

Dissecting anxiety-related QTLs in mice by univariate and multivariate mapping

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To detect genes underlying anxiety-related traits in mice, we performed univariate and multivariate QTL mapping analyses of phenotypes obtained from 71 mice of the BXD recombinant inbred (RI) strains ($n=528$ mice) and their parental strains (C57BL/6J and DBA/2J). Separate and joint mapping analyses were carried out using a linkage map composed of 506 simple sequence repeats (SSRs). The main QTL effects, interactions between pairs of QTLs (epistasis), and their environmental interactions were estimated. The results showed that anxiety-related traits were influenced by multiple QTLs (five main effect QTLs and three epistatic QTLs). Ten potential anxiety-related candidate genes within the QTL intervals on chromosomes 5, 13 and 15 were identified. Some of these genes have been reported previously to be associated with the anxiety response. Based on our results, it is suggested that the multivariate QTL mapping approach improves the statistical power for detecting QTL and the precision of parameter estimation. Moreover, multivariate mapping can also detect pleiotropic QTL effects.

multivariate QTL mapping, mouse, BXD strains, pleiotropy

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Complex traits, including many common diseases, are controlled by multiple loci, each of which can be detected as a quantitative trait locus (QTL). Most approaches map QTLs separately by a phenotype-driven method focusing on an individual trait [1]. However, complex trait data usually contain observations on multiple traits either in multiple environments or under different treatment conditions. Therefore, several potential problems arise; for instance, determining whether multiple traits are affected by a single QTL with pleiotropic effects or by multiple closely linked QTLs. Additionally, the effects of QTLs may vary in different environments or under different treatment conditions. Several studies have suggested that genotype-environment interac-

tions ($G \times E$) play important roles in gene-associated diseases, and thus can be thought of as a potential factor underlying the genetic variation of quantitative traits [2–4].

Recombinant inbred (RI) strains are an important resource that has been used to map complex traits. They provide compelling advantages for mapping complex traits. In mice, the largest RI set, the BXD strains, has been extensively used for mapping traits with complex genetic and environmental effects, including mood and anxiety disorders [5–8]. Previous studies have shown that the BXD RI strains have marked variation in anxiety- and fear-related phenotypes. Differences in these phenotypes are also evident in the parental strains. For example, the C57BL/6J strain exhibits lower levels of anxiety-related and fewer stress-related effects compared with the DBA/2J strain that

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exhibits greater fear-related responses [8–12]. Therefore, the BXD RI strains provide a powerful tool to study the genetic basis of anxiety- and fear-related behaviors in mice [13–16].

Single-trait QTL mapping methods are readily expandable to multiple-trait analysis. Jiang and Zeng [17] previously proposed a multivariate statistical model to conduct multiple-trait analysis. However, the same trait measured in different environments should be considered as distinct traits, and the maximum likelihood method applied in that approach increases the computational complexity. Although principal component and canonical variables have been used in multiple-trait analysis [18,19], the estimates are still incapable of delineating the effect(s) of each QTL. We previously proposed a full-QTL model for modeling the genetic architecture of a complex trait from multi-environment trials, using a mixed linear model approach [20]. By extending this statistical model, here, we performed multivariate QTL mapping of anxiety-related phenotypes under different conditions in an attempt to analyze main QTL effects, epistatic effects, as well as their interaction effects with different treatments. Simultaneously, a separate QTL mapping analysis was carried out for each measurement using a full QTL model based on mixed linear model. When these two mapping results were compared, we found some pleiotropic main and epistatic QTLs, several of which were sensitive to different treatment conditions.

1 Materials and methods

1.1 Mouse strains

In this study, we analyzed anxiety-related behavior in the open quadrants of an elevated zero maze, a standard tool for testing anxiety [21]. Seventy-one mice of the BXD RI strains, 60 to 120-d-old were used in this study. This strain was derived by crossing the C57BL/6J (B6) and DBA/2J (D2) strains to produce the BXD1 through BXD32 strains (26 strains) [22] in the 1970s, and the BXD33 through BXD42 strains (9 strains) [23] in the 1990s. Genotypes of the BXD strains were generated at the University of Tennessee Health Science Center. A total of 3795 markers were genotyped, including 3033 SNPs and 762 SSRs. Many adjacent markers have identical strain distribution patterns; therefore, we selected 2320 markers for the subsequent analysis (1814 SNPs and 506 SSRs). To reduce computation complexity, only the 506 SSRs were used to map causal QTLs.

