Identification of candidate genes and mutations in QTL regions for chicken growth using bioinformatic analysis of NGS and SNP-chip data

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INTRODUCTION

Economically important production traits in domestic animals are generally complex, i.e., determined by factors that may include both genetic and environmental regulators. This is also true for many diseases in humans and animals. Thus, while it is often highly desirable to understand the regulation of specific complex traits, the task can be extremely challenging. For example, regions identified by quantitative trait loci (QTL) analysis will even after fine mapping of the QTL typically indicate regions including millions of base pairs and hundreds of genes that need to be explored to find causative mutations.

In this study our aim was to develop a bioinformatics strategy to mine already identified QTL regions to identify candidate genes for growth trait in chicken. The QTLs have been identified for body weight at 56 days for more than 50 generations. Several quantitative trait loci (QTL) have been mapped in an F2 intercross between the lines, and the regions have subsequently been replicated and fine mapped using an Advanced Intercross Line. We have further analyzed the QTL regions where the largest genetic divergence between the High-Weight selected (HWS) and Low-Weight selected (LWS) lines was observed. Such regions, covering about 37% of the actual QTL regions, were identified by comparing the allele frequencies of the HWS and LWS lines using both individual 60K SNP chip genotyping of birds and analysis of read proportions from genome resequencing of DNA pools. Based on a combination of criteria including significance of the QTL, allele frequency difference of identified mutations between the selected lines, gene information on relevance for growth, and the predicted functional effects of identified mutations we propose here a subset of candidate mutations of highest priority for further evaluation in functional studies. The candidate mutations were identified within the GCG, IGFBP2, GRB14, CRIM1, FGF16, VEGFR-2, ALG11, EDN1, SNX6, and BIRC7 genes. We believe that the proposed method of combining different types of genomic information increases the probability that the genes underlying the observed QTL effects are represented among the candidate mutations identified.

Mapping of chromosomal regions harboring genetic polymorphisms that regulate complex traits is usually followed by a search for the causative mutations underlying the observed effects. This is often a challenging task even after fine mapping, as millions of base pairs including many genes will typically need to be investigated. Thus to trace the causative mutations there is a great need for efficient bioinformatic strategies. Here, we searched for genes and mutations regulating growth in the Virginia chicken lines – an experimental population comprising two lines that have been divergently selected for body weight at 56 days for more than 50 generations. Several quantitative trait loci (QTL) have been mapped in an F2 intercross between the lines, and the regions have subsequently been replicated and fine mapped using an Advanced Intercross Line. We have further analyzed the QTL regions where the largest genetic divergence between the High-Weight selected (HWS) and Low-Weight selected (LWS) lines was observed. Such regions, covering about 37% of the actual QTL regions, were identified by comparing the allele frequencies of the HWS and LWS lines using both individual 60K SNP chip genotyping of birds and analysis of read proportions from genome resequencing of DNA pools. Based on a combination of criteria including significance of the QTL, allele frequency difference of identified mutations between the selected lines, gene information on relevance for growth, and the predicted functional effects of identified mutations we propose here a subset of candidate mutations of highest priority for further evaluation in functional studies. The candidate mutations were identified within the GCG, IGFBP2, GRB14, CRIM1, FGF16, VEGFR-2, ALG11, EDN1, SNX6, and BIRC7 genes. We believe that the proposed method of combining different types of genomic information increases the probability that the genes underlying the observed QTL effects are represented among the candidate mutations identified.

Keywords: candidate genes, growth, functional prediction, genetic divergence, QTL, SNP resequencing

ORIGINAL RESEARCH ARTICLE

doi: 10.3389/fgene.2013.00226

published: 05 November 2013

www.frontierrin.org
November 2013 | Volume 4 | Article 226 | 1
MATERIALS AND METHODS

