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Marker generation for Fine Mapping a QTL in the chicken

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The purpose of this study was to design and test five SNP markers in an inbred chicken cross between Red Junglefowl and domestic White Leghorn of the 8th generation. The markers lie in a region affecting the tonic immobility behaviour which differs significantly between the two species. The markers could be identified by usage of PCR and pyrosequencing. The data obtained were further used in a small scale quantitative trait locus (QTL) analysis. QTL analysis is a statistical method to link phenotypic traits to genotypic data. Four out of five markers could be genotypes and thereby, made it possible to proceed with the QTL analysis. The results showed that there is no QTL associated with the markers identified. The two flanking markers were closest to a significant difference between genotypes and it is therefore a possibility that a QTL lies close further down or up the searched region. From the line map it is indicated that there is little recombination in the marker region.

Nyckelord/Keyword:

Behavior, Red Junglefowl, Single Nucleotide Polymorphism marker, Tonic immobility, White Leghorn, QTL analysis

Table of Contents

1 Abstract

The purpose of this study was to design and test a series of SNP markers in an inbred chicken cross between Red Junglefowl and domestic White Leghorn of the 8th generation. The markers lie in a region affecting the tonic immobility behaviour which differs significantly between the two species. The markers could be identified by usage of PCR and pyrosequencing. The data obtained were further used in a small scale quantitative trait locus (QTL) analysis. QTL analysis is a statistical method to link phenotypic traits to genotypic data. The results show that there is no QTL associated with the markers identified. The two flanking markers were closest to a significant difference between genotypes and it is therefore a possibility that a QTL lies close further down or up the searched region.

2 Introduction

Tonic immobility is a phenomenon found in several species, for example in chickens. The tonic immobility is triggered by holding down an animal on a flat surface for a short time which causes a period of physical restrain in the animal (Gallup, 1974). This effect then wears off after an amount of time which can be measured and used in studies between wild and domesticated animals. Different phenotypes in individuals can vary due to both environmental factors and the gene that affects such a trait (Wray&Visscher, 2008). It also depends on the interactions between them. It has been shown that domestic animals differs significantly in the time they spend in tonic immobility (Schütz *et al*. 2003) compared to their wild ancestors. Generally Red Junglefowl has a more active reponse in fearful situations than the domestic species which indicates a genetically induced difference in behavior (Schütz *et al*. 2002, Schütz *et al*. 2001)

Quantitative trait locus (QTL) analysis is a statistical method that links two types of information—phenotypic data (trait measurements) and genotypic data (molecular markers) (Miles&Wayne, 2008). Molecular markers are unlikely to affect the trait of interest and are therefore preferred for genotyping (Miles&Wayne, 2008). There are several types of markers that can be used in a QTL analysis but in this study, Single nucleotide polymorphisms (SNPs) were used. The basic methodology for mapping QTLs involves arranging a cross between two inbred strains that differs distinctly in a quantitative trait: segregating progeny are scored both for the trait and for a number of genetic markers (Lander&Botstein, 1988). Data for this experiment was obtained from the 8th generation of an inbred chicken intercross between Red Jungle fowl and the domesticated White

Leghorn. The markers looked for in the progeny are known in both parents. Therefore, depending on what SNP base appears in an individual one can tell from which parent it derives from. If the individual does not possess the phenotypic trait from that parent one can conclude that the SNP does not participate in that quantitative trait loci.

Previous studies on tonic immobility have been performed resulting in an identified QTL on chromosome 1 (Schütz *et al*. 2003). The purpose of this project was to fine-map a Quantitative trait locus, by designing and testing a series of flanking markers. A small-scale QTL analysis was also conducted since time permitted. It was important to obtain valid results from the design and marker genotyping in order to proceed with the QTL analysis. The SNP markers identified are located at 114 mega bases (Mb), 118Mb, 121Mb, 123Mb and 125Mb on chromosome 1. With a polymerase chain reaction (PCR), DNA samples can be amplified to a manageable amount of wanted DNA fragment containing the particular SNP markers. Since the SNP markers are just one nucleotide there is a very small region that needs to be sequenced and therefore, it is convenient to continue with pyrosequencing.

3 Materials & Methods

3.1 Tonic immobility and DNA isolation

The data from tonic immobility tests had already been collected from individuals of the same age and of 8^{th} generation. Each chicken was placed on its back for 10 seconds in a specific cradle and then the time spent immobilized were recorded. Each individual was tested twice, and the maximum time spent in tonic immobility was then used for each.

