Fine mapping of QTL for primary antibody responses to KLH in laying hens.

M. Siwek [1#] ,S. Cornelissen[1&], L.L. Janss[1], E. F. Knol[2], M. Nieuwland[1], H. Bovenhuis[1], M. A. M. Groenen[1] , H. K. Parmentier[1], J.A.M. van Arendonk[1], J van der Poel[1].*
[1]Animal Breeding and Genetics Group, [2] Adaptation Physiology Group, Wageningem Insitute of Animal Sciences, Wageningen University, P.O. Box 338, NL – 6700AH Wageningen, The Netherlands, [2] IPG Institute for Pig Genetics, P.O. Box 43, 6640 AA Beuningen, The Netherlands [#]current address: Department of Biotechnology, University of Technology and Agriculture, Mazowiecka St 28, 85-225 Bydgoszcz
@: siwek@atr.bydgoszcz.pl

Introduction

A total genome scan has been applied to identify quantitative trait loci (QTL) in an experimental population that was created from High (H) and Low (L) selection lines, where the H and L lines were divergently selected for primary immune response to Sheep Red Blood Cells (SRBC). A QTL for KLH (keyhole limphet hemocyanin) was detected on GGA14 (Siwek et al, 2003). KLH is a novel antigen for birds and, therefore, a suitable antigen to study antibody (Ab) response as part of the Immune Response. In the whole genome scan approach microsatellite markers were used to identify QTL. However, when all available microsatellite markers were used, the identified region on GGA14 still spanned over 50cM. A promising approach in fine mapping a QTL region is combining information obtained by linkage analysis (LA) and linkage disequilibrium analysis (LD) (Meuwissen and Goddard, 2000). In the LA approach information transmitted from the parents to their offspring is traced with DNA markers. If LA (roughly within family information) is combined with LD (roughly between family information), additional knowledge about historic recombination events is utilized. The LD or LDLA approach requires an increase in marker density as useful LD is expected to extend only over limited distances. This implies that additional markers are needed for fine-mapping a QTL in order to point out positional candidate genes. The most suitable markers for finemapping analysis are SNP markers. SNP are far more abundant than microsatellites; in chicken a SNP frequency of 1 SNP per 100bp is observed (Vignal et al., 2000, ICGSC, Nature 2004). This SNP abundance makes it very suitable for haplotype analysis.

Material and methods

Chicken Population.

The H/L F_2 population originated from a cross (ISA Warren, medium heavy layers) between two divergently selected lines for either high (H line) or low (L line) primary antibody response to SRBC. Selection was based on the individual antibody titre at 5 d after primary intramuscular immunisation with SRBC at 37 d of age (Van der Zijpp and Nieuwland, 1986). Reciprocal crosses with birds from the 18th generation were made to generate F_1 animals. From the F_1 generation, an inter-cross was made to produce 672 individuals in 6 hatches of the F_2 experimental population. For the QTL detected on GGA14 in the F_2 generation, using within family regression, only one family showed the significant founding for QTL effect.

All birds were housed in brooder cages with free access to water and feed (152 g/kg CP and 2,817 kcal/kg ME). Birds were not beak-trimmed and each individual bird was marked with a wing-band. All birds were vaccinated against Marek's disease, infectious bronchitis, and infectious bursal disease at hatch, and at 2 and 15 d of age, respectively. The Ethical Committee On Animal Care and Welfare of Wageningen University, The Netherlands approved the experiment.

Phenotyping of the generations F_2 and F_4.

Total antibody responses to KLH were measured in individual plasma samples obtained at 7 d after s.c. immunization with 1 mg KLH (Cal Biochem-Novabiochem Co., La Jolla, CA) in 1 mL PBS (pH 7.2) at 12 wk of age. Antibody titers to KLH of all birds were measured by an indirect ELISA as described by Sijben et al. (2000), and as performed in the previous experiment (Siwek et al., 2003). Titers were expressed as the $\log_2$ values of the highest dilution giving a positive reaction.

