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Confirmation and Fine Mapping of Ethanol Sensitivity QTLs, and Candidate Gene Testing in the LXS Recombinant Inbred Mice

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Nonstandard abbreviations: ILS, Inbred Long Sleep; ISS, Inbred Short Sleep; LORE, loss of the righting reflex due to ethanol; *Lore*, QTL for LORE; LXS, RI panel derived from ILS x ISS cross; NET, norepinephrine transporter; RI, recombinant inbred; SERT, serotonin transporter

ABSTRACT

In previous studies we have mapped quantitative trait loci (QTLs) for hypnotic sensitivity to ethanol using a small recombinant inbred (RI) panel and a large F_2 backcross. Alcohol sensitivity is a major predictor of long-term risk for alcoholism. We remapped hypnotic sensitivity using a new set of 75 RI strains, the LXS, derived from Inbred Long Sleep (ILS) and Inbred Short Sleep (ISS) strains. We expected to improve mapping resolution in the QTL regions and to identify novel QTLs for loss of the righting reflex following ethanol (LORE). We used three common mapping algorithms (R/qtl, QTL Cartographer, and WebQTL) to map QTLs in the LXS, and compared the results. Most mapping studies use only a single algorithm, an approach which may result in failure to identify minor QTLs. We confirmed most of our previously reported QTLs, although one major QTL from earlier work (*Lore2*) failed to replicate, possibly because it represented multiple linked genes separated by recombination in the RI strains. We also report narrowed confidence intervals, based on mapping with a new genetic resource of over 4000 polymorphic SNP markers. These narrowed confidence intervals will facilitate candidate gene identification, and assessment of overlap with human regions specifying risk for alcoholism. Finally, we present an approach for utilizing these RI strains to assess evidence for candidate genes in the narrowed intervals, and apply this method to a strong candidate, the serotonin transporter.

INTRODUCTION

Initial sensitivity to the intoxicating effects of ethanol is a major risk factor for subsequent development of alcoholism in family-history positive males (Shuckitt, 2000). The inbred Long Sleep (ILS) and inbred Short Sleep (ISS) mouse strains are an excellent resource for addressing the genetic bases of this behavior, as they were derived by selection for differential sensitivity to a sedative dose of alcohol (McClearn and Kakihana, 1981). The ILS and ISS, and their progenitors, have been widely used in alcohol research, and they have been cited in some 400 publications over the past 30 years.

In previous work we mapped QTLs for sensitivity to an intoxicating dose of ethanol (*Lores*: loss of righting due to ethanol), using a small panel of 25 RI strains (LSXSS) derived from the non-inbred Long Sleep (LS) and Short Sleep (SS) (Markel et al., 1996) mice, and subsequently, a large F_2 intercross between ILS and ISS of over 1000 mice (Markel et al., 1997). The latter cross confirmed four of the putative QTLs from the LSXSS, and identified three novel regions. We confirmed and captured four of the major QTLs for this trait, on chromosomes 1, 2, 11 and 15, in reciprocal congenic strains (Bennett et al., 2002a), and narrowed the interval surrounding the QTL on two chromosomes to less than 12 Mb (Bennett et al., 2002b).

There are compelling reasons to use a large RI set such as the recently created LXS set of 75 strains (Williams et al., 2004) for genetic mapping (Belknap, 1998; Chesler et al., 2005; Williams et al., 2004): (1) a fourfold map expansion provides improved map resolution; (2) trait heritabilities are higher in RI than in F_2 or backcross animals from equivalent crosses, allowing the mapping of traits with lower h^2; (3) genotyping is done only once; and (4) phenotypic data from many different laboratories and experiments can be combined to derive genetic correlations and assess gene-environment (GXE) interaction. The study of GXE interactions is uniquely suited to RI sets, and is likely to be enormously important in understanding and dissecting complex diseases (Churchill et al., 2004).

Haughey et al. (2005) used the LXS, along with binding assays and molecular evidence from ILS and ISS, to identify the norepinephrine transporter (NET) gene on chr 8 as a candidate for a *LORE* QTL. Here, we present new haplotype data further supporting this candidate.

The serotonin transporter (SERT) is an attractive candidate for ethanol sensitivity in both mice and humans. In a human population, a polymorphism in SERT resulted in a GXE interaction such that stressful events elicited more serious responses in one genotype (Caspi et al., 2003). Mice with one or two null alleles show more anxiety and greater increases in stress hormones following stressful stimuli (Murphy et al., 2001). The gene is located at the peak of the *Lore4* QTL on chr 11 (Markel et al., 1997) at 76.7 Mb. The SERT inhibitor fluoxitine has differential effects in ILS and ISS on MK-801-induced activity, indicating that 5-HT affects activity induced by NMDA receptor blockade in these mice (Hanania et al., 2002). An inverse relationship between ethanol consumption and serotonin level has been observed in mice (Kelai et al., 2003), and treatment with selective serotonin reuptake inhibitors (SSRIs) has been reported to decrease drinking in alcohol-dependent humans (Lejoyeux, 1996). Here, we tested, and ultimately rejected, SERT as a candidate for *Lore4*.

All LORE testing in the LXS panel, reported here, was done in three independent cohorts over a 1-year period. This experimental design was initially developed to spread out specific environmental effects on phenotypic variability so as to reduce the impact stemming from a single cohort. We found a striking impact of nongenetic variation, such that strain means and, hence, QTLs varied by cohort. Here, we report QTLs based on analyses of the individual cohorts, and the combined dataset. Although the multiple cohort design did not minimize the effect of nonspecific environmental variation, it did allow us to use the second cohort to estimate effect size more accurately (Bennett and Carosone-Link, 2005). Several QTLs which replicated across cohorts, and previous studies, attained high combined significance levels, confirming them as important genomic regions for followup work in gene identification.

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METHODS

Animals. The LXS RI panel was generated in the specific-pathogen-free (SPF) facility at the Institute for Behavioral Genetics, Boulder, CO, from crosses between the ILS and ISS (Bennett et al., 2002a; Williams et al., 2004). The number of consecutive filial matings currently ranges from F_{27} to F_{33}, with 75 strains still extant. At 25 days of age, mice were weaned, tail clipped, and transferred to male-only or female-only cages, 2-5 per cage. Mice were maintained on a 12-hour light/dark cycle, and were given food and water *ad libitum* (Breeder Diet [9% fat, Harlan Industries, Indianapolis, IN]). All procedures followed guidelines developed by the National Institutes of Health (NIH) and the Institutional Animal Care and Use Committee.

Loss of Righting Reflex due to Ethanol (LORE). All testing was conducted in the SPF facility. Mice were tested for alcohol sensitivity first at 55 to 65 days (trial 1) and again seven days later (trial 2) by intraperitoneal (ip) injection of a 4.1 g/kg dose of ethanol (20% w/v solution in saline). Blood ethanol concentration (BEC) at awakening was determined using a spectrophotometric assay (Smolen and Smolen, 1989). All testing was done between 0900-1400 hrs, during the light cycle. If the mouse still had not lost the ability to right itself, after ten minutes, the injection was considered faulty, and a retest was done one week later. Approximately 10% of all mice injected failed to lose the righting response on one occasion; this is generally attributed to a misplaced or leaky injection, and is a typical occurrence for ip injections (Crabbe et al., 2005; Markel et al., 1995). Blood ethanol concentration was determined in 66% of these animals, ten min post-injection; to corroborate this conclusion; these data are presented here. Duration of LORE was determined using the method of McClearn and Kakihana (1981), modified as reported by Markel et al. (1997), such that all mapping was done using the mean LORE duration from trials 1 and 2. Briefly, righting response was lost when the mouse could not right itself three times within one minute. ISS and ILS mice were injected as controls for environmental variability in LORE.

