (D2) inbred mouse strains. It is well known that the C57BL/6J mice drink alcohol whereas the DBA/2J mice avoid it. The genetic differences between these two strains are the best characterized in pharmacogenetic research (Phillips *et al*., 1994). Therefore, they have proven invaluable for identifying chromosomal loci that may influence alcohol related phenotypes. However, an inbred strain samples only a fraction of the genetic variance that was present in the original population from which the strains were derived. Thus, a QTL found in a single inbred cross does not represent the total number of QTL that can segregate with a phenotype, nor is it a guide to which of the QTL are most common in an outbred population (Flint and Mott, 2001). Therefore, QTL derived from a few inbred crosses cannot adequately represent the homologous locations in humans.

Another approach would be to perform QTL analysis in a population in which the genetic heterogeneity has been preserved. A good example would be to use non-inbred lines that have been selectively bred for the phenotypic trait of interest. The selection process maximizes differences in trait-relevant alleles, while minimizing differences between lines in alleles unrelated to the selection phenotype (Grahame *et al*., 1999). And, because non-inbred lines are developed from a more heterogeneous background, they offer substantially more genetic diversity than would be found in most inbred strains and would thereby increase the number of trait-relevant loci (QTLs) upon which selection can act.

One preference drinking animal model developed by selective breeding is the non-inbred High- and Low-Alcohol Preferring (HAP and LAP) mice that differ greatly in voluntary consumption of 10% (v/v) ethanol in a standard two-bottle choice (ethanol *versus* water) procedure (Grahame *et al*., 1999). The HAP and LAP mice originated from a colony of HS/Ibg mice that were maintained at the Institute for Behavioral Genetics in Boulder, Colorado. This heterogeneous stock, with a wide range of alcohol preference, was developed by intercrossing eight inbred strains of mice (McClearn *et al*., 1970). The HAP1/LAP1 mice have shown a substantial response to selection. Replicate lines (HAP1/LAP1; HAP2/LAP2) were independently developed for the alcohol preference phenotype. The HAP and LAP mice are the only mice extant that are selectively bred for differences in alcohol preference. Therefore, they provide a unique resource for QTL detection and mapping.

Two independent but complementary studies to identify QTL influencing alcohol preference in the non-inbred HAP/LAP lines were carried out and will be reported in this paper. The purpose of the first study was to confirm QTL that have been previously identified by others to be important for alcohol preference. The purpose of the second study was to perform a genome wide survey to both confirm those chromosomal regions identified in the initial study and to identify novel loci that might be segregating in the HAP and LAP mice.

The first study utilized a powerful source for identifying QTL information and one that has often been overlooked. The strategy is to observe the divergence of allele frequencies in two oppositely selected lines in the early generations of selection, when the magnitude of random genetic drift is small (Belknap *et al*., 1997; Falconer and Mackay, 1996; Keightley and Bulfield, 1993; Lebowitz *et al*., 1987; Nuzhdin *et al*., 1998). Chromosomal regions selected for genotyping and QTL analysis were based on prior alcohol preference QTL research in the BXD RI strains, backcrosses, selected lines, and F2 studies derived from the B6 and D2 inbred mouse strains. This choice was made because of the abundance of published data (eight independent studies) from these two progenitor strains (reviewed by Belknap and Atkins, 2001), and also because these two strains were among the eight strains ancestral to the HS stock from which the HAP/LAP lines were derived. Moreover, among all eight inbred strains ancestral to the HS and thus HAP/LAP mice, the B6 strain showed the highest preference behavior by a large margin compared to the other seven strains, while the D2 strain was among the lowest (Belknap *et al*., 1993; Rodgers, 1972). This greatly increases the probability that alleles from these two strains (especially B6) will also be important in determining differences between the HAP and LAP lines. We therefore hypothesized that many (but not all) of the same QTLs reported by others in B6- and D2-derived populations would also exist in our selected lines. In all cases, only significant QTLs reported by others ($p < 0.0001$), or in the meta-analysis of Belknap and Atkins (2001) across all eight studies, were tested in our selected lines. We also included significant QTLs for alcohol preference drinking from recent rat studies using crosses between P and NP, and also the HAD and LAD selected lines (Bice *et al*., 1998; Foroud *et al*., 2000). For the rat studies, we tested chromosomal regions in the mouse that were syntenic with the reported map location of the rat QTLs.

The second study utilized an F2 intercross to perform a genome-wide screen for QTL that may influence alcohol consumption. To identify the QTLs in a non-inbred population such as the HAP/LAP mice, the segregation of markers and QTLs must be studied in several generations and across several matings. Such studies would typically be performed by crossing HAP and LAP founders to create F1 progeny, which are then intercrossed to generate the F2 sample. Because the parental generation is often heterozygous for the microsatellite markers tested, the F1 animals will not be genetically identical as they are in an inbred study design. This study design is completely analogous with the collection of human pedigree data, in which markers are segregating in all generations and individuals are never homozygous for all marker loci. One difference between the human data and the non-inbred HAP/LAP model is the assumption regarding QTL fixation. Whereas it is extremely unlikely that a human population could be found in which all or even most of the relevant QTLs for a phenotype have been fixed, selection for alcohol preference in the HAP/LAP model is likely to have fixed most of the QTLs of major effect, increasing the power for QTL detection.

MATERIALS AND METHODS

Sample

The sample for *Study 1* consisted 96 HAP1/LAP1 mice in the phenotypic extremes of the 12th generation. They included 48 HAP1 mice (24 males and 24 females) with ethanol drinking scores ranging from 11.6 to 22.25 g/kg/day and 48 LAP1 mice (24 males and 24 females) with ethanol drinking scores ranging from 0.63 to 1.09 g/kg/day. Additionally, HAP2/LAP2 mice in the phenotypic extreme of the 3rd and 6th generations were also used. Because the allele frequency of the parents provides an unbiased estimate of the frequency expected in the offspring, only the frequency of the breeders was examined for the 3rd and 6th generations. The mice in the 3rd generation included 22 HAP2 mice with ethanol drinking scores ranging from 5.47 to 16.64 g/kg/day and 26 LAP2 mice with ethanol drinking scores ranging from 0.87 to 1.63 g/kg/day. The animals in the 6th generation included 24 HAP2 mice with ethanol drinking scores ranging from 6.69 to 19.33 g/kg/day and 20 LAP2 mice with ethanol drinking scores ranging from 0.82 to 1.26 g/kg/day.