SNP detection

Animals

The SNP discovery panel consisted of eight animals. All of them originated from one F_1 family which had the highest contribution to QTL effect. In the panel: F_1 sire, two F_1 dams, and 5 F_2 individuals, ancestors of F_4 generation were present.

Primer design

In total 58 STSs were tested for amplification and sequencing. Primers were designed from end sequences of BAC clones that contained loci mapped on GGA14 or based on the Beijing SNP database (Wong et al., in press). All primers were designed using Primer3 through the web interface (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

PCR and sequencing

PCR reactions were carried out in 24µl volumes. Reactions were performed as described by Jungerius et al. (2003). After PCR reaction the excess of primers was removed by running the samples over a column of BioRad P-100 (BioRad, Hercules CA , USA) in a Multiscreen MAHV N45 plate (Millipore, Billerica, MA, USA) in 96-well plate format. From each sample, 2µl was checked on agarose gel to estimate the DNA concentration. Sequencing reactions were performed with the forward and the reverse amplification primer. The sequencing reactions contained 100 – 400 ng of purified PCR product, 2µl of Big Dye Terminator Rtmix (Perkin – Elmer, Foster City, CA , USA), 2µl of Half Big Dye Buffer (Genetix, New Milton, UK) and 0.8 pmol of either primer in a final volume of 10µl. Excess dye terminator was removed by running the samples over a column of Sephadex G-50 (Amersham Pharmacia, Uppsala, Sweden) in Multiscreen MAHV N45 plate format. Subsequently, the samples were dried using a Speed Vac and analysed with DT3100POP6 module, 96 well plate on an ABI3100 sequencer.

SNP identification

Sequencing gel images were analysed using the Sequencing Analysis Software (ABI) for lane tracking and trace file extraction. All trace files were analysed with the Pregap4 program of the Staden software package (Bonfield & Staden, 1996; http://www.mrc-lmb.cam.ac.uk/pubseq).

Genotyping strategy of the F_2 H/L population.

Genomic DNA was isolated using the Gentra Generation Capture Plate™ Kit from the whole blood according to the Capture Plate™ Kit protocol (Gentra Systems, ver. 5.00, Minneapolis, MN, USA). The genotyping strategy of the QTL region consisted of three steps:

Step 1 (M1). microsatellite markers used in the whole genome scan approach, genotyped on the entire F_2 population (6 half – sib families)

Step 2 (M2) First set of SNP markers was genotyped on one F_2 half–sib family (i.e. the one that contributed the most to the QTL detected on GGA14). Based on this family, SNP markers were selected that were within the desired marker brackets and that were most informative based on marker variance component analysis (data from one half – sib family).

Step 3 (M3) pre-selected markers (step 2) were combined with additional, new SNP markers in one set of 16 SBE primers and used to genotype the entire F_2 population (6 half – sib families). This marker information is used in LDLA analysis.

In total 9 additional SNP markers were developed and included in the final set for step 3 (16 SBE primers).

SNP Analysis with SnaPshot kit

To generate the template for the SNP analysis, PCR amplification was performed in a total volume of 12 µl with multiple primers. The exact volume of primers was defined experimentally for each combination. PCR reactions were performed with Accuprime ©. Each reaction contained: 5 µl of DNA of a final concentration 10 ng / µl, 1 µl of ddH2O, 10 µl Accuprime, 4 µl of primer mix of final concentration 200 nM. The Accuprime PCR amplification program was: 1 cycle of 2 min at 94C, 40 cycles of 30 sec at 94C, 30 sec at annealing temperature (50C, 55C or 60C), 3 min at 68C, followed by 1 cycle of 2 min at 68C. Subsequently the PCR products were checked on an agarose gel. After the amplification PCR products were pooled up to the final volume of 15 µl. In the next step, all PCR products were cleaned from primers and dNTPs with SAP/ExoI treatment. Before the Snapshot reaction all primers were premixed to a concentration of 0.2 µM for each primer. The Snapshot reaction was performed in a total volume of 10 µl consisted of: 3 µl of pooled and treated PCR products, 2 µl of pooled SnaPshot primers, 4 µl of Half Big Dye buffer and 1 µl of SnaPshot Ready Reaction Mix. The program of PCR was: 1 cycle of 5 min at 96C, 40 cycles of 10 sec at 96C, 5 sec at annealing temperature, 30 sec at 60C. After the extension, all samples were treated with SAP enzyme (1 unit / 1 µl) for 1h at 37C, followed by 15 min at 72C to inactivate the enzyme. The sequencing reaction was performed on ABI 3100 (Applied Biosystems).