DNA was collected previously from blood samples from each chicken which was then prepared for further use.

3.2 Design and test run on agarose gel for primer pairs

Using the software Primer3plus, five pairs of primers were designed in combination with some Next Generation Sequence (NGS) data of the parental animals. The SNP markers searched for were 114 Mb, 118Mb, 121Mb, 123Mb and 125Mb, all located on chromosome 1. In order to see if the primers worked and DNA was amplified, a small test run of PCR (Table 1, Table 2) was performed on four DNA samples for each primer par. The products were then run on a 1.5% agarose gel (Table 1). The gel

control was performed in order to avoid further use of primers that did not give results. In that case, it would have been an unnecessary cost to continue with PCR and pyrosequencing.

Table 1. Protocol for 1.5% Agarose gel

Substrate	Quantity
TBE 0.5x	150 ml
Agarose	2.25g
SYBR safe	3 µl

3.3 Polymerase chain reaction (PCR)

Each individuals DNA were prepared in a DNase-free tube with a reaction volume of 25 µl (Table 2). Preparations and PCR were made one time for each primer pairs tested and a necessity before proceeding with pyro sequencing. In this type of PCR, the starting temperature in the annealing step was set very high in order to get the primers to bind correctly to the DNA sequence. The temperature is then lowered to standard PCR temperature, step by step: a "touchdown" PCR (Table 3). 45 cycles were run.

Table 2. Pre-Pyro Touchdown PCR master mix for x1, x26 and x104 samples.

Substrate	x1 (µl)	x26 (µl)	x104 (µl)
Nuclease-free H_2O	18,9	492	1965,6
10X DreamTaq buffer	2,5	65	260
dNTP Mix 2mM	0,5	13	52
Primer → (5 µM)	1,2	31,2	124,8
Primer → (5 µM)	1,2	31,2	124,8
DreamTaq DNA poly.	0,15	3,9	15,6

Table 3. Protocol for Touchdown PCR of 45 cycles

STEP	Temp. ºC	Time	Number of cycles
Initial denaturation	95	3 min	1
Denaturation	95	30 s	
Annealing	Tm-5	30 s	45
Extension	72	1 min/kb	
Final extension	72	15 min	1

3.4 Pyrosequencing

Sequence primer (S1), corresponding with the right PCR-primers, were put on thaw and a PyroMark heating block was put on preheating at 80ºC. The DNA samples were purified in a series of purification steps. 24 tubes of PCR product were purified for each a time. 60 µl of master mix, (Table

4) was added to each PCR-tube, giving a total volume of 85µl. Thereafter, the samples were placed on a Vortex for ten minutes at 1800 rpm.

Table 4. MasterMix PyroMark binding Buffer, quantity of x1 and x26 samples

Substrate	x1 (µl)	x26 (µl)
PyroMark binding buffer	40	1040
H_2O	18	468
PyroMark Beeds	2	52

Thawed S1 primer [5 µM] was diluted to a final concentration of 0.3 µM (1:17 dilution) by mixing 39 µl primer with 611 µl PyroMark Annealing buffer. 25 µl of the mix were added to all wells on a 24 sample PyroMark plate. The plate was placed on the PyroMark®Q24 Vaccum Workstation next to the PCR-products. The PCR-products were loaded to the PyroMark plate through the cleansing steps at the workstation following its´ given instructions. The PyroMark plate was placed on the heating block for two minutes and then put to rest for ten minutes at room temperature.

The pre-run information was collected in the PyroMark Q24 2.0.0 software and the dispersion well was prepared according to the information given. Dispersion well and PyroMark plate were then run on the PyroMark Q24 pyrosequencer.

3.5 Fine mapping and statistics

Genotypic and phenotypic values were also used for fine mapping in R. R is a statistical package for mapping QTLs in experimental crosses. Associations were tested using a one-way ANOVA test.

4 Results

All primers showed amounts of product and could therefore be used in the study. However, SNP marker 125Mb gave invalid results from pyro sequencing and could not be identified. The marker was therefore not included in the analysis.

4.1 Genotypic analysis

Due to identical IDs, six individuals were eliminated which resulted in a total number of 90 individuals. Table 5 shows a summation of collected genotypes for each marker.