In *Study 2*, for the genome-wide screen, 4 reciprocal crosses of the HAP1 and LAP1 mice, taken from generation 23, were performed. Six pairs of F1 progeny from each of the 4 crosses were intercrossed to produce 432 F2 animals. The progenitors, F1, and F2 mice were genotyped for this study.

Phenotypic Measure

To determine ethanol consumption scores (*Studies 1 and 2*), mice at 45 days of age were individually caged and allowed to drink from two 25 mL-graduated cylinders. One cylinder contained 10% ethanol (v/v) and the other contained distilled water. Food was available *ad libitum*. The volumes consumed were measured three times per week, and the cylinders containing fluids were switched to control for position bias. Mice were weighed on the first Monday of each week during testing. Testing continued for a period of 4 weeks. Consumption scores were averaged over a period of 4 weeks (for details see Grahame *et al*., 1999).

Chromosomal Regions Selected for Genotyping

In *Study 1*, we chose to examine chromosomal regions that have already been demonstrated to harbor significant QTLs in other studies in mice and rats. For the mouse studies, we looked at QTLs identified in studies of C57BL/6 (B6) and DBA/2 (D2) mice because there were eight independent studies of alcohol preference in the literature to draw upon (reviewed by Belknap and Atkins, 2001). For the rat studies, we tested mouse regions known to show synteny with the rat QTL locations using the Ensembl database (http://www.ensembl.org). Table I shows those QTL regions that met these criteria, and thus were tested in this study.

Genetic Typing Methods

DNA Isolation

For both studies, DNA was isolated using the Puregene kit (Gentra Systems, Minneapolis, MN) according to the manufacturer's protocol.

PCR Reactions

For *Study 1*, the protocol and procedures for the PCR and gel electrophoresis have been published elsewhere (see Bice *et al*., 1998); microsatellite

Table I. Known QTL Regions Based on Prior studies that were Specifically Examined in the HAP1/LAP1 (S12) and HAP2/LAP2 (S3 and S6) Selection Lines

Chromosome	Mapping population	Map position (cM)	QTL	Candidate gene(s)	Reference
Mouse					
1	B6D2F$_2$	peak 60.0	*Ap1q*	*Htr5b, Acrd, Acrg*	Tarantino *et al.* (1998)
2	B6D2F$_2$	peak 34.0	*Ap2q*	*Scn1a-9a*	Tarantino *et al.* (1998)
2	BC to B6	peak 37.0	*Alcp1*	*Scn1a-9a*	Melo *et al.* (1996)
2	B6D2F$_2$	28.0	*D2Mit7*	*Scn1a-9a*	Phillips *et al.* (1998)
3	B6D2F$_2$	peak 49.0	*Ap6q*	*Adh1*	Tarantino *et al.* (1998)
3	Selected Lines	71.8	*D3Mit17*	*Adh1*	Belknap *et al.* (1977)
4	B6D2F$_2$	peak 81.0	*Ap3q*	*Htr1d, Aldh5a1, sac*	Tarantino *et al.* (1998)
9	B6D2F$_2$	9.0	*D9Mit90*	*Htr1b, Drd2*	Phillips *et al.* (1998)
9	B6D2F$_2$	17.0	*D9Mit91*	*Htr1b, Drd2*	Phillips *et al.* (1998)
9	B6D2F$_2$	29.0	*D9Mit4*	*Htr1b, Drd2*	Phillips *et al.* (1998)
9	B6D2F$_2$	35.0	*D9Mit144*	*Htr1b, Drd2*	Phillips *et al.* (1998)
9	B6D2F$_2$	42.0	*D9Mit8*	*Htr1b, Drd2*	Phillips *et al.* (1998)
9	B6D2F$_2$	peak 26.0	*Ap5q*	*Htr1b, Drd2*	Tarantino *et al.* (1998)
Rat					
3 (mouse 2)	P/NP F$_2$		*D3Mit10-D3Rat49*	*Nr4a2, Scn1a-9a*	Bice *et al.* (1998)
4 (mouse 6)	P/NP F$_2$		*D4Rat34*	*NPY, Snca, Crhr2*	Carr *et al.* (1998)
4 (mouse 6)	HAD/LAD F$_2$		*D4Rat34*	*NPY, Snca, Crhr2*	Bice *et al.* (1998)
5 (mouse 4)	HAD/LAD F$_2$		*D5Mgh17*	*Penl1* (proenkephalin)	Foroud *et al.* (2000)
12 (mouse 5)			*D12Rat93*	*Nos1*	Foroud *et al.* (2000)

markers were purchased from Research Genetics (Huntsville, AL). For *Study 2*, microsatellite markers for genotyping were designed by the Center for Inherited Disease Research. They included 134 microsatellite markers that were typed against 54 inbred mouse strains (Witmer *et al.*, 2003). PCR primer sequences were designed for markers selected for multiplexing using the fluorescent dyes FAM, VIC, NED, and ROX. These markers were purchased from Applied Biosystems (Foster City, CA, USA).

The QIAGEN Multiplex PCR Kit was used for all multiplex PCR reactions according to the following protocol: the 11 μL reaction mixture contained: 3.3 μL of 30 ng/μL template DNA, 1.1 μL of primer mix (consisting of a variable number of fluorescently labeled primers diluted to 2 μ*M*, and 5.5 μL of Qiagen Master Mix (Qiagen, Valencia, CA). These reactions were prepared in 384-well plates using a Biomek 2000 Robotic Workstation (Beckman Instruments) and amplified in an iCycler thermocycler (Bio-Rad, Hercules, CA, USA) under the following PCR cycling program: 95_{15m} $(94_{30s}57_{90s}72_{90s})$ $^{35}72_{10m}$.