[^{3}H]Citalopram binding to SERT. Mice were sacrificed by cervical dislocation. Brain regions of interest (prefrontal and remaining cortex, cerebellum, hippocampus, nucleus accumbens, amygdala, ventral midbrain and caudate) were dissected from ILS and ISS mice (N = 6-8 per strain) and frozen at -80^{o}C. On the day of assay, tissues were homogenized in 30 mM sodium phosphate buffer (pH 7.4) containing 0.32 M sucrose and centrifuged at 20,000 x *g* at 4^{o}C for 20 min. The membrane pellets were resuspended in the phosphate-sucrose buffer and incubated in a volume of 0.25 ml with [^{3}H]citalopram (Perkin Elmer Life Sciences, Boston, MA), unlabelled citalopram, and fluoxetine (Sigma-Aldrich, St. Louis, MO) at room temperature for 1 hr. Indirect saturation curves were generated in "remaining cortex" and cerebellum using 4.7 nM [^{3}H]citalopram; concentrations of unlabeled citalopram ranging from 0.03 nM to 1 μM and ~80 μg protein. Non-specific binding was defined with 10 μM fluoxetine. In the other brain regions, specific binding was measured with a single, two-fold higher concentration of [^{3}H]citalopram (9.5 nM -/+ 10 μM fluoxetine) and ~ 75μg protein. The assays were terminated by rapid vacuum filtration over GF/B filters (Brandel Inc., Gaithersburg, MD) and three washes with ice-cold sodium phosphate buffer. The retained radioactivity was measured by liquid scintillation spectrometry. Proteins were determined by the method of Bradford (1976) using bovine serum albumin as the standard. The maximal number of binding sites (B_{MAX}) and affinity (K_D) were determined from the saturation curves with nonlinear fitting (GraphPad Software, San Diego CA).

Heritability Determination and Statistical Analyses. Narrow-sense heritability (h^2) assesses the proportion of the phenotypic variance (V_P), due to additive genetic variance (V_A). h^2 is easily determined in an RI study as the variance of the strain means, relative to the total variance, and was calculated separately for sex and cohort by adjusting r^2 from a oneway ANOVA for varying sample sizes (Belknap et al., 1996). 95% confidence limits on h^2 were determined using the Moriguti-Bulmer procedure for approximating confidence limits (Sokal and Rohlf, 2000). Statistical analyses were done using SPSS for Windows (v. 12.0, SPSS, Inc., Chicago, IL).

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QTL Mapping and Haplotype Analysis. QTL mapping was done in several stages, to identify loci acting individually and QTLs which interacted, either additively or epistatically, to affect each phenotype. Initial analysis was done with MapManager QTX, version 19 (Manly and Cudmore 2001; Manly and Olson, 1999; http://www.mapmanager.org/mmQTX.html), to produce a genetic map which was then used as the input file for R/qtl. The genetic map was based on genotype determined previously for the LXS strains (Williams et al., 2004; available at http://www.genenetwork.org/genotypes/LXS.geno). Strain means for all phenotypes were analyzed using R/qtl (Broman et al., 2003; http://www.biostat.jhsph.edu/~kbroman/qtl/), as described in Bennett et al. (2005). R/qtl was also used to assess sex-specificity of QTL regions by mapping on the mean phenotypic difference between males and females in each strain (K. Broman, pers. comm.). Further mapping was done using a new genetic resource (the Wellcome-CTC Mouse Strain SNP Genotype Set). This genotype set consists of genotypes for 480 strains, including the full LXS panel, and 13,370 successful SNP assays that are mapped to build 34 of the mouse genome; 4834 of these are polymorphic between ILS and ISS (http://www.well.ox.ac.uk/mouse/INBREDS). This genetic map was used in R/qtl and WebQTL (Wang et al., 2003; http://www.webqtl.org/home.html).

Finally, we used several mapping strategies available in Windows QTL Cartographer (Wang et al., 2005; http://statgen.ncsu.edu/qtlcart/WQTLCart.htm). Composite interval mapping (CIM) adds background loci to simple interval mapping (we specified all other significant and suggestive QTL regions) to remove their effects on the target QTL. We also used the multiple trait analysis option of Cartographer, as the individual LORE phenotypes (sex- and cohort-specific) were correlated traits (Table 1). Accounting for this correlation among traits increases power and reduces sampling variance (Jiang and Zeng, 1995).

We mapped in each of three cohorts individually, and in the combined dataset. This combined dataset was also used to investigate cohort as a main effect, using a General Linear Model in SPSS v. 12.0, with the phenotype as dependent variable, and cohort, sex, and strain as

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independent variables. A combined *p*-value (Sokal and Rohlf, 2000) for each QTL identified in multiple cohorts was also determined.

A haplotype is a group of markers retained as a block. We used SNP markers identified in the Wellcome-CTC Mouse Strain SNP Genotype Set, for which ILS, ISS, and the LXS RI were genotyped, at an average density of 45 kb, to generate coarse haplotypes for ILS and ISS. Much higher resolution is possible using the SNPs for A/J (A), C57BL/6J B(6), and DBA/2J (D2) (the three progenitors to ILS and ISS for which complete sequence is available) compiled from Celera Genomics, the Perlegen/NIEHS resequencing project, the Wellcome-CTC SNP Project, dnSNP, and the Mouse Phenome Database (MPD) (http://aretha.jax.org/pub-cgi/phenome/mpdcgi?rtn=docs/home) archived at GeneNetwork (http://www.genenetwork.org/cgi-bin/beta/snpBrowser.py) to determine strain-specific haplotypes. The latter is the denser map, (averaging one SNP per 4 Kb), detailing all SNPs identified by these sequencing efforts; however, our strains of interest are not genotyped for this density. We used the Wellcome SNP set to create a skeleton of markers from the strains fully covered at GeneNetwork. ILS and ISS were placed on this map wherever genotypes permitted, and their haplotypes inferred from comparison with strains with matching flanking patterns. To error check database entries, SNP genotypes from the Wellcome set, MPD, and GeneNetwork were compared. All haplotypes were clearly consistent (data not shown), although not all databases contain all SNPs.

RESULTS

LORE in the LXS. A total of 952 mice in three cohorts were tested for loss of righting due to ethanol (LORE; Table 1). The third cohort included only 206 mice from 57 strains, providing low power for mapping QTLs; consequently, no individual analyses of this cohort are reported, though we did use these data in pooled analyses. Correlations among all three cohorts, along with heritabilities, are given in Table 1, and indicate good phenotypic correspondence in all three cohorts. At the time testing began, there were 77 strains extant; three have since been lost.