Here, we present a bioinformatic strategy that in a structured and objective way helps to prioritize candidate genes for further study in mapped QTL regions by integrating information from multiple sources. First, the region to be evaluated further is narrowed down by, at each SNP-location in the evaluated region, calculating a combined score for the potential that each part of the region harbors a mutation underlying the phenotype. This is done by combining the statistical support from significance of the QTL effect at the particular marker, which is a measurement of the effect of the alternative alleles on the studied phenotype, with two measures of the genetic divergence between the founder lines (i.e., allele-frequency differences) at the particular location, which is an indicator of the direct or indirect selective pressure on the region due to an association with the phenotypes of importance when generating the divergent founder lines. Then, all the polymorphisms in the prioritized region are evaluated in more detail to select the most likely genes affecting the analyzed trait and bioinformatically predict the potential functional effects of each identified polymorphism. The details of the procedure, and its application to our particular chicken dataset, are described with a flowchart in Figure 1 and in the text below.

MAPPED QTL REGIONS TO BE EXPLORED

We studied seven fine-mapped QTL on chicken chromosomes 1–5, 7, and 20, with previously observed effects on body-weight at selection age in a QTL mapping pedigree founded with HWS and LWS chickens from generation 41 (Jacobsson et al., 2005; Besnier et al., 2011). The fine-mapping of the QTL was previously reported by Besnier et al. (2011) where the effect of each SNP in the QTL regions was estimated using a Flexible Intercross Analysis model (Ronnegård et al., 2008). The statistical QTL support curve across the regions from the analysis based on this model (Model B in the original paper) was here used for identification and evaluation of candidate regions.

INDIVIDUAL GENOME-WIDE 60 K SNP CHIP GENOTYPING

Genome-wide 60K SNP chip genotypes of 20 individuals from each of the HWS and LWS lines, generation 41 (Marklund and Carlberg, 2010) was available. We used these genotype data to estimate the allele-frequency differences between the lines across the QTL regions to be explored.

GENOME RESEQUENCING OF POOLED POPULATION-SAMPLES AND SNP-CALLING

Genome resequencing was performed in two separate runs using DNA pools from the HWS and LWS lines. The data from the two experiments were combined to maximize the sensitivity in the SNP detection. For earlier studies DNA from two pools of genomic DNA, one from each of the HWS and LWS lines, were used to generate resequencing data with 5 x average depth coverage for each line. The reads were aligned to the Red Jungle Fowl’s (RIF) reference genome assembly (WUGSC 2.1/galGal3, May 2006; Marklund and Carlberg, 2010; Rubin et al., 2010).

For the current and future studies the second round of resequencing was performed using two new pools of DNA samples.
The individuals selected for each pooled were guided by data from earlier performed 60k SNP-chip genome-wide genotyping. From each line, the eight individuals with the most non-representative genotype pattern in the QTL regions were selected to increase the possibilities for detection of variation within lines and thereby allow improved precision in the fine mapping of regions with high degree of between-line fixation. The ABI SOLiD resequencing was carried out by the Uppsala Genome Center using mate-pair libraries and 50 bases per read with \( \sim 7x \) depth coverage in each line. We aligned the reads to the RJF reference genome assembly (WUGSC 2.1/galGal3, May 2006) using the MOSAIK software (Lee et al., 2013). The resequencing datasets from the two rounds of sequencing were combined for SNP calling based on a total of \( \sim 12x \) depth coverage in each line. However in each line SNP alleles were called at each SNP position as determined using the threshold of three non-RJF reads that we set for SNP detection including the total number of reads from both lines (i.e., \( \sim 24x \) depth coverage) to increase the sensitivity. The GigalBayes software, a newer version of PolyBayes (Marth et al., 1999), was used for SNP calling.

**GENETIC DIVERGENCE ANALYSIS USING THE FLANKING-SNP-VALUE METHOD IN RESEQUENCING DATA**

We applied the flanking-SNP-value (FSV) method (Marklund and Ahsan et al., 2010) to the resequencing data from the HWS and LWS lines across the selected QTL regions. The FSV method computes estimated allele frequency differences between the HWS and LWS lines for each evaluated SNP position based on information from the SNP itself as well as from data of flanking SNPs in both directions within an interval presumed to show a high degree of linkage disequilibrium with the SNP. Thus, the input data for FSV computation are the AB scores at all these positions, which in each line are estimated allele frequency differences between the HWS and LWS populations of these original QTL were selected using the combined information from the QTL analysis and estimates of differences in allele frequencies between the lines inferred from SNP chip genotyping and FSV computation (Table 2).