Fluorescently labeled PCR products were analyzed using the ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the following procedures: (1) 17 μL of a Hi Di Formamide/Rox mixture was prepared (50 μL of Genescan 400 HS Rox size standard per 950 μL of ABI HI-Di Formamide) and aliquoted into a 384-well reaction plate using the Biomek robot, (2) 3 μL of PCR product was added to the plate containing the Formamide/Rox mixture using the Biomek, (3) Plates were denatured for 5 min and then cooled to 4°C for 5 min and (4) Capillary electrophoresis was performed using the ABI 3100 Genetic Analyzer under conditions for microsatellite analysis suggested by the manufacturer. A 36 cm capillary array was used with POP-4 polymer.

To ensure there was no overlap of peaks for size or color, the multiplexing of markers was based on allele sizes and fluorescent label (FAM, VIC, NED). Because markers were typed against 54 inbred strains by the Center for Inherited Disease Research (CIDR), allele size ranges are available and have been published (Witmer *et al.*, 2003).

GENETIC ANALYSIS

Study 1

The QTL analysis method was based on that of Belknap *et al.* (1997) and Lebowitz *et al.* (1987) designed for the early generations of selection that show marked differences in the trait under selection. Evidence for the presence of a QTL was obtained from the difference in relative allele frequencies between the HAP and LAP lines at a marker ($\delta = q_H - q_L$)

exceeding that expected from random drift and sampling error. Most markers showed only two alleles, but when three alleles were encountered, the allele showing the largest value of δ was used in the analysis (its frequency was designated as q), and the frequencies of the other remaining alleles were pooled with frequency, p. The value of Z, the normal deviate, was calculated as follows for each marker and used to test for QTL significance.

$$Z = \delta/\sqrt{[2p_0q_0F + p_Hq_H/2n_H + p_Lq_L/2n_L]}, \quad (1)$$

where the first term in the denominator is the expected random drift variance (Falconer and Mackay, 1996), the second and third terms are the variances due to sampling error in the HAP and LAP selection lines, respectively, n_H and n_L are the sample sizes in each line, p_H, q_H, p_L, q_L are the allele frequencies in each line, F is the inbreeding coefficient at a given selected generation (Falconer and Mackay, 1996) and p_0 and q_0 are the initial allele frequencies in the S_0 founding population. The inbreeding coefficients averaged over both lines were 0.06, 0.12 and 0.23 for the S2, S6 and S12 generations, respectively. Asymptotic LOD scores (df = 2) were derived from p values using the expression: LOD = $-\log_{10}(p)$.

Because no genomic DNA samples exist for the foundation population (S0), we chose to estimate the S0 allele frequencies by using the mean of the HAP and LAP allele frequencies in the earliest available generation. Because q_0 was often the most common allele, pooling the remaining alleles as p_0 led to intermediate frequency estimates for both q_0 and p_0. The range of q_0 was from 0.36 to 0.64 for the markers shown in Table II, which results in values of Z in Eq. (1) changing by less than 3% throughout this range. Even if the actual values of q_0 were very much smaller or very much larger than our estimates, our intermediate estimates of q_0 had the effect of decreasing the value of Z from Eq. (1) and thus decreasing the power of the analysis. In other words, the method we used to estimate p_0 and q_0 is deliberately conservative, and has the effect of underestimating the true QTL effect for some if not most QTLs.

We assumed that only high preference predisposing alleles would increase in frequency in the HAP lines, and the opposite would be the case in the LAP lines. Therefore, the direction of the effect was known and one-tailed tests were used. Because we were trying to confirm in our selection lines the existence of QTLs already known to be statistically significant in other mouse populations, we used $p < 0.01$, as recommended by Lander and Kruglyak (1995). This criterion is for confirmation purposes only, and would not be sufficient to establish the existence of a new QTL. For the latter, approximately $p < 0.0001$ would be required.

Study 2

All genotypic data were initially evaluated for non-Mendelian inheritance in the three generations of marker data utilizing the program CRI-MAP. (Green *et al.*, 1990). All marker inconsistencies were reviewed in the laboratory and resolved. The data from this study were then utilized to construct recombination-based marker maps using the program CRI-MAP (Green *et al.*, 1990). Marker maps were compared to published maps to confirm marker order and distances. The genotypic data from the F2 animals were also reviewed to identify double recombinants, which could inflate inter-marker distances. Due to the variable heterozygosity of the markers, pedigrees and F1 inter-crosses were evaluated to ensure multiple adjacent markers were informative.

Linkage analysis to identify QTLs in the selectively genotyped HAP1×LAP1 F2s was performed using the least squares method developed by Haley *et al.* (1994) specifically for linkage analysis in non-inbred animal lines and implemented in the program QTL Express (Seaton *et al.*, 2002). Analysis was performed at 1 cM intervals along each chromosome and an F test statistic with two degrees of freedom was used to evaluate the evidence for a putative QTL.

Six HAP1/LAP1 progenitors were used to generate the 432 F2 offspring; therefore, each F2 mouse was assigned to one of four pedigrees. Because a regression method of linkage analysis was employed, we included in the model the effect of pedigree as well as gender in order to increase our power to detect QTLs.

In order to evaluate the genome-wide significance of the linkage results in the full sample of F2 progeny, the permutation method of Doerge and Churchill (1996) was utilized. A total of 10,000 replicates were generated with the pedigree structure and marker data held constant while the alcohol consumption scores were randomly assigned among the F2 progeny. The replicates were then analyzed to detect linkage in the same way as was performed in

Table II. Marker Loci associated with 10% ethanol preference drinking in the HAP1/LAP1 or HAP2/LAP2 selectively bred mouse lines