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There was no transgression, that is, no strain means for LORE surpass either of the progenitor strains (Figure 1). This is an optimal situation for genetic mapping of the selected trait (Liu, 1997). LORE in strain 35 (μ = 20.5) was as low as that in ISS (μ = 22.5; $p = 0.84$), and ILS had the longest duration of LORE (μ = 185), significantly greater than the next highest strain (μ = 149; 1-tailed $p = 0.04$). Heritability of LORE, based on the RI strain means in the combined dataset, was 57%. Heritability values by sex and cohort all clustered close to this value (Table 1).

BEC at awakening was determined for 814 mice with successful injections. As previously reported (DeFries et al., 1989; Markel et al., 1997), this measure was significantly negatively correlated with LORE ($r = -0.41$ for the first determination of LORE, $p < 0.001$; $r = -0.43$ for the second, $p < 0.001$). BEC at awakening assesses CNS sensitivity to ethanol: ISS recover the righting response at a much higher BEC than ILS. Any between-strain difference in ethanol metabolism is primarily due to dose and administration (Smolen et al., 1986), neither of which varied here. Heritability of BEC is much lower than for LORE (~0.2 in the present study, due to error variance in the test) making this trait less desirable for mapping. To rule out any confounding effect of BEC in mapping QTLs for LORE, the strain mean was regressed out and residuals correlated with LORE. The correlation was highly significant ($r = 0.97$, $p < 10^{-40}$), indicating that no correction for BEC was necessary.

For all strains but ISS, failure to lose the righting response within ten min was most likely due to a faulty injection. Mean blood ethanol concentration (BEC) for these mice was 323 mg% (Figure 2A), statistically lower ($p < 0.001$) than in mice which did lose the righting response (μ = 357 mg%), although this latter value is BEC at awakening, which will be somewhat lower than BEC at loss of righting. Consequently, data from animals failing to lose the righting response were not used in determining strain mean for LORE. In ISS, a different picture emerged. In a subset of these mice, low BEC undoubtedly followed a misplaced injection (Figure 2B, bars on the left, μ = 199 mg%). In a second group, BEC was significantly higher (μ = 417 mg%; $p < 0.001$), sufficient

to cause LORE in LS (Smolen and Smolen, 1989) and most inbred strains (Crabbe et al., 2005), indicating that selection for resistance to the sedative effect of ethanol in the short sleep mice resulted in a floor effect.

Sex was a significant main effect influencing LORE ($p < 0.001$). Males had a longer LORE duration ($\mu = 81.3 \pm 1.59$ min) than females ($\mu = 75.3 \pm 1.59$ min), which agrees well with previous literature. Because this effect was consistently seen in all strains, there was no significant sex by strain interaction ($p = 0.23$). It is notable that within cohort, correlations always exceeded between-cohort correlations, although all correlations were significant at or below $p < 10^{-7}$ (Table 1).

Identifying LORE QTLs. As within-cohort correlations were larger than between, cohorts 1 and 2 were initially analyzed separately; subsequently, all data was pooled over cohorts. The QTL region on chr 1 was identified in both cohorts (Table 2). Regions on chr 3, 8, 14, and 18 emerged in one cohort but not the other (Table 2). Pooling over cohorts identified all QTL regions albeit with slightly lower LODs. The number of strains tested showed a suggestive correlation with LOD score ($r = 0.34$, $p = 0.09$). No sex-specific QTLs were identified.

All mapping programs gave very similar findings for QTLs (Figure 3: R/qtl (A) and QTL Cartographer (B); Supplemental Figure 1: WebQTL; Supplemental Figure 2: QTL Cartographer). Figure 3 illustrates discrepancies among the mapping programs. For example, R/qtl (Figure 3A) identified the chr 3 QTL as significant ($p = 0.05$), while this region is suggestive in QTL Cartographer (Figure 3B). Similarly, regions on chr 14 and 18 are suggestive in R/qtl (Figure 3A), but not in QTL Cartographer ((Figure 3B).

QTL regions on chr 1, 3, 8, 14, and 18 replicated in one or both previous mapping populations with highly significant combined *p*-values (Markel et al., 1996; 1997). In the LSXSS RI panel, QTLs were identified by single marker *t*-tests, which did not permit estimation of confidence intervals. For these regions, replication is surmised based on inclusion of the point estimate from Markel et al. (1996) in the 1-LOD interval from the LXS. The chr 1 QTL replicated in all three

mapping populations, with the highest combined *p*-value, and also confirmed in congenic strains (Bennett et al., 2002a, b). The 1-LOD intervals reported here (for this chr 1 QTL) don't quite overlap with QTLs from the F_2 intercross (Markel et al., 1997). It is worthwhile noting that the F_2 estimate of the peak LOD is at 90 Mb and the 1-LOD interval spans 74-93 Mb, suggesting that there was an error in determining this interval. Although confidence intervals are not necessarily normal, a less skewed distribution relative to the peak LOD, then would indicate a support interval of approximately 74-106 Mb, and the *Lore* region identified in the F_2 would overlap with that in the LXS.

Replication across mapping programs. We compared suggestive ($p < 0.63$) and significant ($p < 0.05$) QTLs from interval analysis from all three mapping packages (Table 3; full graphics from WebQTL and multiple trait mapping analyses from QTL Cartographer are provided in Supplemental Materials Figures 1 and 2, respectively). Sexes were analyzed separately, pooling data over cohorts 1 and 2, because the data are archived in this format in WebQTL. The QTLs on chr 1, 3, and 14 replicated in all algorithms, although significance levels differed. There was a surprising range of significance levels. For example, regions on both chr 1 (males) and 3 (females) approached significance in R/qtl and Cartographer, but not in WebQTL (Table 3). However, perusal of the LODs partially resolves the dilemma; all LODs are quite close but the reported *p*-values differ. This disparity is likely due to the fact that WebQTL reports only suggestive, significant, or highly significant cutoffs, whereas in the other programs it is possible to obtain a more precise probability. Additionally, WebQTL defaults to 2000 permutations for estimating significance cutoffs whereas in other programs 1000 permutations were done. In R/qtl and Cartographer, there was little difference between the significance cutoffs obtained using 1000 or 2000 permutations. Despite this variation, general trends over packages were very similar, with the largest disparity in WebQTL. For example, the chr 1 QTL in males had lower LOD scores than females in all programs. For chr 3, the direction of the difference was reversed, with males having higher LODs than females. The peak position and support interval for QTLs on chr 1 and 3 were

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extremely similar among all mapping programs, although the chr 14 region varied considerably. A suggestive region on chr 18 was identified in males only in R/qtl, and suggested in both sexes by a significant GXE interaction determined by multiple trait mapping in Cartographer (Supplemental Figure 2). A suggestive region on chr 19 was identified by both WebQTL and Cartographer. In all analyses of variance, strain contributed significantly ($p < 0.001$) to LORE. Although 2-3 different investigators tested LORE in each cohort, this variable did not have a significant effect on LORE for either sex.