1.2 Anxiety assay

Anxiety-related behavior was examined in animals under five conditions: one, animals that were acutely restrained and received ethanol; two, animals that were acutely restrained and received saline; three, animals that received

only a saline injection; four, animals that received only an ethanol injection; and five, animals that were not restrained and did not receive any injection. Acutely restrained animals were placed in an immobilization tube for 15 min. Animals receiving injections received either ethanol (1.8 g/kg) or saline, and were returned to home cages. Three variables were recorded during the test session: Open-5, time spent in the open quadrants during the first 5 min of the session; Open+5, time spent in the open during the last 5 min of the session; and Open10, time spent in the open during the entire test session.

Separate QTL mapping analyses were performed for each measurement (Open-5, Open+5, and Open10) to identify significant main and epistatic QTLs. Because any genetic relationships among these three behavioral measures are not taken into account in single-trait mapping computations, we also performed joint mapping of the QTL in an effort to determine pleiotropic effects, or common genetic influence on multiple traits. The two analyses (single and joint mapping) were carried out in QTLNetwork, a software package (http://ibi.zju.edu.cn/BCL/softwareNetwork_GPU.html) that can detect individual and epistatic interacting QTLs for individual and multiple traits [20]. In both analyses, the five experimental conditions were considered as different environments. Because the univariate mapping approach has already been described in detail by Yang et al. [20], and the multivariate mapping method will soon be published elsewhere, here we only briefly introduce the multivariate mapping approach.

1.3 Statistical model for the multivariate mapping approach

Individual and epistatic interacting QTLs were detected using the following statistical model including general QTL effects and QTL by environment interaction effects,

$$y_{ijk} = \mu_i + \sum_l a_{il}x_{lj} + \sum_{l<h} aa_{ilh}x_{lj}x_{hj} + e_k + \sum_l \sum_k ae_{ilk}u_{lj} + \sum_{l<h} \sum_k aae_{ilh}u_{lj}u_{hj} + \varepsilon_{ijk}, \quad (1)$$

where, for the i th trait ($i \in [1, m]$, m is the total number of traits), y_{ijk} is the phenotypic value of the j th line in the k th environment; μ_i is the population mean of the i th trait; a_{il} is the additive effect of the l th QTL (Q_l), fixed effect; x_{lj} is the coefficient of the parameter a_{il} ; aa_{ilh} is the additive by additive effect between Q_l and Q_h , fixed effect; e_k is the random effect of the k th environment; ae_{ilk} and aae_{ilh} are the additive by environment and additive \times additive by environment interaction effect, respectively, and are both random effects; and ε_{ijk} is the random residual effect.

For the i th trait, the statistical model in matrix notation is

$$y_i = \mathbf{1}\mu_i + \mathbf{Xb}_{Ai} + \mathbf{Xb}_{AAi} + \mathbf{Ie}_{Ei} + \mathbf{U}_{AEi} \mathbf{e}_{AEi} + \mathbf{U}_{AAEi} \mathbf{e}_{AAEi} + \mathbf{Ie}_{ei}. \quad (2)$$

For multiple traits, the multivariate model in matrix notation is

$$\begin{aligned}
 \mathbf{Y} &= \mathbf{1}_n \mathbf{b}_\mu^T + \mathbf{X}_A \mathbf{B}_A + \mathbf{X}_{AA} \mathbf{B}_{AA} + \mathbf{U}_E \mathbf{E}_E + \mathbf{U}_{AE} \mathbf{E}_{AE} + \mathbf{U}_{AAE} \mathbf{E}_{AAE} + \mathbf{E}_\varepsilon \\
 &= \mathbf{X} \mathbf{B} + \sum_{u=1}^{r+1} \mathbf{U}_u \mathbf{E}_u, \tag{3}
 \end{aligned}$$

where \mathbf{Y} is a $(n \times m)$ matrix of the phenotypic value y_{ijk} ; \mathbf{b}_μ is a $(m \times 1)$ column vector of the population mean μ_i ; \mathbf{B}_A is a $(s \times m)$ matrix of the additive effect a_{il} ; \mathbf{B}_{AA} is a $(t \times m)$ matrix of the additive by additive effect aa_{ilhk} ; \mathbf{X}_A and \mathbf{X}_{AA} are the coefficient matrix of \mathbf{B}_A and \mathbf{B}_{AA} , respectively; \mathbf{E}_E is a $(p \times m)$ matrix of environmental effect e_k ; \mathbf{E}_{AE} and \mathbf{E}_{AAE} are the random matrix of ae_{ilk} and aae_{ilhk} , respectively; and \mathbf{E}_ε is a $(n \times m)$ matrix of the random residual effect ε_{ijk} .