		HAP1/LAP1	HAP2/LAP2	HAP2/LAP2	Combined *p*(LOD)	
Locus	cM	S12 *p* (Freq *q*)	S3 *p* (Freq *q*)	S6 *p* (Freq *q*)	S12/S3	S12/S6
D1Mit506	86.6	$p=0.075$ (0.583/0.635)	**$p=0.035$ (1.0/0.771)**	$p=0.035$ (0.958/0.550)	$p=0.018$ (1.2)	$p=0.018$ (1.2)
D2Mit37	45.0	$p=0.17$ (0.479/0.585)	$p=0.0039$ (0.409/0.904)	$p=0.056$ (0.604/0.925)	**$p=0.005$ (1.7)**	$p=0.053$ (0.81)
D3Mit349	66.2	$p=0.09$ (0.656/1.0)	$p=0.129$ (0.25/0.08)	$p=0.057$ (0.348/0.025)	$p=0.06$ (0.75)	$p=0.032$ (1.0)
D3Mit19	87.6	$p=0.012$ (0.198/0.948)	$p=0.227$ (0.295/0.346)	$p=0.230$ (0.386/0.275)	$p=0.019$ (1.2)	$p=0.019$ (1.2)
D4Mit54	66.0	$p=0.038$ (0/0.531)	$p=0.094$ (0.182/0.404)	$p=0.063$ (0.146/0.525)	$p=0.024$ (1.1)	$p=0.017$ (1.2)
D4Mit256	82.7	$p=0.019$ (0.826/0.125)	$p=0.113$ (0.500/0.538)	$p=0.143$ (0.750/0.474)	$p=0.015$ (1.3)	$p=0.019$ (1.2)
D9Mit90	9.0	$p=0.014$ (0.073/0.814)	**$p=0.0085$ (0.159/0.615)**	**$p=0.009$ (0.104/0.700)**	**$p=0.001$ (2.3)**	**$p=0.001$ (2.3)**
D9Mit330	26.0	$p=0.043$ (0.083/.135)	$p=0.099$ (0.091/0.231)	$p=0.034$ (0/0.375)	$p=0.027$ (1.1)	$p=0.011$ (1.4)
D9Mit4	29.0	***p=0.004 (0.01/0.896)***	$p=0.15$ (0.773/0.577)	$p=0.306$ (0.729/0.825)	***p=0.005 (1.7)***	***p=0.009 (1.5)***
D9Mit35	52.0	$p=0.048$ (0.813/0.250)	$p=0.307$ (0.205/0.269)	$p=0.094$ (0.292/0.275)	$p=0.076$ (0.68)	$p=0.025$ (1.0)
D9Mit114	52.0	$p=0.040$ (0.813/0.219)	$p=0.27$ (0.225/0.269)	$p=0.044$ (0.283/0.275)	$p=0.059$ (0.77)	$p=0.012$ (1.3)
D11Mit219	43.0	$p=0.06$ (0/0.436)	$p=0.201$ (0.409/0.250)	$p=0.187$ (0.229/0.263)	$p=0.047$ (0.85)	$p=0.107$ (0.57)

The *p*-values in boldface are significant at $p < 0.01$.
All Microsatellite markers used in this study include: Chr. 1:94, 196, 14, 506, 16, 145, 80, 48, 135, 387, 449; Chr. 2:61, 91, 14, 7, 156, 241, 379, 37; Chr. 3:43, 42, 86, 17, 12, 349, 19; Chr. 4:12, 72, 54, 256, 148, 42; Chr. 6:183, 240, 69, 93; Chr. 9:289, 274, 12, 35, 114, 100, 90, 91, 330, 4, 144, 8; Chr. 11:219, 40, 195, 113, 356.

the original data (Seaton *et al*., 2002), and the distribution of the results from these replicates was used to derive the empiric 5% and 1% threshold of genome-wide significance for the linkage test statistic.

RESULTS

Study 1

Response to Selection for the HAP1/LAP1 Mouse Lines

The response to selection for the HAP1/LAP1 lines through generation 12 can be seen in Figure 1a. By the 12th generation, the HAP1 animals consumed an average of more than 12.0 g/kg/day of 10% ethanol (v/v), while the LAP1 mice consumed an average of less than 1.5 g/kg/day. The cumulative realized heritability estimate of alcohol preference for the HAP1/LAP1 mice through generation 10 has been estimated to be $0.20 + 0.04$ (for details, see Grahame *et al*., 1999).

Preference for alcohol in the HAP1 mice was considerable. By the 10th generation, nearly 70% of total fluid consumption was alcohol; LAP1 mice drank only 8% of their daily fluid as alcohol (Grahame *et al*., 1999). Figure 1b shows the mean preference ratio for 10% ethanol (expressed as percentage total fluid consumption) for the HAP1/LAP1 lines over each generation. Bars indicate standard errors.

Response to Selection for the HAP2/LAP2 Mouse Lines

The HAP2 and LAP2 lines are replicate lines of the HAP1/LAP1 lines; however, selection is approximately 9 generations (or, a little over 2 years) behind the HAP1/LAP1 mice. The alcohol testing protocol is identical to that used for the HAP1/LAP1 lines and the criteria for selection are the same. Figure 2a shows the response to selection for the HAP2/LAP2 lines through generation 6. The mean preference ratio for 10% ethanol for the HAP2/LAP2 mice through generation 6 can be seen in Figure 2b. Bars indicate standard errors.

To calculate the realized heritability for the HAP2/LAP2 lines, the cumulative realized response to selection (R) was regressed on the cumulative selection differential (S)—see Falconer and Mackay (1996) for further details of this analysis. The cumulative realized heritability estimate for the HAP2/LAP2 mice through generation 6 has been estimated to be $0.13 + 0.03$ (the standard error was also estimated after Falconer and Mackay, 1996).

Differences in Allele Frequency

Differences in allele frequency were examined between the HAP1 and LAP1 lines from generation 12, and between the HAP2 and LAP2 lines from generations 3 and 6. Significant differences in allele