1-LOD intervals are reduced in the LXS. The 1-LOD support intervals in the RI panel (LXS columns) were reduced relative to those obtained in the F_2 map (Table 2), with the exception of the chr 1 region. (And as noted above, the determination of this interval in the F_2 is suspect.). These intervals were reduced even further when the denser SNP map, archived on the WebQTL site, was used (Table 4). On average, using the denser SNP map reduced the 1-LOD support interval fourfold, relative to the microsatellite map. The range in absolute values was quite remarkable: the chr 14 region showed a support interval of 2.9 Mb in males (down from 40.8 Mb using microsatellites), while the chr 3 region, 18.3 Mb by microsatellite mapping, was reduced to approximately 9 Mb using the SNP map.

Composite interval and GXE mapping. Controlling for all suggestive and significant background QTLs reported in Table 2 (composite interval mapping in QTL Cartographer) resulted in a large increase in the significance of the QTLs on chr 1 and 3 in both sexes, such that both of these regions showed a highly significant probability of containing a QTL for LORE ($p < 0.001$ for females and $p < 0.01$ for males). Although a number of other QTL regions were suggestive by this method, none of them surpassed the significance cutoff determined by 1000 permutations. Including independent sex- and cohort-specific LORE phenotypes in a multiple trait mapping model, corroborated both the chr 1 region ($p < 0.01$) and the chr 8 region ($p < 0.05$) at significant levels. The multiple trait mapping analysis also provides a likelihood ratio statistic for GXE interaction, as the same phenotype was measured in different environments (i.e. cohorts and

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sexes). Significant GXE was seen on chr 4, 8, 10, 11, and 15, indicating genomic areas which affected LORE in specific environments (Supplemental Figure 2).

For each cohort, a model was developed including interacting loci which together accounted for a sizeable portion of the phenotypic variance (Table 5A). These loci were identified from a two-way scan of strain means and locus genotypes in R/qtl, and selected on the basis of permutation tests for joint LODs. None of these interactions exceeded the permutation cutoff for epistasis, indicating only additive effects among the loci, which were the same in each cohort (Table 5B). For cohort 1 these three loci, on chr 1, 3, and 8, acting additively, accounted for over 40% of the phenotypic variance, while for cohort 2, this value was 30%.

Replication of LORE QTLs. We asked if previously identified QTLs from our initial LSXSS RI (27 strains; Markel et al., 1996) or the subsequent ILSXISS F_2 intercross (Markel et al., 1997) would replicate in this panel. Four of the five regions identified in the LXS replicated regions identified in the LSXSS panel (chr 1, 3, 18; Table 2), and the ILSxISS F_2 (chr 1, 8, 18; Table 2). Each replication gave significant combined p-values, using Fisher's test (Table 2; Sokal and Rohlf, 2000), although the chr 1 and 3 regions were more significant by several orders of magnitude. Two QTLs identified from the F_2 genome scan, on chromosomes 11, and 15, replicated in a multiple trait model (which entered mean values for LORE, by sex and in each cohort, as separate independent variables), with a significant GXE interaction. For the chr 11 region, there was a strong GXE interaction (LOD = 2.6), stemming from the fluctuation of the additive effect value, with both sexes in cohort 1 showing an opposite trend from mice in cohort 2. This can be seen in Supplemental Figure 2. The chr 15 region shows a more complicated picture, which could be due to a similar phenomenon producing a GXE interaction (LOD = 2.2), break up of the QTL into multiple, linked QTLs (as suggested by analysis of interval-specific congenic strains; Bennett et al., 2002b), or a combination of these factors.

SERT Testing. A preliminary analysis in the LXS showed that the 46 strains with an ILS region spanning the serotonin transporter gene (*Slc6a4)* on chr 11, near the peak of a QTL

identified in an F_2 mapping study (Markel et al., 1997), had longer LORE (μ = 82.7) than the 28 strains with ISS genotype (μ = 74.8; 1-tailed *p* = 0.1). Genotypes of microsatellites near the gene were polymorphic between ILS and ISS (data not shown), suggesting *Slc6a4* may have been polymorphic and thus a potential candidate for LORE.

The following experiments were done to test the hypothesis that *Slc6a4* was a candidate gene underlying some of the difference in LORE between ILS and ISS. Specific binding of [^{3}H]citalopram, a highly selective SERT inhibitor (Hyttel, 1994), to crude membranes prepared from brain regions of interest, was used to determine if there were any regional differences in the number of SERTs between the ILS and ISS mice. Full saturation curves in cerebral cortex (minus prefrontal cortex) and cerebellum showed a single binding site with high affinity (K_D values = ~3 nM) and B_{MAX} values of ~500 fmol/mg protein, but no strain differences. Likewise, specific binding of 9.5 nM [^{3}H]citalopram to membranes prepared from other brain regions showed the regional differences in levels of SERTs (in fmol/mg protein: ventral midbrain ~500, nucleus accumbens ~480, amygdala ~480, dorsal striatum ~360, hippocampus ~320, prefrontal cortex ~230) but no differences between the ILS and ISS mice.

Haplotype analysis suggested that ILS and ISS are not polymorphic through *Slc6a4* (bold, Table 6). Although ILS and ISS were not genotyped in the gene itself, they and all other strains but B6, share the same haplotype for almost 600 Kb upstream of the gene. In the three strains completely sequenced (A, B6, D2) which were ancestral to ILS and ISS, there are two distinct haplotypes over the 107 SNPs in the SERT gene. The B6 is different from the other 2 strains. The divergence of B6 both upstream (10 SNPs) and in the gene suggests that ILS and ISS don't possess this haplotype, but instead share a nonpolymorphic haplotype with the other strains, thus ruling out *Slc6a4* as a candidate.

Haplotype analysis of 78 SNPs in the NET gene (*Slc6a2*) revealed substantial polymorphism (Figure 4). ILS, D2, and A share one haplotype. B6 has a different haplotype upstream and in the proximal region of the gene, through the distal portion of the gene (exon 4 and 11), all strains but

ISS share a common haplotype. ISS is B6-like in the proximal region of the gene, but has a unique SNP pattern in exons 4 and 11.

DISCUSSION

As expected (Belknap, 1998), heritability for LORE in the LXS was substantially higher (Table 1) than our previous estimate of ~0.4 (Markel et al., 1997). Increased h^2 facilitates genetic mapping, but variability in behavioral phenotypes can cause problems in QTL analysis. A cohort design may amplify environmental effects not common to both cohorts, resulting in identification of different QTLs due to specific GXE interactions. This approach may be necessary because of limitations on space or resources, and is preferable to testing all individuals of a given strain in one block. This effect is well-known in the plant literature (Beavis, 1994), but less so in mammalian QTL work where independent mapping populations are less common. We suggest that the slight differences in mapping results shown in Table 2, identified in cohorts 1, 2, and 3, are due to this effect (Bennett and Carosone-Link, 2005). Despite a few between-cohort differences, in general, the QTLs replicated remarkably well considering that each accounted for less than 10% of the phenotypic variance. This replication indicates that the QTLs are real, however, *p*-values (or LODs) are not overwhelming as the effect size of each is small.