**RESULTS**

In an earlier study, Besnier et al. (2011) fine-mapped a number of QTL affecting body-weight at 8 weeks of age (Table 1; Figures 2A–E). The evaluated QTL regions are located on chicken chromosomes 1–5, 7, and 20 and cover in total 121.4 Mb of the genome.

Using the prioritizations strategy described above, 44.7 Mb of these original QTL were selected using the combined information from the QTL analysis and estimates of differences in allele frequencies between the lines inferred from SNP chip genotyping and FSV computation (Table 2).

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**Table 1** | Fine-mapped growth QTL regions with significance according to Besnier et al. (2011),

| GGA | QTL | Region name | Start (Mb) | End (Mb) | Size (Mb) |
|-----|-----|-------------|------------|----------|-----------|
| 1   | Growth1 | C1G1 | 169.6 | 181.0 | 11.4 |
| 2   | Growth2 | C2G2 | 479 | 65.4 | 175 |
| 3   | Growth4 | C3G4 | 24.0 | 88.0 | 43.9 |
| 4   | Growth6 | C4G6 | 13 | 13.5 | 12.1 |
| 5   | Growth8 | C5G8 | 55.0 | 39.0 | 5.3 |
| 7   | Growth9 | C7G9 | 10.0 | 35.4 | 24.5 |
| 20  | Growth12 | C20G12 | 71 | 13.8 | 6.7 |
| Total |       |         |           |          | 121.4 |

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1GGA: Gallus gallus Assembly; 2QTL names as in Besnier et al. (2011).
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**FIGURE 2** (A–E) Five of the fine-mapped growth QTL regions based on model B (QTL Support curve), and their significance threshold (QTL Sign. Threshold line) as in Besnier et al. (2011). The FSV curve represents FSV computations from resequenced NGS data from the HWS and LWS lines (Marklund and Carlborg, 2010), the SNP chip curve represents allele frequency differences between HWS and LWS from SNP genotyping, and the combined data score curve represents the formulated score from all of the above stated dataset curves. The Selected Region line represents the selected candidate regions for bioinformatic analysis of genes and mutations.

**Table 2** Candidate regions selected based on QTL data and allele frequency differences between the lines inferred from SNP chip genotyping and FSV computation from resequencing. The selected percentages of the QTL regions significant with model B, are given (Besnier et al., 2011).

| Region name | Start Mbp¹ | End Mbp² | Size (Mbp) | QTL support² | Ensembl genes³ |
|-------------|------------|----------|------------|--------------|----------------|
| C1G1        | 169.6      | 175.0    | 5.4        | 5.4          | 97             |
| C2G2        | 59.7       | 65.4     | 5.7        | 2.1          | 52             |
| C3G4        | 24.1       | 35.8     | 11.7       | 10.3         | 142            |
| C4G6        | 10.6       | 12.9     | 2.3        | 0.0          | 62             |
| C5G8        | 34.2       | 36.8     | 2.6        | 0.0          | 20             |
| C5G9        | 38.2       | 39.0     | 0.8        | 0.0          | 16             |
| C7G9        | 20.4       | 35.4     | 15.0       | 4.3          | 209            |
| C20G12      | 8.3        | 9.5      | 1.2        | 1.2          | 38             |
| **Total**   | 44.7       | 23.3     | 636        |              |                |

¹Coordinates based on the Chicken (Gallus gallus) assembly v.2.1/galGal3; ²Size of the selected regions significant with QTL model B (Besnier et al., 2011); ³Number of Ensembl genes in the initial list in the selected regions.
Table 3 | The variant effect predictor summary of SNPs in selected candidate segments of the QTL regions (according to Table 2).