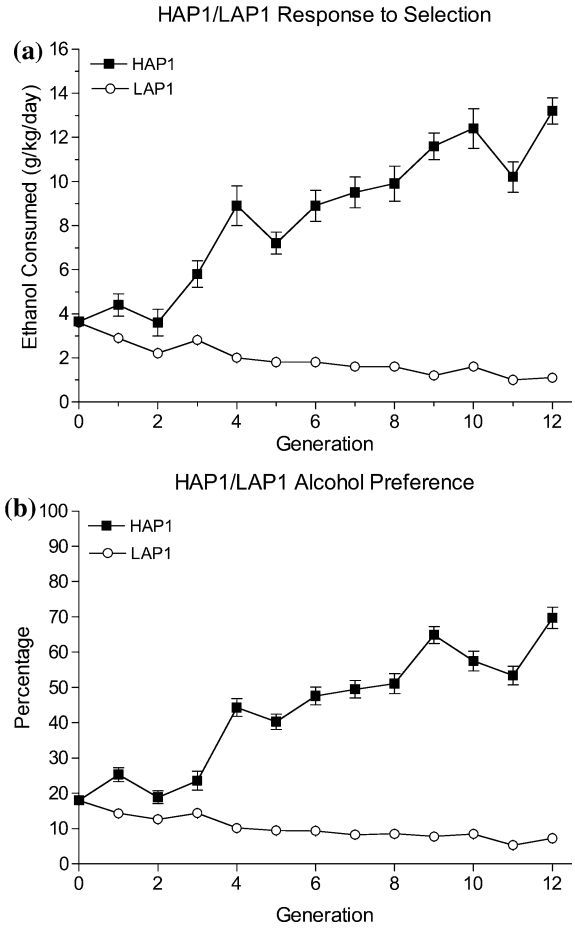

Fig. 1. (a) Each point represents the mean (±SEM) amount of 10% ethanol consumed (g/kg/day) in two-bottle choice tests by the HAP1 and LAP1 mice over 12 generations of selective breeding. (b) Each point represents the mean (±SEM) preference ratio for 10% ethanol (expressed as total fluid consumption) for the HAP1 and LAP1 lines over 12 generations of selective breeding.

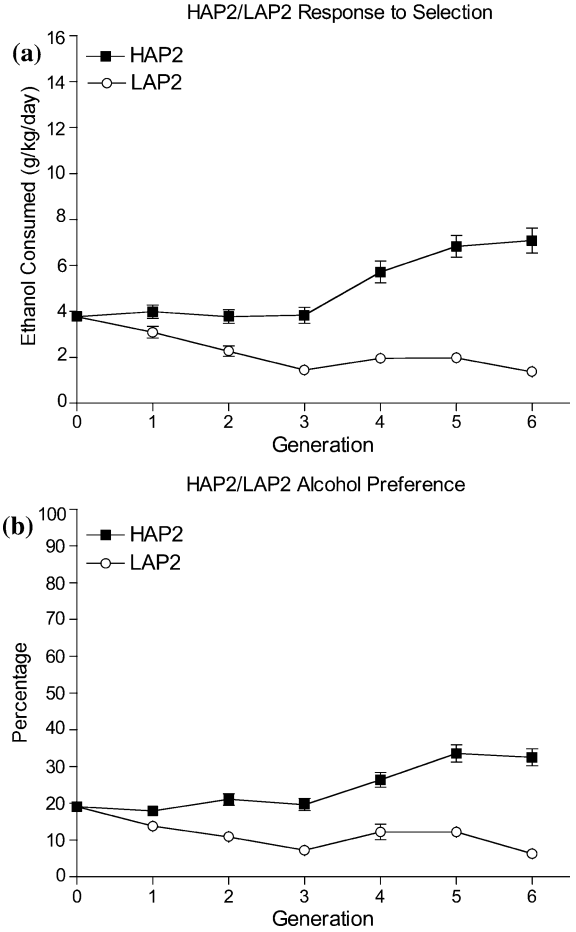

Fig. 2. (a) Each point represents the mean (±SEM) amount of 10% ethanol consumed (g/kg/day) in two-bottle choice tests by the HAP2 and LAP2 mice over six generations of selective breeding. (b) Each point represents the mean (± SEM) preference ratio for 10% ethanol (expressed as total fluid consumption) for the HAP2 and LAP2 lines over six generations of selective breeding.

frequency at a marker are indicative of the presence of nearby QTLs associated with alcohol preference. The *p*-values and LOD scores for those chromosomal regions can be seen in Table II. For *Study 1*, *p*-values that refer to a single test (single point) are reported.

For the HAP1 and LAP1 mice, differences in allele frequency ($p < 0.01$) among those markers examined were found on chromosome 9. In the HAP2 and LAP2 mice, differences ($p < 0.01$) were found on chromosomes 2 and 9.

One of the most promising regions in the HAP1/LAP1 lines was marker *D9Mit4* ($p = 0.004$). This marker is located at 29 cM near the *Drd2* locus (28 cM) on chromosome 9. For the HAP2/LAP2 mice, from generation 3, the most promising region was the marker *D2Mit37* ($p = 0.004$), located on chromosome 2 (45.0 cM) near *Scn1a-9* and *Nr4a2* located at 34.5 cM. The marker *D9Mit90* was also significant in the HAP2/LAP2 mouse lines (S3, $p = 0.0085$; and S6, $p = 0.009$). *D9Mit90* is located at 9.0 cM on chromosome 9 and is approximately 19 cM from *Drd2*. This marker was also a putative QTL identified in the short-term selection study performed by Belknap (1997).

COMBINED *p*-VALUES

For each marker, the *p*-values were combined across the HAP1/LAP1 and HAP2/LAP2 studies using Fisher's method (Sokal and Rohlf, 1995) to produce an overall *p*-value for the difference in allele frequency. This was performed only when the

direction of the QTL effect, that is, which allele was associated with higher preference scores, was the same in both HAP1/LAP1 and HAP2/LAP2 analyses. A significant difference in allele frequency would be indicative of a QTL for a particular chromosomal region. The goal of combining the analyses or meta-analysis, as described by Belknap and Atkins (2001), is to increase statistical power for two or more independent studies in which the hypothesis being tested is the same. In this study, the hypothesis was whether a QTL exists near a given marker. It is not appropriate to combine the *p*-values for the S3 and S6 generations because they were from the same selection lines, and therefore are not completely independent. Therefore, the *p*-values for S12 (line 1) and S3 (line 2) were combined, and the *p*-values for S12 (line 1) and S6 (line 2) were combined. For each marker listed in Table II, the combined *p*-values attained $p < 0.01$, the criterion for confirmation (Lander and Kruglyak, 1995).