Mapping using the combined data resulted in replication of virtually all QTLs from the individual cohort analyses, with slightly lower LOD scores (Table 2) and effect sizes (Bennett and Carosone-Link, 2005). Environmental effects specific to a single cohort, were averaged over the unaffected mice in the other cohorts, decreasing the significance of cohort-specific QTLs (Table 2). Thus, this approach may provide more robust identification of QTLs operating across most conditions. A preferred approach involves resampling the pooled data, randomly assigning all values to one of two cohorts, the first for estimating QTL location, the second for estimating effect size (Melchinger et al., 1998). All but one of the QTLs identified in the original dataset replicated in

the resampling design with higher LODs and larger effect sizes (Bennett and Carosone-Link, 2005).

Although QTL regions on chr 1 and 3 were significant for one sex but only suggestive for the other, there were no completely sex-specific QTLs. This result is not surprising, given the consistent difference between LORE in males and females, reported in previous studies (Markel et al., 1995). This differential sensitivity is due, in part, to differences in body fat and aqueous compartments between the sexes, which alter ethanol distribution (Goldstein, 1983), and slightly higher elimination rates in females (Owens et al., 2002).

The QTL regions on chr 14, 18, and 19 illustrate two issues relating to non-replicability, which are important to recognize for follow-up confirmation studies. Although the chr 14 region was identified in cohorts 1 and 3 (Table 2), and in all three mapping packages (in at least one sex), the support intervals do not overlap. This ambiguity makes it difficult to pursue this QTL, despite its overlap with a human QTL for ethanol sensitivity (Schuckitt et al., 2005). The chr 18 and 19 regions illustrate a different problem. The chr 18 region was identified by R/qtl in cohort 1, and previous mapping studies (Table 2), as well as by Cartographer. However, WebQTL did not find this region even suggestive, highlighting the importance of using multiple approaches for mapping. R/qtl did not pick up the suggestive region on chr 19, identified in females by WebQTL and Cartographer.

All but one (on chr 14) of the LORE QTLs reported here replicated regions identified in previous mapping populations (Table 2). This replication, assessed by overlapping 1-LOD support intervals, allowed us to combine *p*-values for each QTL. For the regions on chr 1 and 3, these *p*-values were highly significant ($p < 0.001$). Composite interval mapping also identified these two QTLs as significant, as did the additive model shown in Table 5. In both cohorts analyzed separately, the same three loci, on chr 1, 3, and 8, explained much of the phenotypic variance. One of these intervals, on chr 1, also contains one of fifteen genes with significant differences in expression between ILS and ISS in cerebellum (MacLaren et al. 2006) and ventral tegmentum

(Dr. M. Miles, pers. comm.). *Xrcc5* (X-ray repair complementing defective repair in Chinese hamster cells) has a 2.5-fold higher expression in ISS, and a human ortholog in a genomic region linked to ethanol sensitivity in humans. A study using data from over 700 individuals collected by the Collaborative Study on the Genetics of Alcoholism (COGA; http://www.niaaa.nih.gov/ResearchInformation/ExtramuralResearch/SharedResources/projcoga.htm) identified several markers on Chromosome 2 linked to sensitivity in this population. *XRCC5*, the human ortholog of *Xrcc5*, maps within 2 Mb of the marker with the highest linkage score (Schuckit et al., 2001).

Both F_2 mapping (Markel et al., 1997) and interval-specific congenic strain (ISCR) confirmation (Bennett et al., 2002b) identified a slightly more proximal region on chr 1 than did the LXS. Of six ISCR, four carried a proximal region of ILS on an ISS background, while two carried the more telomeric ILS region, identified in the LXS. All ISCR showed the same pattern, of increasing LORE, however, larger sample size in the four proximal strains, provided stronger support for this region. The LXS results reported here, suggest a second, linked QTL for LORE. The abundance of support for the chr 1 and 3 regions: replication in all cohorts, and all mapping programs, as well as significant LOD scores by composite interval mapping, multiple trait mapping for chr 1, and differential expression for *Xrcc5*, favor these two as worthwhile of follow-up study.

It is clearly desirable to use the dense SNP map (archived on the Wellcome Trust site: http://zeon.well.ox.ac.uk/rmott-bin/strains.cgi). LOD scores were somewhat higher (Table 3), but the main advantage to using the SNP genotypes is the large (on average, 72%) reduction in the confidence interval surrounding the QTL. This reduction will facilitate congenic (Bennett et al., 2002a) and ISCR strain (Bennett et al., 2002b) construction.

Two of the previously confirmed QTLs for LORE, on chr 11 and 15 (Markel et al., 1997; Bennett et al., 2002a), were identified in the LXS only in a multiple trait model, which gains increased power from correlations among individual traits. The significant GXE interactions for these regions indicates their specificity to as-yet unknown environmental effects. An RI panel of

75 strains can reliably detect QTLs accounting for 10 (Valdar et al., 2003) - 20% of V_G (Belknap, 1998); thus, it is not surprising that not all QTLs replicated in all mapping populations.

The QTL on chr 8 was initially identified in a large F_2 intercross (*Lore3*; Markel et al., 1997), and replicated in two of the three LXS cohorts (Table 2) by all three mapping packages. The 1.5 LOD support interval included the norepinephrine transporter (NET) gene (*Slc6a2*), at 92.2 Mb. Knockout mice lacking dopamine β-hydroxylase cannot synthesize norepinephrine (NE), and are hypersensitive to the sedative effects of ethanol (Weinshenker et al., 2000), making it a reasonable candidate for *Lore3*. This effect is blocked by acute replacement of central NE. ILS and ISS differ in a number of NET characteristics, including *Slc6a2* haplotypes, [^{3}H]NE uptake, NET binding and mRNA levels, which are consistently 30-50% lower in ILS (Haughey et al., 2005). NET genotype significantly (p = 0.04) affected LORE in the LXS RI strains, explaining 5.6% of the phenotypic variance, with strains ILS in the NET region sleeping an average of 14 min longer than strains with an ISS genotype. This relatively small effect is likely the reason that *Lore3* was the only QTL region from the F_2 mapping that failed to confirm in reciprocal congenic strains (Bennett et al., 2002a).

The SERT gene is located at the peak of the *Lore4* QTL and numerous studies have implicated serotonin in ethanol related-behaviors. A suggestive difference in LORE in the LXS further supported SERT as a candidate. The initial SNP analysis suggested that ILS and ISS were polymorphic through the gene region, but more recent data argues against this conclusion. The denser haplotype (Table 6), suggests that there are no ILS/ISS polymorphisms in the gene, however, as no SNPs in the gene were typed in these strains, this conclusion is inferred based on flanking regions. This, and the lack of binding differences rule against SERT as a candidate. NET remains as a strong candidate, based on the numerous polymorphisms between ILS and ISS in the gene, particularly in the unique haplotype seen in exons 4 and 11.

LORE constitutes a mouse model with strong face validity to a major risk factor for alcoholism in humans (Schuckit, 2000). Our mapping results in the LXS RI panel provide additional support for the heritable nature of this trait in mice, and replicated many previously identified QTLs. Several of these have now emerged as candidates for intense follow-up to pursue the underlying gene(s) based on their replicability, significance, and interval reduction.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Strain distribution for duration of loss of righting post-ethanol (LORE) in minutes in the LXS and the two parental strains. Data from all cohorts, and both sexes, were combined, as not all strains were tested in each cohort. ILS and ISS are shown with striped bars. Error bars represent standard error of the mean.