The mapping strategy adopted was similar to the full-QTL model proposed by Yang et al. [20]. It is divided into two steps: one, a 1D genome scan to map individual QTLs; and two, a 2D genome scan to detect all possible epistatic interactions. In the mapping approach, we used the Wilks' Lambda test instead of the F -test to determine the existence of QTLs because of the relationship among the multivariables.

2 Results

To explore the relationships among the three behavioral measures, we carried out correlational analyses for Open-5, Open+5 and Open10. The results showed that there were highly significant correlations among the phenotypes ($r_p \approx 0.78-0.94$) and among the genotypes ($r_G \approx 0.82-0.96$), suggesting that common or shared mechanisms may regulate the three related behavioral traits.

To identify the QTLs underlying these three behavioral measures, we mapped the QTLs for the individual QTL

effect and for the epistatic effect of two QTLs, using both the univariate mapping (UVM) and multivariate mapping (MVM) approaches. The positions and additive effects of QTLs estimated by the UVM and MVM analyses are listed in Table 1.

As shown in Table 1 and Figure 1, two additive QTLs on chromosome 15 for Open-5 (Q15-19 at 46.1 cM) and Open10 (Q15-20 at 47.1 cM) were detected by UVM analysis. The close proximity of these QTLs suggests that the loci associated with these QTLs are closely linked. Interestingly, MVM analysis detected a single QTL (Q15-19) on chromosome 15 at 46.1 cM associated with all three behavioral measures. In addition, three QTLs, in close proximity, on chromosome 13 at 28.4, 36.4, and 34.4 cM were identified by UVM in Open-5, Open+5 and Open10 respectively. MVM analysis confirmed only one QTL (Q13-9) at 31.4 cM on chromosome 13. Because we obtained three separate but closely linked QTLs on chromosome 13 in the univariate analysis, and a single QTL when joint mapping was carried out, we suspected that there was actually a single QTL with pleiotropic effects on the behavioral traits. Again, when MVM and UVM were used to analyze the epistasis QTL, we found that MVM could detect pleiotropic effect from linked QTLs (Table 2). Together, these results indicated that MVM based on a mixed linear model approach could distinguish pleiotropic QTLs from closely linked QTLs.

Our findings show that the MVM approach has greater power in detecting QTLs than UVM analysis (Tables 1 and 2). UVM analysis detected a single QTL, Q5-53 for Open10 ($a \hat{=} -1.85$, $P < 0.001$, $ae_4 \hat{=} 1.65$, $P < 0.05$), however, MVM analysis revealed three different QTLs associated with each of the three behavioral measures (Open10 $a \hat{=} -0.90$, $P < 0.05$, $ae_4 \hat{=} 1.63$, $P < 0.05$; Open+5 $a \hat{=} -1.15$, $P < 0.01$; Open-5 $ae_4 \hat{=} 1.76$, $P < 0.05$). Similarly, UVM analysis detected a single QTL, Q13-20, associated with Open10,

Table 1 QTL positions and additive effects obtained by UVM and MVM analyses^{a)}

Method	QTL (position in cM)			Additive effect (A)		
	Open-5	Open+5	Open10	Open-5	Open+5	Open10
UVM			Q5-53 (91.3)			-1.85 [‡]
	Q13-9 (28.4)	Q13-9 (36.4)	Q13-9 (34.4)	-2.53 [‡]	-2.39 [‡]	-3.06 [‡]
			Q13-20 (64.3)			-1.90 [‡]
	Q15-19 (46.1)		Q15-20 (47.1)	-4.21 [‡]		-3.56 [‡]
			Q4-38 (74.3)			
MVM	Q5-53 (91.3)	Q5-53 (91.3)	Q5-53 (91.3)		-1.15 ^{**}	-0.90 [°]
	Q13-9 (31.4)	Q13-9 (31.4)	Q13-9 (31.4)	-3.32 [‡]	-3.76 [‡]	-3.54 [‡]
	Q13-20 (64.3)		Q13-20 (64.3)	-1.73 [‡]		-1.13 ⁺
	Q15-19 (46.1)	Q15-19 (46.1)	Q15-19 (46.1)	-3.10 [‡]	-1.45 [‡]	-2.28 [‡]

a) UVM, univariate mapping; MVM, multivariate mapping. Qm-c is the QTL located at the left side of marker m on chromosome c. *, **, + and ‡ represent 0.05, 0.01, 0.005 and 0.001 significant levels (P values), respectively. The empty cells in the additive effect column indicated that the additive effect is insignificant.