Table 5. Marker genotypes (114Mb, 118Mb, 121Mb, 123Mb) and their percental distribution calculated from 90 individuals.

Genotype	114Mb	118Mb	121Mb	123Mb
Red Junglefowl	48.90	48.90	32.20	47.80
White Leghorn	23.30	13.30	12.20	8.90
Heterozygote	27.80	35.60	51.10	43.30
Lack of data	0.00	2.20	4.40	0.00

In all markers except 121Mb, Red Junglefowl homozygous occurs more frequently than both heterozygous and White Leghorn homozygous. There is larger difference in frequency between heterozygous and White Leghorn homozygous than between Red Junglefowl homozygous and heterozygous. This includes all markers except 114Mb, where Red Junglefowl homozygous is by far the most common.

4.2 Single marker QTL Analysis

The genotype was connected to the phenotype of each individual. Due to lack of phenotypic data, seven individuals were excluded. Final number of individuals was 83. In all four markers, Red Junglefowl generally spends the most time in tonic immobility. The values are represented by an unequal number of individuals per marker (Table 6) and (Figure 1).

Table 6. Mean value and standard deviation of the maximum time spent in toni immobility connected to the genotype of each marker.

Genotype	114mb	118mb	121mb	123mb
Red Junglefowl	176.4 ± 22.9	165.2 ± 20.0	174.3 ± 26.1	177.7 ± 18.5
White Leghorn	215.9 ± 40.1	164.8 ± 51.1	169.8 ± 54.4	75.6 ± 19.5
Heterozygote	129.8 ± 17.4	161.2 ± 24.6	155.4 ± 19.1	166.7 ± 25.2

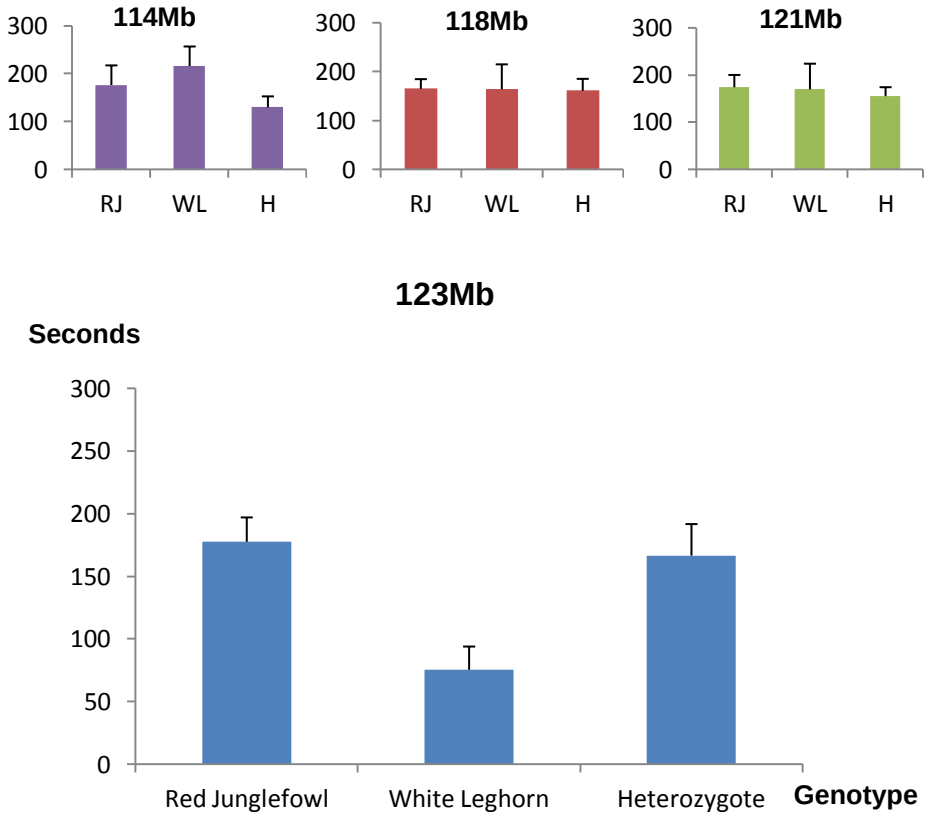

Figure 1. Means and standard errors of maximum time spent in tonic immobility for each genotype at SNP marker 114Mb, 118Mb, 121Mb and 123Mb.