Figure 2. BEC (mg%) in mice which failed to lose the righting response. (A) The less sensitive, non-ISS strains were pooled. Mean BEC was significantly less than BEC at awakening in mice in which LORE occurred. (B) The resistant ISS were analyzed separately. Two groups emerged: those consistent with a faulty injection (on left), which had significantly lower BEC than those apparently resistant (right side) to the sedative effect of the administered dose (4.1 g/kg).

Figure 3. Interval mapping results for LORE from R/qtl (A) and QTL Cartographer (B), sexes and cohorts combined.

Figure 4. Three haplotype blocks in *Slc6a2* (NET gene). ILS and ISS are polymorphic for all SNPs typed in the gene and 150 Kb upstream. ILS, D2, and A share one haplotype, which differs from the B6 haplotype in the proximal region of the gene. ISS is similar to B6 in exon 1, and in upstream and downstream regions. In exons 4 and 11, a unique haplotype is seen in ISS.

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TABLES

Table 1. Heritability[a] by sex and cohort and correlations (*p*-value) for LORE.

	Males, coh 1	Males, coh 2	Females, coh 1	Females, coh 2	Males, coh 3	Females, coh 3
Males, coh 1	0.68	0.59*	0.76*	0.70*	0.72*	0.75*
Males, coh 2		0.53	0.68*	0.82*	0.77*	0.72*
Females, coh 1			0.66	0.71*	0.77*	0.81*
Females, coh 2				0.71	0.77*	0.81*
Males, coh 3					0.56	0.75*
Females, coh 3						0.79
N (strains, mice)	(76,182)	(70, 193)	(73, 194)	(66, 177)	(45, 99)	(47, 107)

[a] Heritabilities are given on the diagonal.

*Correlations significant at $p < 10^{-7;}$ all others $p > 0.05$.

For combined cohorts, $h^2 = 0.51$ for males, 0.63 for females, and 0.57 for combined sexes.

Table 2. QTLs for LORR identified in LXS (Cohorts 1, 2, and pooled) using R/qtl, and previous mapping populations.

	LXS Cohort 1		LXS Cohort 2		Pooled Cohorts[a]			F_2[b]		LSXSS RI[c]	
Chr, Mb pos	LOD (*p*-value)	[1-LOD Interval Mb]	LOD (*p*-value)	[1-LOD Interval Mb]	LOD	(*p*-value)	Adjusted Effect[d]	Peak Mb (*p*-value)	[1-LOD Interval Mb]	Peak Mb (*p*-value)	Combined *p*-value[g]
1, 134.22[e,f]	2.88 (0.1)	[103.4 – 154.1]	2.41 (0.15)	[103.4 – 173.1]	2.8	(0.1)	10.5	90 (0.01)	[74.2 – 92.8]	93 (0.002)	3.00×10^{-6}
3, 143.60[f]	3.28 (0.04)	[133.4 – 151.7]	1.41 (> 0.63)		3.1	(0.05)	11.5	68 (0.63)	[0 – 147]	150 (0.03)	8.00×10^{-4}
8, 118.34[e]	(NS)	--	1.87 (0.63)	[102.0 – 128.3]		(NS)	9.0	116 (0.05)	[87 – 123]	(NS)	0.019
14, 90.66	2.77 (0.15)	[71.0 – 97.7]	(NS)	--	2.3	(0.3)	10.0	(NS)	--	(NS)	0.008
18, 89.33[e,f]	2.78 (0.15)	[77.0 – 89.2]	(NS)	--	2.3	(0.3)	10.0	77 (0.6)	[46 to 89.2]	82 (0.03)	0.003
Strains (mice)	76 (376)		72 (370)			77 (952)			(1073)	77 (1114)	

[a]Data from cohorts 1 and 2 were pooled prior to analysis.

[b]Markel et al., 1997

[c]Markel et al., 1996

[d]Effect size adjusted by analysis in two cohorts, one for location and the second for effect size (see Bennett and Carosone-Link, 2005 for details).

[e]Confirmed (Chr 1, 18) or identified (Chr 8) in F_2 population.

[f]Identified in LSXSS RI

[g] Fisher's test; independent populations combined (Cohorts 1, 2, F_2 and LSXSS RI, see text for explanation of determination of overlap)

Table 3. Comparison of the mapping programs using LORE from pooled cohorts[a] to identify QTLs.

		WebQTL					R/qtl					QTL Cartographer				
Chr	Sex	SNP or Marker	Mb	LOD	*p*-value	[1-LOD]	Marker	Mb	LOD	*p*-value	[1-LOD]	Marker	Mb	LOD	*p*-value	[1-LOD]
1	M	*rs13476091*	123.6	2.6	<0.63	[113.9 – 136.7]	*D1Mit139*	129	2.3	0.3	[107-157]	*D1Mit100*	147	2.8	0.1	[142 – 157]
	F	*rs13476134*	138.2	4.1	<0.001	[132.4 – 151.2]	*D1Mit30*	136	3.6	0.02	[107-175]	*D1Mit100*	147	3.8	0.01	[141-175]
3	M	*rs13477466*	143.4	3.4	<0.001	[137.8 – 149.6]	*D3Mit127*	147	3.0	0.06	[136 – 156]	*D3Mit86*	152	3.0	0.05	[150 – 156]
	F	*rs3657112*	147.3	3.0	<0.63	[145.6 – 149.6]	*D3Mit127*	147	2.7	0.1	[136 – 156]	*D3Mit323*	156	2.2	0.2	[147-162]
8	M				NS					NS		*D8Mit148*[b]	126	2.9	0.1	[120-telo]
	F	*D8Mit47*[c]	106.1	2.2	<0.63	[100.6 – 113.0]	*D8Mit200*	116	2.3	0.2	[104-telo]	*D8Mit120*[b]	120	3.5	0.02	[116-128]
14	M	*rs3677985*	98.7	2.3	<0.63	[88.3 – 100.1]	*D14Mit228*	92	2.4	0.2	[53-99]	*D14Mi97*	111	2.9	0.1	[110-telo]
	F	*rs13482325*	91.4	2.3	<0.63	[74.1 – 95.4]				NS		*D14Mit266*[b]	114	2.1	0.2	[99-telo]
18	M				NS		*D18Mit128*	91	2.4	0.2	[76-telo]	*D18Mit86*[b]	25	2.9	0.1	[20-26]
	F				NS					NS		*D18Mit86*[b]	25	2.1	0.3	[22-32]
19	F	*mCV23069037*	51.9	2.1	<0.63	[51.6 – 55.1]				NS		D19Mit86	26	2.3	0.63	[21-29]

[a] Strain means computed over pooled cohorts 1 and 2, separately by sex

[b] This region did not reach the suggestive cutoff when sexes were pooled.

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Table 4. Lore QTL 1-LOD support intervals for SNP and microsatellite marker analysis.