QTL Analysis using Regression Interval Mapping.

Prior to the QTL paternal linkage regression analysis, phenotypic data were adjusted for the systematic hatch (H_i) and sex (S_j) effects using the PROC GLM Procedure (SAS Institute, 1995).

$$Y_{ijk} = \mu + H_i + S_j + e_{ijk} \qquad (a)$$

Where µ is the grand mean, H_i is the effect of the i_{th} hatch (i = 1, 2,...., 6), S_j is the gender effect and e_{ijk} represents residual effects. The hatch is referred to as a group of individuals hatched at one time.

Regression interval mapping was used for QTL detection. A paternal half-sib analysis model was applied (Knott et al., 1996; De Koning et al., 1999). In the paternal half-sib model no assumptions were made concerning the allele frequencies in the founder lines and number of QTL alleles. The F_2 animals were treated as unrelated half-sib families using the statistical model:

$$Y_{ij} = m_i + b_i P_{ij} + e_{ij} \qquad (b)$$

where Y_{ij} is the trait score of individual j, originating from sire i; m_i is the average effect for half-sib family i; b_i is the substitution effect for a putative QTL; P_{ij} is the conditional probability for individual j of inheriting the first paternal gamete, and e_{ij} is the residual effect.

QTL analysis using combined Linkage and Linkage Disequilibrium (LDLA) .

The LDLA method compares the expected covariances between haplotype effects given a postulated QTL position to the covariances that are found in the data. The expected covariances between the haplotype effects are proportional to the probability that the QTL position is identical by descent (IBD) given the marker haplotype information. The joined data of microsatellite- and SNP markers (SNP set 2) was analysed in the combined Linkage

Disequilibrium and Linkage (LDLA) Analysis approach. Haplotypes were derived using SimWalk2 (Sobel & Lange, 1996). The phenotypic records of F_2 animals were analysed using the statistical model:

$$Y = Xb + Zh + e \qquad (d)$$

Where *Y* is the vector of records, *b* is the vector of fixed effects (sex, batch), *h* is the vector of random effects of the haplotypes, *e* is the vector of residuals; *X* and Z are known incidence matrices for the effects in *b* and *h* respectively.

Two genetic inheritance models were tested in the LDLA analysis: one single additive genetic variance component (1 variance, combined sire and dam component) and a separate sire and dam genetic variance component (2 variances). The 2-variances model was tested against the 1- variance model in order to detect parent-of-origin effects. In the larger marker brackets (more than 11cM) the QTL was fitted at several positions within the interval. In smaller marker brackets, the QTL position was assumed to be in the middle of the bracket.

Results

Best position for the QTL

1. Results of paternal half-sib regression analysis (One half-sib family)

In Figure 1 the results of the regression approach are given for the original microsatellites (M1), microsatellites plus first set of SNP's (M2) and the final analysis with the extra set of SNP's added (M3). Adding more markers (M2, M3) decreased the confidence interval but also decrease the significance of the detected QTL. The highest test statistic equalled 18.2 for the microsattelite markers (M1), 10.9 for analysis with the first set of SNP markers (M2), and 13.7 for analysis with the final set of SNP markers. The a-posteriori positions of the second group of SNP's (M3), based on observed recombination fractions was different from the design based on the draft map. The 9 SNP's were chosen to be at less than 1 cM distance from one another, but turned out to be dispersed over the distal part of GGA14.