The markers *D9Mit90* and *D9Mit4* were found to be the most promising markers when the *p*-values were combined (Table II). In each case, the meta-analysis increased the power to detect the QTL and confirmed the importance of the region. For *D9Mit90*, the power to detect the differences in marker alleles was increased by the meta-analysis, as shown by the *p*-values ($p = 0.001$, LOD 2.3 for both S3 and S6 compared to the S12). Therefore, the meta-analysis enhanced the power to detect important differences in marker allele frequency between the HAP/LAP mice in both the line 1 and line 2 studies.

Study 2

Mean alcohol consumption scores for the 6 HAP1/LAP1 progenitors and 432 F2 animals are shown in Table III. The genome screen was carried out using 134 microsatellite markers with an average intermarker distance of 11.2 cM in 6 HAP1/LAP1 progenitors, 36 F1 breeders, and 432 F2 mice.

One region on chromosomes 9 was found to meet the criteria for genome-wide significance, producing a LOD score of 5.04; $p = 0.0012$ (Fig. 3). This QTL accounts for 11% of the phenotypic variance associated with the phenotype and best fits an additive model of inheritance. The linked region is between the markers *D9Mit2* and *D9Mit355*. Gender specific analyses supported a greater effect of the QTL among female mice (LOD score = 5.19; $p = 0.0008$) than the male mice (LOD score = 1.19; $p = 0.715$). The 95% confidence intervals were estimated for the chromosome 9 QTL based on the latest mouse genome build (#34.1). Similar to most F2 studies, we have identified a broad QTL region, and the 95% confidence interval for the female sample is 48.52–96.56 Mb. For the full sample, it is 32.40–96.56 Mb.

Table III. Alcohol Consumption in the Progenitor and F2 Animals

Sample	*n*	Mean	(SEM)*
HAP1 progenitors	3	22.41	(0.50)
LAP1 progenitors	3	0.84	(0.01)
F2	432	6.18	(0.29)

All values are in g/kg/day; SEM = standard error of measurement.

Two other QTLs, with LOD scores greater than 2.0, were identified on chromosomes 2 and 5; however, these QTLs did not reach the genome-wide threshold level for significance. The QTL on chromosome 5 has a maximum LOD score of 2.93; $p = 0.14$. This QTL is at approximately 44 cM, near the marker *D5Mit201* (Fig. 4). The QTL on chromosome 2 has a LOD score of 2.32; $p = 0.40$ (Fig. 4). This QTL is at 107.0 cM, between the markers *D2Mit113* and *D2Mit266*.

DISCUSSION

This study utilized a non-inbred mouse model for the identification of QTLs underlying alcohol consumption. We report two studies with very different approaches to identify QTL for alcohol preference.

In *Study 1*, evidence for the presence of QTLs was gained when the allele frequencies at a marker diverged significantly more in the oppositely selected HAP and LAP lines than would be expected from genetic drift and allele frequency estimation error (Belknap *et al.*, 1997). Because the regions examined had already been established as significant in various B6- and D2-derived populations, this study provides further evidence and confirmation that at least two of these QTLs influence alcohol preference in the HAP/LAP lines.

The two most promising QTL regions that were identified in this study (Table II) were near the markers *D9Mit90* (9 cM) and *D9Mit4* (28 cM) on distal to mid Chromosome 9. Because this region appears to be unusually broad, and the middle marker shows a lower LOD score than the markers at either end of this region, there may be more than one QTL here, as has been suggested by others

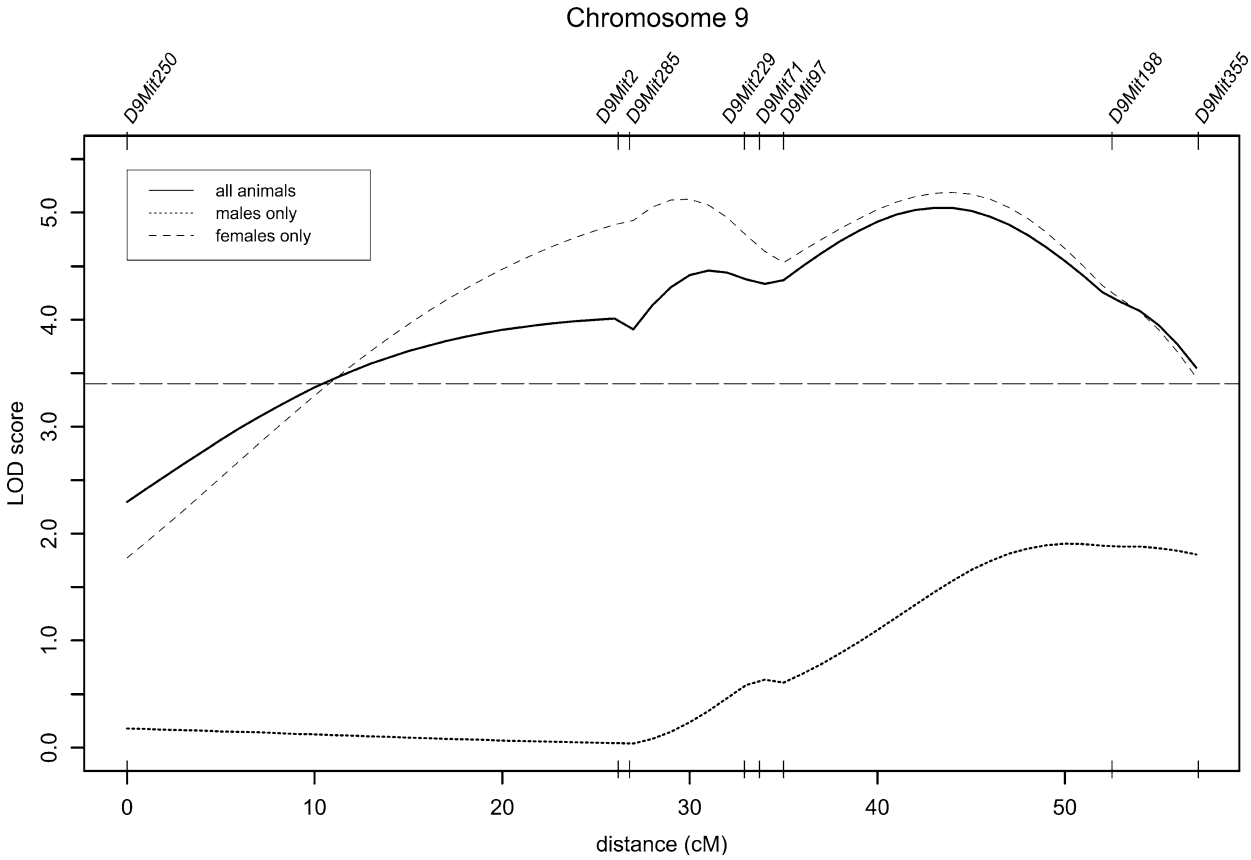

Fig. 3. Lod scores computed for alcohol consumption for chromosome 9 in a sample of 432 F2 progeny. This figure shows the lod scores computed separately for males only, females only, and male and female combined. The thick dashed line indicates the 5% threshold level for significance.