			SNP		Microsatellite
Chr	Sex	Peak (Mb)	1-LOD Interval Size (Mb)	Peak (Mb)	1-LOD Interval Size (Mb)
1	M	123.4	18.5	127.3	50.7
1	F	138.1	11.9	134.2	69.7
3	M	143.5	8.8	142.2	18.3
3	F	152.6	9.6	142.2	18.3
8	F	100.6	12.4	116.0	31.6
14	M	98.7	2.9	93.9	40.8
14	F	91.7	7.0	93.9	40.8

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Table 5. Multiple QTL models for LORE from individual cohorts.

A. Full model summary for each cohort.

Cohort	df	SS	LOD	$\%V_{exp}$	*p*
1	3	29102	9.51	43.8	4.51×10^{-9}
2	3	18985	5.64	30.3	1.78×10^{-5}

B. Additive interactions among the same three loci combine to produce the observed phenotype in both cohorts.

Position (cM)	df	SS	LOD	$\%V_{exp}$	F	*p*
Chr1 (44)	1	13973	5.25	21.0	26.9	1.86×10^{-6}
Chr3 (62)	1	12438	4.74	18.7	24.0	5.78×10^{-6}
Chr8 (36)	1	4519	1.89	6.8	8.7	4.26×10^{-3}

Table 6. SNP genotypes for *SERT* (*Slc6a4*) region for ILS, ISS, and their 8 progenitor strains[a].

SNP	position	**ILS**	B6	A	C3H	RIIIS/J	D2	IS/CamRkJ	**ISS**	AKR/J	BALB/cJ
rs3700578	75931235	**CC**	TT	CC	CC	TT	CC	CC	**CC**	CC	CC
rs3700353	76080641	**AA**	GG	AA	AA	GG	AA	AA	**AA**	AA	AA
UT_11_75.985289	76139782	**GG**	AA	GG	GG	AA	GG	AA	**GG**	GG	GG
Nes08468269	76152228	**AA**	GG	AA			AA		**AA**		
rs13481109	76320812	**TT**	AA	TT	TT	AA	TT	AA	**TT**	TT	TT
Nes08472956	76651107	**AA**	AA	AA			AA		**AA**		
rs3662476	76673210	**TT**	CC	TT	TT	CC	TT	CC	**TT**	TT	TT
Nes08472234	76702102		CC	CC			CC				
Nes08471845	76718718		GG	GG			GG				
Nes08471846	76718974		GG	AA			AA				
Slc6a2 [b]	76,724,257-76,758,001		ZZ	XX			XX				
rs6263121	77047432	**AA**	AA	AA	AA	AA	AA	AA	AA	AA	GG
rs13481112	77366647	**CC**	CC	TT	CC	CC	CC	CC	CC	CC	CC
rs6384104	77518400	**CC**	CC	CC	CC	CC	CC	CC	TT	CC	TT

[a] Based on Ensembl Build 34

[b] All strains genotyped for multiple (107) SNPs through the SERT gene showed identical haplotypes (XX or ZZ) over the entire 33744 bp.