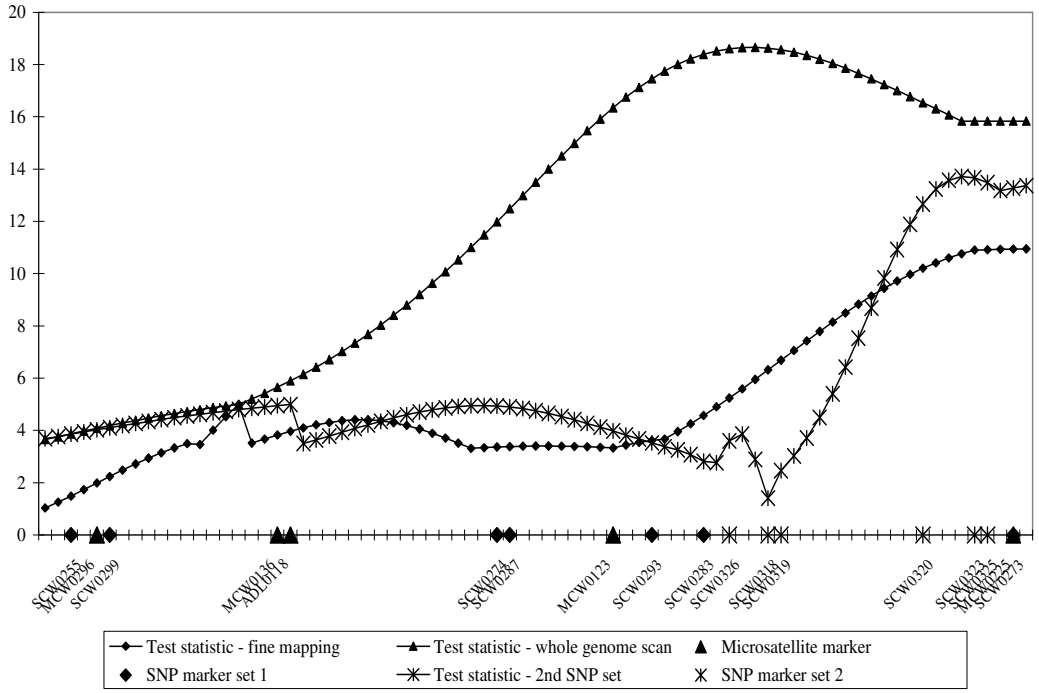

Figure 1. Regression interval mapping of primary antibody response to KLH on GGA14. Analysis of one family.

2. Results of LDLA analysis of entire F_2 population.

The entire F_2 population was genotyped for 5 microsatellite markers (M1) and 16 SNP markers. The combined analysis LDLA was performed, for both LDLA 1 variance and LDLA 2 variances model. The LDLA 2 variances model was tested against LDLA 1 variance to check for imprinting. In Figure 2 results are given for the combined LDLA analysis. In analysis, the highest test statistic was found around position 76 at the distal end of GGA14. The maximum test statistic for the LDLA 2 variances analysis was 7.69 ($p = 0.005$). Significance thresholds derived from the log-likelihood ratio test indicated that both approaches found QTL affecting KLH response. The QTL was narrowed down to the region below 1 cM. The QTL peak falls in the marker bracket: SCW0325 and MCW0225. The estimated distance between these two markers is 0.1cM. The LDLA analysis with the 2-variance component model explained the data better than the single variance model, the LR test equal to 7.69.