(Phillips *et al.*, 1998). For both the HAP1/LAP1 lines, the progenitor population starts out close to floor, while the ceiling in alcohol consumption is indeterminate. However, there is evidence that a common set of genes underlie both high and low intake. In a short term selective breeding study, Belknap and colleagues (Belknap *et al.*, 1997) demonstrated that in lines that diverged far less than the current HAP and LAP mice, markers associated with a QTL on Chromosome 9 (perhaps the same QTL as observed in the present study) as well as other QTL loci showed a bidirectional response to selection in both the High- and Low-selected directions after four generations of selection. Because those lines had not yet reached floor for alcohol intake, it may mean that although the phenotype and selection limit is perhaps different for high and low preference, a common set of alleles may contribute to both phenotypes.

The QTL region on chromosome 9 includes the candidate gene *Drd2* located at 28 cM. These markers were close to a large effect QTL identified by Phillips *et al.* (1998), Tarantino (1998) and in the review of all studies by Belknap and Atkins (2001). The *Drd2* gene codes for the dopamine D2 receptor, and has been implicated in the rewarding effects of ethanol (Blum *et al.*, 1996; Dyr *et al.*, 1993; Koob and Bloom, 1988; Samson *et al.*, 1993; Wise and Bozarth, 1987). Animal studies have provided evidence for dopamine's involvement in ethanol self-administration (Hodge *et al.*, 1997; Samson *et al.*, 1990) and stimulant effects on locomotion (Phillips and Shen, 1996). The D2 dopamine receptor antagonists have been found to reduce ethanol consumption (Dyr *et al.*, 1993) and to reduce operant responding for ethanol (Samson *et al.*, 1990, 1993) in high alcohol drinking (HAD) rats. Dopamine D2 receptor knock-out mice with a B6 background have also been studied. Phillips *et al.*

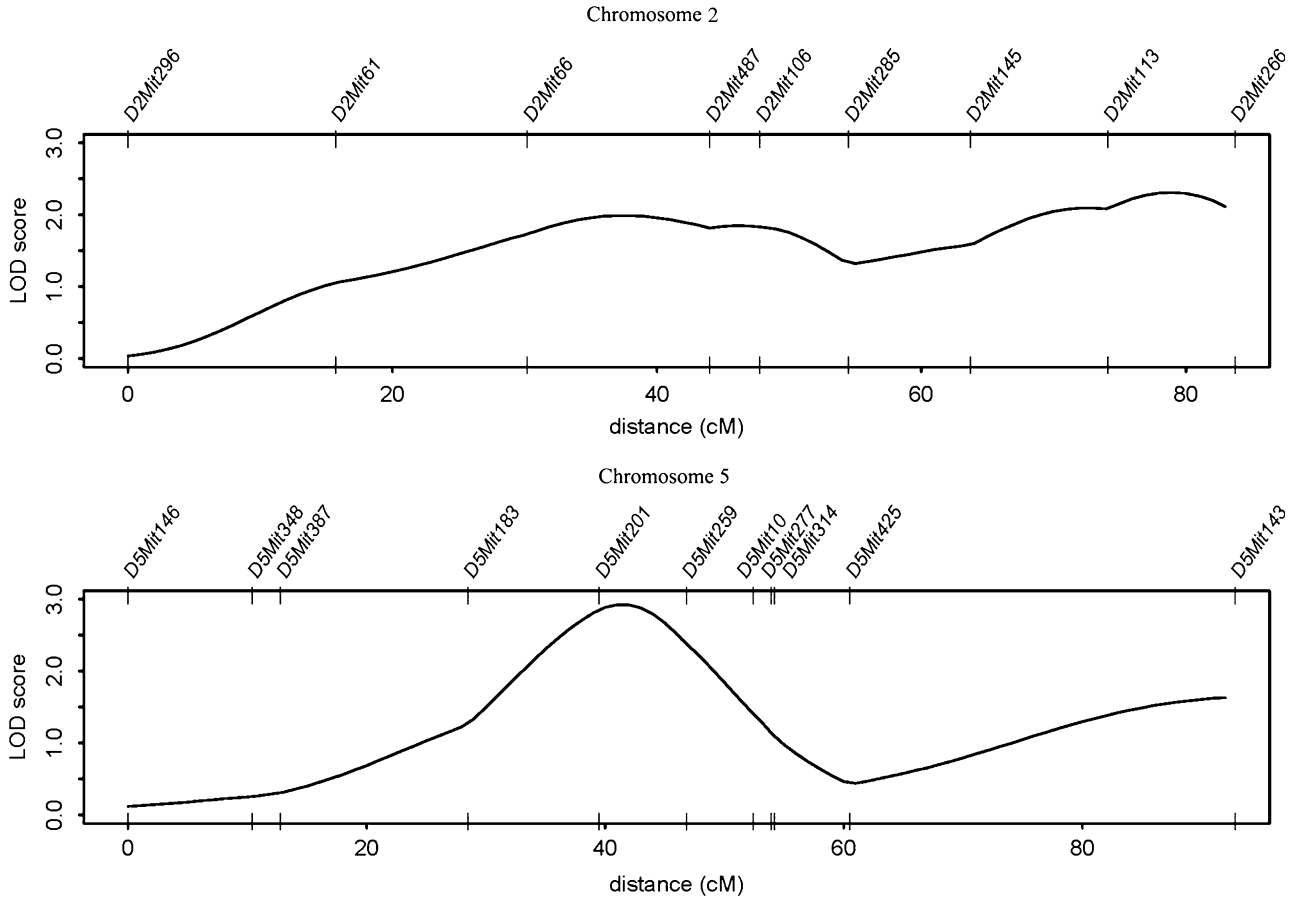

Fig. 4. Lod scores computed for alcohol consumption for chromosome 2 and 5 in a sample of 432 F2 progeny. The lod scores for these QTLs were greater than 2.0 but below the genome wide threshold level of significance (for chromosome 2, $p = 0.4052$; for chromosome 5, $p = 0.1414$).