Figure 1

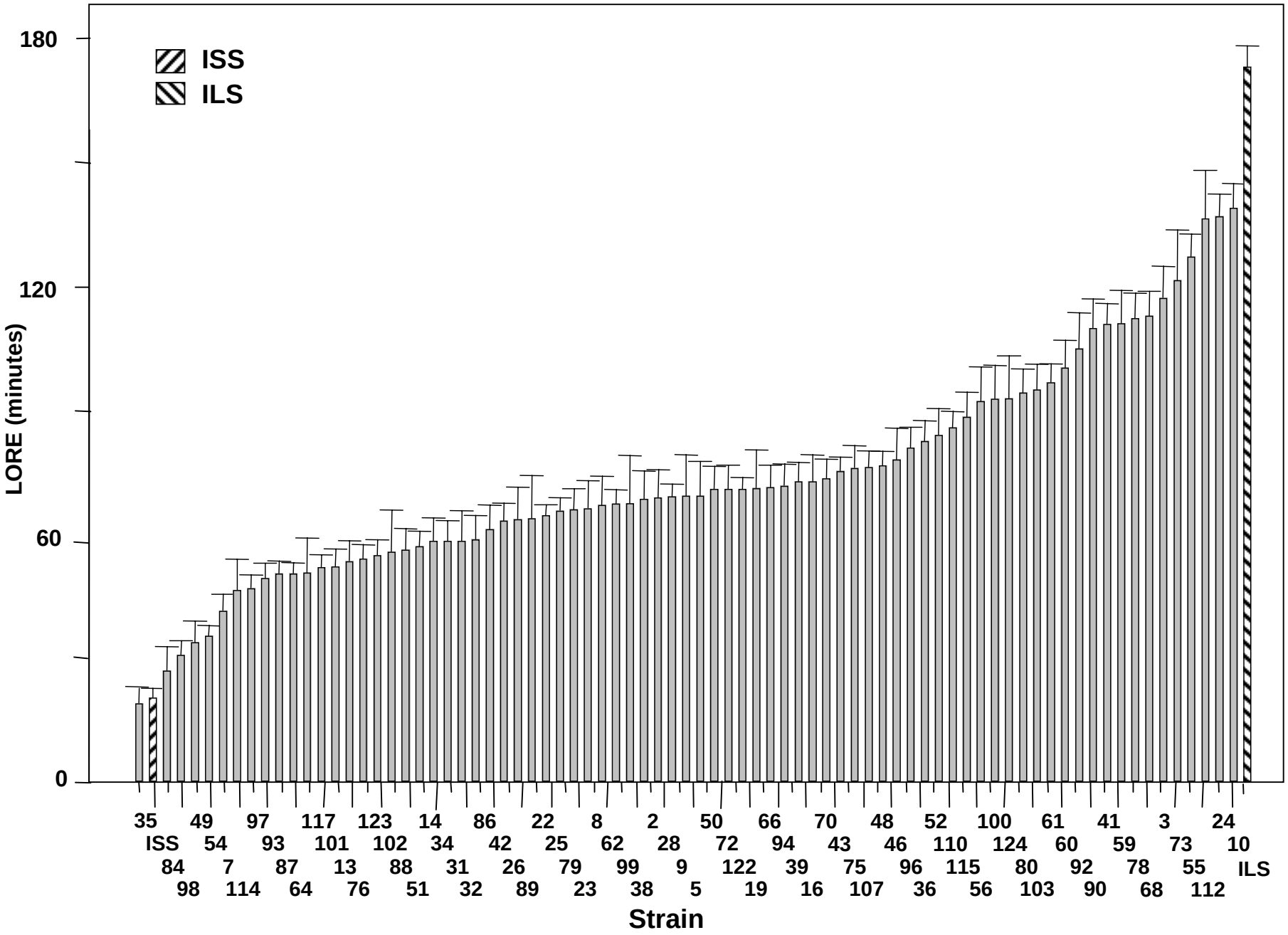

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Figure 2

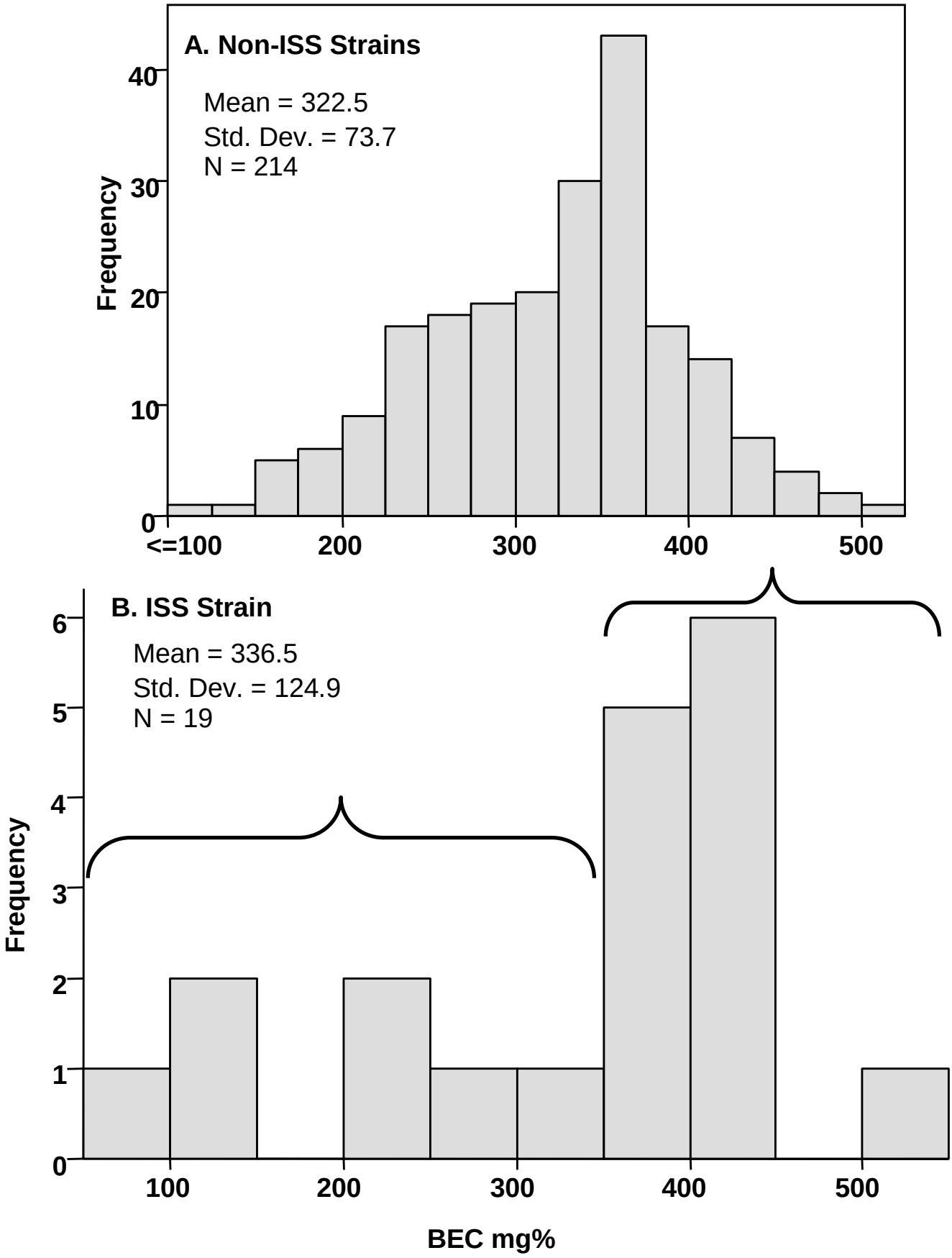

Figure 3

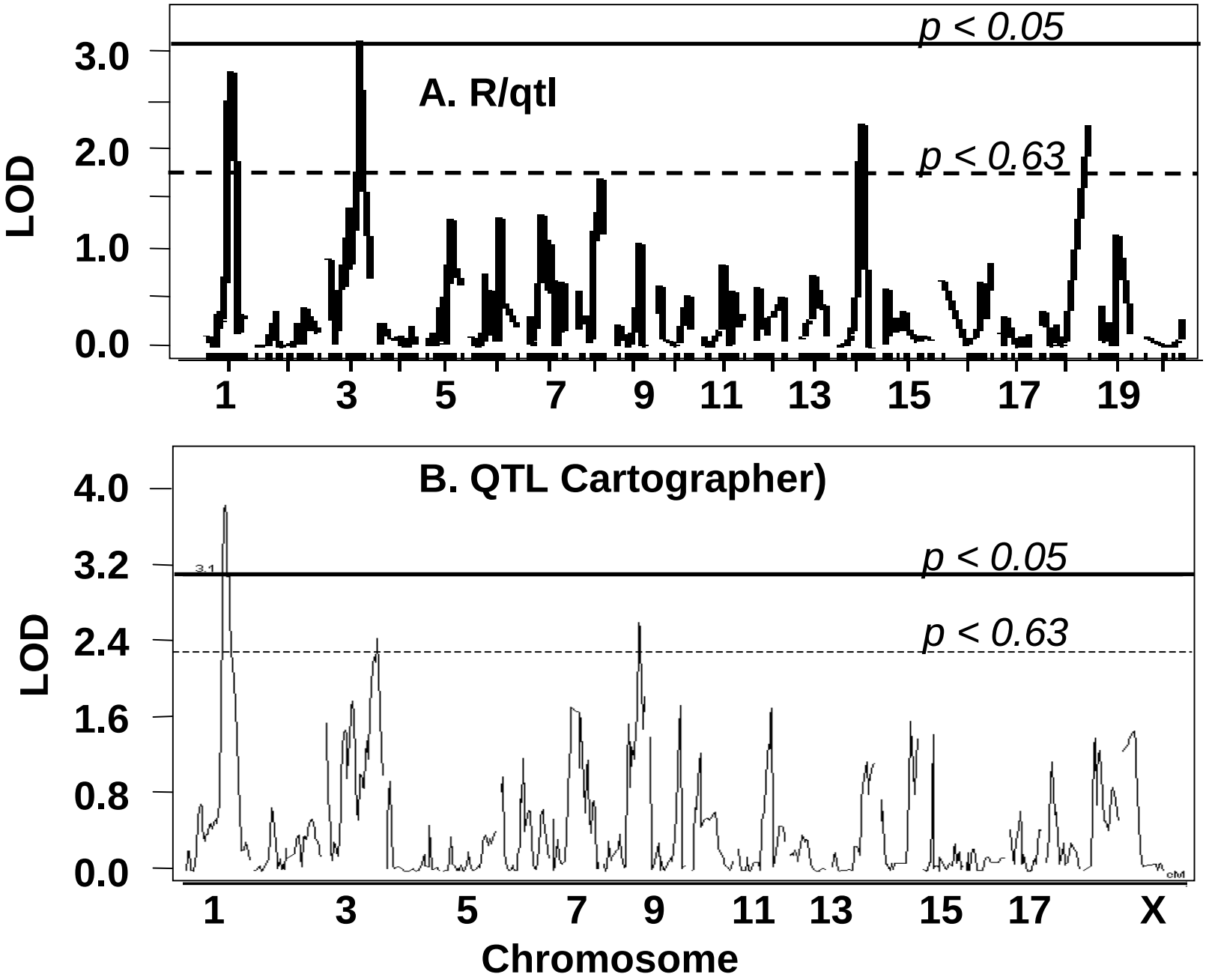

Figure 4