| Location within gene | Region |
|----------------------|--------|
|                      | C1G1   | C2G2   | C3G4   | C4G6   | C5G8   | C7G9   | C20G12  | Total   |
| 3Prime UTR           | 200    | 93     | 200    | 153    | 73     | 348    | 75      | 1142    |
| 3Prime UTR, Splice site | 1      | 1      | 1      | 2      |        |        |         |         |
| 3Prime UTR           | 22     | 9      | 44     | 20     | 3      | 50     | 28      | 176     |
| 3Prime UTR, Splice site | 1      | 1      | 4      | 5      |        |        |         |         |
| Coding unknown       | 5      |        |        |        |        |        |         |         |
| Downstream           | 6118   | 2636   | 5318   | 2373   | 1395   | 7930   | 1384    | 27154   |
| Essential splice site | 2      | 3      | 6      | 1      | 1      | 4      | 3       | 20      |
| Non-synonymous coding | 215    | 82     | 255    | 92     | 60     | 470    | 80      | 1254    |
| Non-synonymous coding, Splice site | 6 | 4 | 8 | 5 | 3 | 17 | 1 | 44 |
| splice site, Intronic | 78     | 37     | 133    | 33     | 24     | 191    | 31      | 527     |
| Stop gained          | 5      | 7      | 3      | 2      | 10     |        | 27      |         |
| Stop gained, Non-synonymous coding | 1 |       |        |        |        |        |         |         |
| Synonymous coding    | 350    | 208    | 543    | 165    | 59     | 1113   | 159     | 2637    |
| Synonymous coding, splice site | 9 | 9 | 12 | 5 | 6 | 20 | 12 | 73 |
| Upstream             | 5506   | 2626   | 5755   | 2570   | 1200   | 8312   | 1479    | 27488   |
| Within mature miRNA  | 1      |        |        |        |        |        |         |         |
| Within non-coding gene | 4    | 2      | 1      | 3      | 12     | 4      | 26      |         |
| Within non-coding gene, splice site | 1 |       |        |        |        |        |         |         |
| Total                | 12516  | 5708   | 12284  | 5422   | 2870   | 19484  | 3256    | 60540   |

In Table 3, we provide a summary of the results obtained using the Ensembl VEP tool. Nearly 61,000 SNPs (excluding intergenic and intronic SNPs) were found to be located within functional elements across the selected candidate segments in this analysis. In Table 4, we provide a selection of one or two of the best candidate mutations in each region.

**DISCUSSION**

In this study we have developed and applied a bioinformatic strategy to search for candidate mutations affecting body weight at 36 days in several QTL regions that were previously identified and fine-mapped in an intercross between two divergently selected chicken lines. Given the 40 generations of divergent selection for body weight it is reasonable to assume that many of the underlying functional mutations will display a relatively large allele frequency difference, or complete fixation, between the lines. This assumption is supported by earlier work with the lines that many regions across the genome have been driven to fixation for alternative alleles in the lines and that most selection has been on standing genetic variation present in the common base-population at the onset of selection (Johansson et al., 2010). At a smaller number of selected loci mutations might have arisen after the initiation of selection. It is, however, unlikely that the QTL evaluated here are due to such new mutations as they are identified using a statistical analysis that assumes that the crossed lines are fixed for alternative QTL alleles.

To narrow down the target regions and identify the most plausible mutations, we used several independent sources of information. First, measurements of the genetic divergence between the founder lines of the intercross were used as indicators of regions that have been under strongest selection. Both individual SNP chip genotyping and genome resequencing of pools of individuals were used to provide stability and high-resolution in the estimates of the allele frequency difference between the lines. The potential functional impact of genes and SNPs located within the target regions was bioinformatically evaluated to identify a set of candidate mutations to be further tested and evaluated in functional studies. In regions where there exist several possible candidate genes, our use of a combined and objective selection criteria helped to localize the most promising candidate genes and mutations. The genes and mutations listed in Table 4 qualified as the strongest candidates underlying the observed QTL. Among these, the glucagon (GCG) gene on chromosome 7 (C7G9 region) is perhaps the most obvious candidate gene due to its well-documented effect on appetite (Suzuki et al., 2010), a trait for which the HWS and LWS lines show an extreme difference. No non-synonymous mutations were found in the glucagon gene, but a mutation was identified in a downstream CpG island with a large (0.87) estimated allele frequency difference between the lines (AFD), and possibly a regulatory effect on glucagon gene expression. The C7G9 region also included mutations in CpG islands with even larger AFD estimates and possibly regulatory roles in genes that in turn can regulate other genes with effects...
Table 4 | Candidate mutations identified in the evaluated QTL regions.