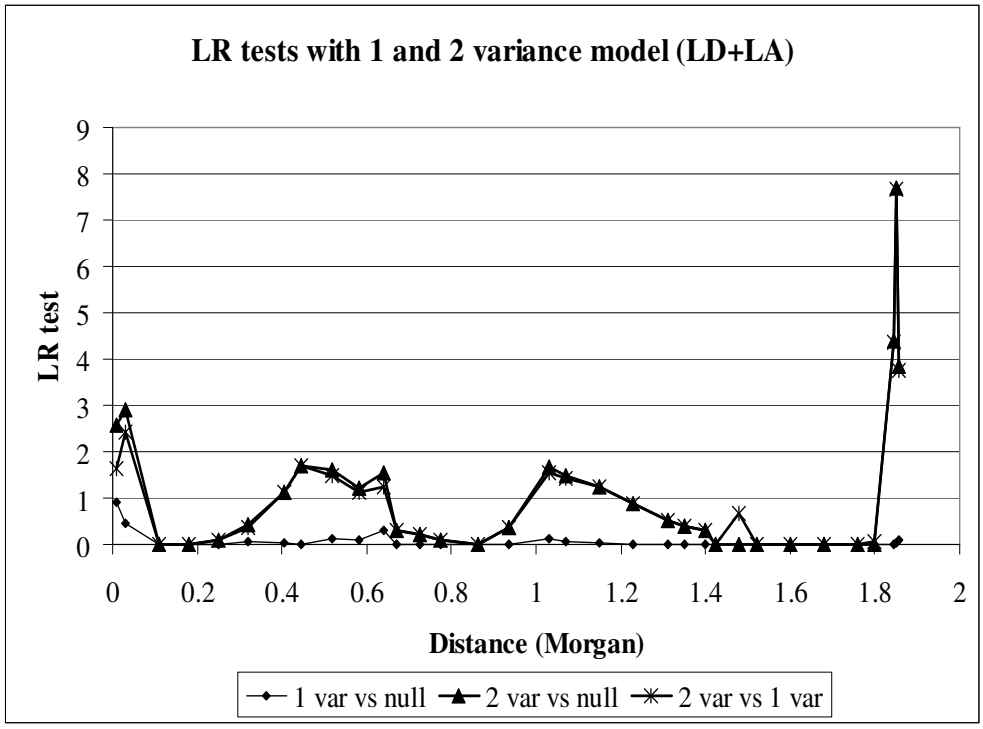

Figure 2. Combined Linkage and Linkage Disequilibrium analysis of primary antibody response to KLH on GGA14.

3. Effect of segregating haplotypes in F_2 generation.

Based on LDLA results, the most significant contribution was defined and consists of last four markers. Three sires share the same haplotype related with high KLH effect. The high KLH effect was in range from 0.52 to 0.59. In the other group, the low KLH effect clear haplotype grouping could not be done.

Discussion

QTL for KLH on GGA14 was initially reported at microsatellite MCW0225 at 77 cM. To narrow down the QTL region additional SNP markers were added to the data set. Additional marker information narrowed down the QTL peak, but also decreased the test statistic. Despite the additional markers, confidence interval was still too big to be able to come up with positional candidate genes in QTL region. Based on the marker variance component (data not shown) information for each of the SNP markers, the most suitable set of SNP markers was created, and based on the Benjing SNP data base information additional SNP's were detected. Marker variance component approach evaluated the potential relevance of a particular SNP and decreased the number of SNP necessary for further analysis. This type of approach was suggested by Shifman and Darvasi (2004) in their simulation study on mouse inbred strain. The most informative markers in term of QTL effect were: MCW0225, and two neighbouring SNP, one before the MCW0225 marker (SCW0325) and the other at the end of the chromosome (SCW0273). No recombination between SCW0325 and MCW0225 were found. The original approach dealing with the data set was based at classical linkage analysis. In current study, linkage disequilibrium was added to investigate whether or not between historic information on LD could be used to map this QTL more precise.

The application of LDLA approach to narrow down the QTL was very successful. The QTL detected with microsatellite markers and paternal half – sib regression analysis spanned over 50cM. The fine mapping data and LDLA analysis narrowed down QTL region below 1cM.

In summary, the results of QTL fine mapping are presented. The study suggests novel analysis approach to evaluate the potential relevance of particular SNP to the dissected trait. Successful application of relevant SNP markers and combined LDLA analysis allowed us significantly narrow down QTL region. The haplotype block related with high KLH effect was defined and uncovered in the F_1 sires.

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