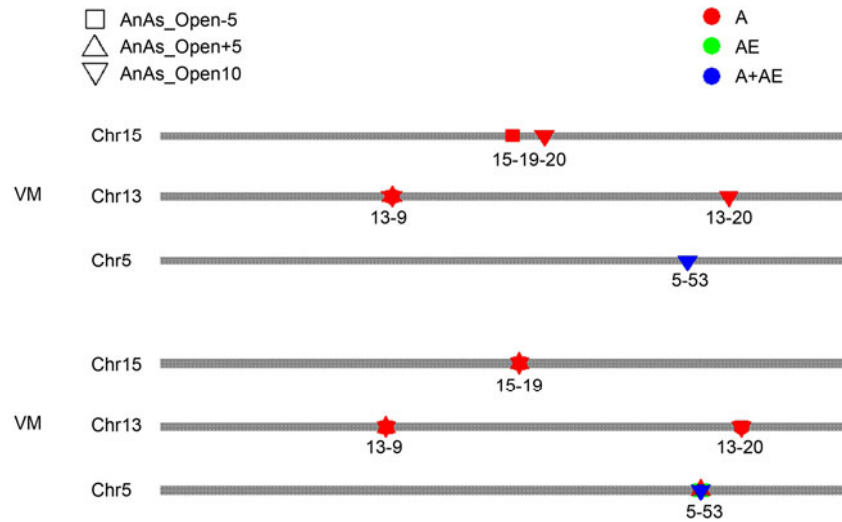


Figure 1 Positions of the main QTLs detected by separate and joint QTL mapping. UVM, univariate mapping (individual trait mapping); MVM, multivariate mapping (joint-trait mapping). AnAs_Open-5, AnAs_Open+5 and AnAs_Open10 refer to the three mice anxiety assay measures: testing for the first 5 min, testing for the last 5 min, and testing the whole 10 min. Red represents a QTL with only additive effects; green represents a QTL with additive \times treatment interaction effects (AE); and blue represents a QTL with both additive and additive \times treatment interaction effects (A + AE).

Table 2 Positions and epistatic effects of two QTLs obtained by UVM and MVM analyses^{a)}

Method	QTL \times QTL (position in cM)			Additive \times Additive effect (AA)		
	Open-5	Open+5	Open10	Open-5	Open+5	Open10
UVM		Q7-33 \times Q14-7 (77.6/21.8)			3.94 \ddagger	
	Q9-9 \times Q17-19 (26.1/52.5)	Q9-8 \times Q17-18 (24.5/50.9)		3.62 \ddagger	3.21 \ddagger	
	Q7-33 \times Q14-7 (77.6/21.8)	Q7-33 \times Q14-7 (77.6/21.8)	Q7-33 \times Q14-7 (77.6/21.8)	3.03 \ddagger	3.56 \ddagger	3.29 \ddagger
MVM	Q7-33 \times Q14-14 (77.6/29.4)	Q7-33 \times Q14-14 (77.6/29.4)	Q7-33 \times Q14-14 (77.6/29.4)		1.11 \ast	
	Q9-10 \times Q17-20 (33.7/54.7)	Q9-10 \times Q17-20 (33.7/54.7)	Q9-10 \times Q17-20 (33.7/54.7)	3.28 \ddagger	3.17 \ddagger	3.22 \ddagger

a) UVM, univariate mapping; MVM, multivariate mapping. Q_m-c is the QTL located at the left side of marker m on chromosome c . \ddagger represents 0.001 significant levels (P values). The empty cells in the additive \times additive effect column indicated that the epistatic effect is insignificant.

while MVM analysis detected the same Q13-20 associated with both the Open10 and Open-5 measures. Similarly, UVM analysis only detected an interaction between Q7-33 and Q14-7 for Open+5 ($aa \hat{=} 3.94$, $P < 0.001$), but MVM analysis estimated that these QTLs were associated with all three measures (Open-5 $aa \hat{=} 3.03$, $P < 0.001$; Open+5 $aa \hat{=} 3.56$, $P < 0.001$; Open10 $aa \hat{=} 3.29$, $P < 0.001$). Finally, the interaction between Q9-10 and Q17-20 was detected only when MVM analysis was performed (Open-5 $aae_4 \hat{=} -2.96$, $P < 0.005$; Open+5 $aa \hat{=} 1.11$, $P < 0.05$; Open10 $aae_4 \hat{=} -1.69$, $P < 0.05$) and not with UVM.