| Region | SNP (bp) | Gene                                      | Location of SNP | No of AA reads; depth coverage | Qual | AFD | PC Score | EC Score | PE Score |
|--------|----------|--------------------------------------------|-----------------|-------------------------------|------|-----|---------|---------|---------|
| C1G1   | 174634021 | Asparagine-linked glycosylation 11 homolog (ALG11) | Cpg island, upstream | 7: 10 72 | 0.97 | N/A | N/A |
| C2G2   | 63823523  | Endothelin 1 (EDN1)                         | Cpg island, upstream | 3: 13 53 | 0.95 | N/A | N/A |
| C2G3   | 20678270  | G protein-coupled receptor-like 1 (GPR1)     | Protein code, NS | 16: 19 182 | 0.97 | 0.67 | 0.63 | 0.42 |
| C4G6   | 12044024  | Similar to receptor tyrosine kinase 6 (FRK)   | Cpg island, upstream | 4: 8 82 | 0.97 | N/A | N/A |
| C4G6   | 12902414  | Fibroblast growth factor 16 (FGF16)          | Cpg island, downstream | 8: 16 175 | 0.95 | N/A | N/A |
| C5G8   | 58319301  | Sorting nexin 6 (SNX6)                      | Cpg island, upstream | 8: 14 142 | 0.97 | N/A | N/A |
| C7G9   | 21689625  | Growth factor receptor-bound protein 14 (GRB14) | Cpg island, downstream | 3: 12 52 | 0.97 | N/A | N/A |
| C7G9   | 22711910  | Glucagon (GCG)                              | Cpg island, downstream | 3: 9 46 | 0.87 | N/A | N/A |
| C7G9   | 24002616  | Insulin-like growth factor binding protein 2 (IGFBP2) | Protein code, synonymous | 4: 8 69 | 0.95 | N/A | N/A |
| C7G9   | 26000638  | Baculoviral IAP repeat-containing 7 (BIRC7)  | Protein code, N/S | 5: 8 65 | 0.97 | 0.29 | 0.14 | 0.04 |

1 Coordinates based on the Chicken (Gallus gallus) assembly v2.7 (galGal2). Location of the SNP in gene and location of SNPs in case of non-synonymous SNPs.2 Total number of reads in both lines representing the alternate allele divided versus the total depth coverage across the SNP position. The Phred scaled probability that a REF/ALT polymorphism exists at this site given sequencing data. Because the Phred scale is $10^{-10 * \text{log}(1 - p)}$, a value of 10 indicates a 1 in 100 chance of error, while a 100 indicates a 1 in 10$^{10}$ chance.4 Allele frequency difference between the chicken lines as estimated using the GigaBayes software.5 Combined score of PC and EC of amino acid substitution calculated using PASE (Li et al., 2013).6 Evolutionary conservation score of amino acid substitution calculated using PASE (Li et al., 2013).
on body weight. Such mutations were found in the insulin-like growth factor binding protein 2 (IGFBP2) and the growth factor receptor-bound protein 14 (GRB14; e.g., Holt and Siddell, 2005) genes. The IGFBP2 gene is also located in this target region but at this stage we have not found sufficient support for any strong candidate mutation in that gene. The IGFBP2 gene can specify the actions of insulin-like growth factors which have key roles in vertebrate growth and development (e.g., Wood et al., 2005). Interestingly, the possibly regulatory IGFBP2 mutation reported here is located in a coding sequence that is a part of a CpG island. Even though it is a synonymous mutation it may affect IGFBP2 expression through mechanisms of codon usage, GC content and/or mRNA stability and folding (reviewed by Shabalina et al., 2013). Overexpression of IGFBP2 has been shown to reduce postnatal body weight gain in transgenic mice (Hoeflich et al., 1999). The GRB14 gene encodes a cellular adapter protein that can bind to receptor tyrosine kinases and intracellular proteins and thereby be involved in various processes. For example, it can bind and modify the signals from the insulin receptor and insulin-like growth factor 1 and its implication in growth regulation has been shown (reviewed by Holt and Siddell, 2005).