(1998) found that D2 receptor-deficient mice showed a marked aversion to ethanol compared to wild-type controls. Taken together, this evidence supports the role of the *Drd2* gene as an important candidate for alcohol preference behavior in animal studies.

In human studies, there has been a great deal of controversy as to whether the *DRD2* receptor gene is an important candidate for alcoholism. Blum and colleagues have provided evidence that the *DRD2* gene may be associated with severe alcoholism, particularly as it may play a role in the "reward deficiency syndrome" (Blum *et al.*, 1995, 1996, 1997, 2000). Other evidence contradicts these findings (Edenberg *et al.*, 1998; Gelernter *et al.*, 1993; Goldman, 1993, 1997).

Another QTL region suggested in this study is on mid chromosome 2. The marker *D2Mit37* is located at 45 cM and is within the QTL region suggested by Belknap and Atkins (2001) based on several B6- and D2-derived crosses. *D2Mit37* is close to a family of brain-expressed sodium channel alpha-subunit genes, *Scn1a, 2a, 3a, 7a*, and *9a*, located on chromosome 2. This QTL is also close to the candidate gene *Nr4a2*. *Nr4a2* encodes a member of the nuclear receptor subfamily (Nurr-1), a transcription factor that is expressed in dopamine cells. Dopamine, which is essential in the mediation of motivation, locomotion, and reward (Wise and Bozarth, 1987), is released in the nucleus accumbens after acute administration of most drugs of abuse, including ethanol (Di Chiara and Imperato, 1988). Mutations in *Nr4a2* affect transcription of the gene encoding tyrosine hydroxylase, and can cause dopaminergic dysfunction. Because of the key role of Nurr1 for midbrain dopamine neurons, it is possible that Nurr1 may be important for rewarding behaviors. Finally, the marker *D2Mit37* is syntenic (shows linkage homology) to a QTL region identified on chromosome 3 in the P and NP rats with a maximum LOD score of 2.5 (Bice *et al.*, 1998).

In *Study 2*, the linkage result on chromosome 9 is the largest effect QTL identified in this genome screen, which coincides with our results in *Study 1*. The most likely candidate genes within the QTL region are *Drd2* and *Htr1b*. A possible role for *Drd2* as a candidate gene for alcoholism has been described previously in *Study 1*. *Htr1b* is located at 46 cM and encodes the 5-HT_{1B}-serotonin receptor. Crabbe and colleagues (1996) have shown that null mutant mice lacking the 5-HT_{1B} receptor gene 5-HT_{1B}(−/−) not only display enhanced aggression but also drank twice as much ethanol as wild-type mice, voluntarily ingesting solutions containing up to 20% ethanol in water. They also found that the 5-HT null mutant mice showed less sensitivity to ethanol-induced ataxia and developed tolerance more slowly than wild-type controls. In studies involving human subjects, the role of *HTR1B* and its association with alcoholism has been equivocal. Several studies have provided evidence that the *HTR1B* receptor gene is associated with alcohol dependence, in particularly, alcoholism with antisocial impulsive features (Fehr *et al.*, 2000; Hill *et al.*, 2002; Lappalainen *et al.*, 1998; Sun *et al.*, 2002); others have found no association (Cigler *et al.*, 2001; Gorwood *et al.*, 2002; Hasegawa *et al.*, 2002; Kranzler *et al.*, 2002).

Additionally, there were two QTLs identified on chromosomes 2 and 5 in *Study 2* that are suggestive at the genome-wide threshold level. This result for chromosome 2 was supported by the results in *Study 1*, adding credence to the possibility that this well-established QTL in B6- and D2-derived populations exists in the HAP/LAP lines as well, although to a smaller degree. The QTL identified on chromosome 2 is at approximately 107 cM. This QTL is in the same location as *Alcp5* and *Alcp6*, which are QTLs for alcohol preference identified by Gill *et al.* (1998). The peak on chromosome 5 is at approximately 44.0 cM near the marker *D5Mit201*. This QTL is located near a cluster of genes that encode gamma-aminobutyric acid (A) ($GABA_A$) receptor subunits $alpha_2$ (*Gabra2*), $beta_1$ (*Gabrb1*), and $gamma_1$ (*Gabrg1*) located between 36 cM and 41 cM on mouse chromosome 5. Several lines of evidence, both from human and animal studies, have shown that there is a genetic association between alcohol dependence and the $GABA_A$ alpha-2 subunit (Cagetti *et al.*, 2003; Carr *et al.*, 2003; Covault *et al.*, 2004; Edenberg *et al.*, 2004; Grobin *et al.*, 1998; Lister and Linnoila, 1991; Low *et al.*, 2000; Rudolph *et al.*, 1999; Saba *et al.*, 2001; Tauber *et al.*, 2003). Collectively, this evidence supports a role for the $GABA_A$ receptor subunit in the risk for alcoholism.

CONCLUSION

In this paper, we report two independent studies with very different approaches, whose results are complementary. In the first study, we examined differences in allele frequency between the HAP and LAP mice from the early generations of selection and confirmed QTLs on chromosomes 2 and 9 that had been identified by others. In the second study, a genome-wide screen using F2 mice, identified a QTL on chromosome 9 with a significant LOD score of 5.04 (combined for males and females). This confirms our results for the allele frequency study and provides additional evidence that a region on mid chromosome 9 is important for alcohol preference. The most likely candidate genes for the QTL on chromosome 9, in both of our studies, are *Drd2* and *Htr1b*.

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