To look for potential candidate genes within the QTL intervals, we listed all the known genes which underly the ten most significant QTLs and are reported to be related to anxiety. We identified ten potential candidate genes: one, *Tmem132d*, in the Q5-53 interval; four, *Dtnbp1*, *Drd1a*, *Cplx2*, and *Grk6*, in the Q13-9 QTL interval; two, *Pik3rl* and *Htr1a*, in the Q13-20 QTL interval; and three, *Arc*, *Apol3*, and *Hsfl*, in the Q15-19 interval. Interestingly, two of these genes (*Dtnbp1* and *Drd1a*) are known to be associ-

ated with dopamine systems and, among other things, dopamine is known to act upon receptors in the brain that can alter the way the brain reacts to normal social situations, leading to anxiety. Previous studies have indicated that *Dtnbp1* is a risk gene and a potential genetic basis for schizophrenia and affective disorders such as bipolar or major depressive disorder (MDD) [24–26]. Similarly, a *Drd1a* polymorphism has been associated with the presence of psychosis and aggressive behavior in patients with Alzheimer disease (AD) [27–29].

3 Discussion

In most biological experiments, multiple phenotypes are observed and measured simultaneously in the same individuals. Such phenotypes are usually highly correlated with one another and may be under the control of common genetic mechanisms. As described above, two different approaches can be used to examine pleiotropic QTLs, either

by mapping each trait separately (UVM) or by joint analysis of traits (MVM). Because the MVM approach takes into account covariance in the traits studied, it has four distinct advantages over the UVM approach. The MVM approach: (1) increases the statistical power of QTL detection; (2) improves the precision of estimation for QTL intervals; (3) allows for hypothesis testing involving relationships among the multiple traits, and (4) detects pleiotropic QTLs [17,30–33]. In addition, the MVM approach can detect additive and epistatic QTLs with significant genetic effects which generally are not detected with UVM methods.

Although the MVM approach offers several advantages over the UVM approach, there is a limitation that should be considered. The MVM approach may identify what appears to be a single pleiotropic QTL, but if this approach alone is used, it can be difficult to determine whether or not the QTL is actually a single QTL or two QTLs in very close proximity to one another. This problem, however, can be resolved by separately testing specific genomic regions within the region identified by joint analysis to distinguish between separate and pleiotropic QTLs [17].

C57BL/6J (abbreviated B6) mice showed lesser anxiety- and stress-related behaviors than strain DBA/2J (abbreviated D2) mice [8–12] and the reported continuously distributed observations of the BXD lines were assumed to be driven by interactions between multiple genes. In this study, we used the MVM mapping approach to detect QTLs involved in anxiety-related behaviors in the mouse. This approach proved to be an accurate and powerful tool to detect pleiotropic additive and epistatic interacting QTLs. The MVM approach is useful in identifying candidate genes involved in multiple anxiety-related traits. The five individual QTLs and three epistatic interactions detected by MVM approach were found to have similar positions and effects. Based on these estimated QTLs, we investigated the genetic basis of the observed anxiety-like behavior in the mice. The individual QTL Q5-53 seemed to have a negative additive effect in two of the test measures (Open+5 $a \hat{=} -1.15$, $P < 0.01$; Open10 $a \hat{=} -0.90$, $P < 0.05$), suggesting that Q5-53 may be associated with enhanced fear or anxiety. However, we noted that alcohol may reduce the anxiety by a small amount, because QTL Q5-53 exhibited a significant positive additive by environment effects when the mice were acutely restrained and received ethanol. Similarly, the effects of the epistatic interactions allowed us to deduce how the identified QTLs might be associated with the fear-like behaviors. For instance, the interaction between QTLs Q9-10 and Q17-20 showed positive additive \times additive effects for the three behavioral measures (Open-5 $aa \hat{=} 3.28$, $P < 0.001$; Open+5 $aa \hat{=} 3.17$, $P < 0.001$; Open10 $aa \hat{=} 3.22$, $P < 0.001$), indicating that their interaction may moderate the anxiety response. Furthermore, we investigated these QTLs that were found to significantly modulate the anxiety or fear response, and identified candidate casual genes located in the significant support interval which is defined by

F -value drop. A recent report identified Tmem132d as a candidate gene for anxiety-related behavior [34]. This gene, located in the region of Q5-53, has been reported to be significantly associated with panic disorder in humans. Besides, the mouse experiments suggest Tmem132d is an important gene for anxiety-related behavior. Previous studies have revealed that *Dtnbpl* [35], located in the support interval of Q13-9, is a candidate gene for risk of schizophrenia, by causing depression and anxiety.

In the present study, ten significant QTLs detected by MVM have been shown to be potential casual loci for the anxiety-related response. We concluded that, compared with UVM, the MVM approach was more powerful in uncovering the genetic and genomic basis of multiple co-related complex traits.

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