Strong candidate genes and mutations were also found in QTL regions on chromosome 3 (C3G4) and 4 (C4G6). In the C3G4 region, the gene encoding the cysteine rich transmembrane BMP regulator 1 (CRIM1), showed a non-synonymous mutations with large allele frequency difference between the lines and high PE scores (i.e., combined PC and EC scores; Table 4) with the PASE tool. CRIM1 interactions with growth factors may be important for the development of the central nervous system (CNS) and other organs (Kolle et al., 2000). Perhaps most interesting is the impact the CRIM1 gene possibly has on the CNS because Ka et al. (2009) reported genes that regulate neuronal plasticity to be differentially expressed between the HWS and IWS lines in the brainstem and hypothalamus. Moreover, electrolytic hypothalamus lesions has been shown to increase appetite in the IWS but not in the HWS line which further supports that CNS is highly involved in the differences between these chicken lines (Buckhart et al., 1983).

In the C4G6 region, candidate CpG island mutations were identified within the fibroblast growth factor 16 (FGF16) and vascular endothelial growth factor receptor 2 (VEGFR-2) genes. FGF16 is known to be involved in embryonic development and cell growth (Antoine et al., 2006) whereas the VEGFR-2 gene has been reported to be of importance for angiogenesis (Patterson et al., 1995). In the chromosome 1 QTL region (C1G1) we also found a candidate mutation, possibly regulatory, in the asparagine-linked glycosylation 11 homolog (AGAL11) gene. AGAL11 has been reported to be involved in biosynthetic processes and required for normal growth in yeast (Cipollo et al., 2001).

The chromosome 2 QTL region (C2G2) showed CpG island mutations at the endothelin 1 (EDN1) gene with the two chicken lines fixed for opposite alleles. EDN1 is known for roles in blood pressure and development (Kurhara et al., 1994). In the regions on chromosome 5 (C5G8) and 20 (C20G12) the genes found in the analysis were less obvious candidates. However, such genes may still have key roles in processes with complex and indirect effects on growth-related traits. Keeping this in mind, we consider mutations identified in the sorting nexin 6 (SNX6; Caldwell et al., 2005), C5G8 region and baculoviral IAP repeat-containing 7 (BIRC7; Kasof and Gomez, 2001; C20G12 region) genes are of most interest to investigate further.

In conclusion, the described combination of data from QTL mapping, next-generation sequencing, SNP chip genotyping and bioinformatic analysis has provided a list of plausible candidate genes and mutations that will facilitate further verification and experimental evaluation. The support for this list from different types of data and analysis enhances the probability that the selected genes and mutations underlying the QTL effects are an unbiased selection of genes and that the contributing gene(s) are included in the set. Further studies based on this list may therefore reveal mutations which underlie the observed QTL effects and can increase our understanding of growth regulation as well as be more emphasized in animal breeding programs with genomic selection.

AUTHORS CONTRIBUTIONS

Muhammad Ahsan and Xidan Li carried out the region-targeted computation and analysis using the different sources of data and took part in the planning of the study. Marcin Kierczak and Andreas E. Lundberg performed the assembly of the SOLID resequencing datasets. Stefan Marklund initiated and planned the study. Paul B. Siegel and Orjan Carlborg contributed with comments and advice. Muhammad Ahsan and Stefan Marklund drafted the manuscript and all co-authors contributed to the final version.

ACKNOWLEDGMENTS

We would like to thank the USDA Chicken GWMAS Consortium, Cobb VanTress, and Hendrix Genetics for access to the developed 60K SNP Illumina iSelect chicken array, DNA landmarks for 60K array genotyping and the Uppsala Genome Center for ABI SOLID sequencing.

This work was financially supported by a EURYI award to Orjan Carlborg and a Future research leader grant to Orjan Carlborg from the Swedish Foundation for Strategic Research. The contribution of Muhammad Ahsan was supported by his scholarship from the Higher Education Commission of Pakistan (HEC).

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