From the One-way ANOVA test it is shown that there is no significant difference between the genotypes of each marker, hence no association to a QTL (Table 7).

Table 7. F-value and significant difference in genotypic mean values in each markers.

Marker	F-value	P-value
114Mb	1.92	P = 0.15
118Mb	0.05	P = 0.95
121Mb	0.03	P = 0.97
123Mb	1.95	P = 0.15

4.3 Line Map

From programming in the R software, following values were obtained (Table 8).

Table 8. SNP markers identified in this study and SNP markers obtained from previous experiments and their position at chromosome 1. Distance in Centimorgan (cM).

SNP	Position (cM)
105Mb	0
114Mb	55
116Mb	114
117Mb	170
118Mb	188
120Mb	190
121Mb	200
123Mb	219

5 Discussion

5.1 Genotypes

The results of this study show that Red Junglefowl homozygous is the most commonly occurring genotype. All three genotypes show a broad variation in the phenotypic analysis. Mean values are almost higher in all cases for Red Junglefowl. Heterozygous genotypes tend to have the minimum time spent in tonic immobility but mean values do not vary much between markers. In another study on tonic immobility, Red Junglefowl males stayed in the paralyzed state for 190.7 ±79.3 seconds (Schütz *et al*. 2003) and females of the same species were paralyzed for 155.9 ± 47.8 seconds (Schütz *et al*. 2003). This corresponds quite well with the mean values for Red Junglefowl (around 174 seconds) in this study, considering that no gender division was regarded. The same tendency can be seen for the White Leghorn.

5.2 QTL identification

According to (Fallconer&Mackay, 1996), the presence of a QTL can be inferred if there is as difference in mean phenotypes among the genotypes at a particular marker. From the results, markers 114Mb and 123Mb were the closest to a significant difference in mean phenotype, but fell below the threshold for significance ($P < 0.05$). Therefore it appears that if there is a QTL in this region it is either at 114Mb or lower, or 123Mb or higher. The phenotypic data is only connected to the individual´s genotype. It would probably give a more accurate result if more factors were involved, such as body weight, gender and influences from other, connected QTLs.

It could be interesting to investigate further if one can see if the other markers seem to adopt some kind of pattern when one marker is fixed at a certain genotype. Markers close to the fixed one should be of the same genotype if no recombination has occurred. A different genotype would however indicate that recombination had occurred. By then connecting the observations to the QTL fine mapping and the distance between markers one should obtain an accurate result of where recombination occurs in this particular region. According to the line map (Table 8) there is a low percent of recombination between markers 117Mb, 118Mb, 120Mb and 121Mb which further confirm that there is no QTL located at that region.

5.3 Methods of choice

PCR is by far the most commonly used method to amplify DNA to a manageable amount (Morisset *et.al* 2008) and was therefore essential to proceed with this experiment. However alternative methods are developing (Morisset *et.al* 2008). Pyrosequencing is an easy way to interpret the DNA sequence and to see which kind of nucleotides that exist at the markers looked for, since it is possible to tune the dispensation order of nucleotides. It is a quick, time saving way to see what kind of SNP marker that has been inherited and thereby the genotype of the individual. Results were obtained in the most efficient way.

5.4 Societal & ethical considerations

There are no ethical difficulties for this particular study. The tonic immobility test and blood samples had already been obtained under correct ethical permit before this experiment. In a broader sense one can question the fact that this is based on animal testing but such studies are vital to understanding of the genetics of the model organisms themselves and by extension all organisms. Small eukaryotes, like *Drosophila melanogaster* (fruitfly), can serve as model systems for research on developmental and cellular processes in larger, more complex eukaryotes, for example humans.

6 Conclusion

Genotypic data was successfully obtained at four out of five SNP markers and it was therefore possible to proceed with the small-scale QTL analysis.

Genotypic and phenotypic data were connected in 83 out of 96 individuals of chicken. None of the markers had genotypes that differed significantly and therefore, one can draw the conclusion that there is no QTL associated with the markers tested. Marker 114Mb and 123Mb were closest to a significant difference between genotypes and it is therefore a possibility that a QTL lies close to 114Mb or lower, or close to 123Mb or higher. More clarity might be if genotypes at marker 125Mb could be identified. This study did not meet any ethical difficulties in the process.

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