Genome-wide Linkage Analysis Identifies Loci for Physical Appearance Traits in Chickens

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LOCI FOR CHICKEN PHYSICAL APPEARANCE TRAITS

Key words: chicken, physical appearance traits, linkage analysis, feather-crested head, beard, comb, wattles, feathered feet

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ABSTRACT Physical appearance traits, such as feather-crested head, comb size and type, beard, wattles size and feathered feet, are used to distinguish between breeds of chicken, and may also be associated with economic traits. In this study, a genome-wide linkage analysis was used to identify candidate regions and genes for physical appearance traits and to potentially provide further knowledge of the molecular mechanisms that underlie these traits. The linkage analysis was conducted with an F2 population derived from Beijing-You chickens and a commercial broiler line. Single nucleotide polymorphisms (SNPs) were analyzed using the Illumina 60K Chicken SNP Beadchip. The data were used to map quantitative trait loci (QTLs) and genes for six physical appearance traits. A 10 cM/0.51 Mb region (0.0-10.0 cM/0.00-0.51 Mb) with 1% genome-wide significant level on LGE22C19W28_E50C23 linkage group (LGE22) for crest trait was identified, which is likely very closely linked to the HOXC8. A QTL with 5% chromosome-wide significant level for comb weight, which partly overlaps with a region identified in a previous study, was identified at 74cM/25.55 Mb on chicken (Gallus gallus) chromosome 3 (GGA3). For beard and wattles traits, a same region 11 cM/2.23 Mb (0.0-11.0 cM/0.00-2.23 Mb) including WNT3 and GH genes on GGA27 was identified. Two QTLs with 1% genome-wide significant level for feathered feet trait, one 9 cM/2.80 Mb (48.0-57.0/13.40-16.20 Mb) region on GGA13 and another 12 cM/1.45 Mb (41.0-53.0 cM/11.37-12.82 Mb) region on GGA15 were identified. These candidate regions and genes provide important genetic information for the physical appearance traits in chicken.
INTRODUCTION

There are 1077 reported local chicken (*Gallus gallus*) breeds in the world (Rischkowsky and Pilling, 2007). Physical appearance traits are often used to distinguish between breeds of chicken and include feather-crested head, comb size and type, beard, wattles size and feathered feet. The physical appearance traits are also associated with growth, reproduction and other important economic traits. For example, the crested head gene has significant negative effects on body weight (Magothe *et al.*, 2010), comb size has an influence on sexual characters and viability (Von Schantz *et al.*, 1995); whilst the rose-comb mutation cause both altered comb morphology and defective sperm motility (Imsland *et al.*, 2012).

The genetic mechanisms that underlie these physical appearance traits have gradually been revealed. For example, feather-crested head is a prominent feature exhibited in several breeds of chicken, such as Silkies and Beijing-You chickens. A previous study has shown that the crest phenotype is associated with ectopic expression of the *HOXC8* gene. This is located on the E22C19W28 linkage group, is expressed in cranial skin and was identified using linkage analysis, genome-wide association and expression analysis from 26 different crested and non-crested breeds (Wang *et al.*, 2012).

For the feathered feet trait, two loci were identified in a previous study (Somes, 1992). Other feather traits were investigated by Noorai *et al.* (2012) who found that the rumpless and ear-tufted traits loci were located on chicken chromosomes 2 and 15, respectively. The positional candidate genes, *Irxl* and *Irx2* for the rumpless trait, and *TBX1* and *GNB1L* for the ear-tufted trait were identified through genome-wide association and haplotype analyses.
A major quantitative trait locus (QTL) for bisexual expression of comb mass and several QTLs specific to female comb mass have been identified (Wright et al., 2008). Linkage analysis and identical-by-descent (IBD) mapping proved that copy number variation (CNV) of intron 1 of the \textit{SOX5} gene causes the pea-comb phenotype in chickens (Wright et al., 2009). In a further study, a locus that affects comb mass has been identified using three separate intercrosses between wild and domestic chickens. The locus contains two tightly-linked genes, \textit{BMP2} and \textit{HAO1}, which together produce the range of pleiotropic effects observed (Johnsson et al., 2012). For the duplex-comb trait, Dorshorst et al. (2015) found that a genomic 20 Kb duplication contains regulatory elements that affect EOMES expression in the embryonic chicken comb and two duplex comb phenotypes (V-shaped and Buttercup).

The Beijing-You (BJY) chicken is a color-feathered, slow-growing Chinese indigenous breed with a feather-crested head, beard and feathered feet (Zheng, 1988). In a previous study, an F2 resource population was constructed from a cross between BJY and a commercial rapidly-growing broiler line (Cobb-Vantress). This population was used to identify loci and candidate genes for meat quality traits (Sun et al., 2013), shank length (Sun et al., 2013), polydactyly (Sun et al., 2014) and growth related traits (Liu et al., 2015) through genome-wide association studies (GWAS) or combined GWAS and linkage analysis (LA) methods.

In this study, we used the linkage analysis for six physical appearance traits (feather-crested head, beard, comb weight, wattles weight and length, and feathered feet) to provide insight into their genetic basis.
MATERIALS AND METHODS

Animals and phenotypes

This research complied with the Guidelines for Experimental Animals established by the Ministry of Science and Technology (Beijing, China). The Chinese Academy of Agricultural Science (CAAS) F2 chicken population was described in previous studies (Sun et al., 2013a; Sun et al., 2013b; Sun et al., 2014; Liu et al., 2015). It was derived from a cross between Beijing-You (BJY) chickens and Cobb broilers (CB).

In this study, a total of 400 chickens were used from three generations, which included 367 F2 chickens from 20 full-sib families in five batches. Blood was collected into acid citrate dextrose anticoagulant tubes from the brachial vein on d 56. At 93 days, F2 chickens were weighed and killed by stunning and exsanguination. Before they were killed, the feather-crested head, beard and feathered feet traits of each chicken were recorded. The comb and wattles of each chicken were weighed after they were killed. The length of the wattles between the upper and the lower point of the organ were measured with a micrometer.

Genotyping and SNP quality

Genomic DNA (gDNA) was extracted from blood samples using the phenol-chloroform method and 50ng/µl were used for genotyping with the Illumina 60K Chicken SNP Beadchip. This was performed by DNA LandMarks Inc., Saint-Jean-sur-Richelieu, PQ, Canada, and is detailed in a previous study (Sun et al., 2013a). A total of 39 samples were excluded due to sample call rate < 90% and 13,985 SNPs were removed for failing to meet one or more of the following conditions: SNP call rate < 90%, minor allele frequency (MAF) < 3%, Hardy-Weinberg equilibrium (HWE) test p of < 10^{-6} and SNPs with no assigned chromosome or linkage group. After quality control measures,
42,585 single nucleotide polymorphisms (SNPs) distributed among 28 chromosomes, linkage group (LGE22) and the Z chromosome, were used for analysis. The average physical distance between two neighbouring SNPs was approximately 20.4 Kb (Sun *et al.*, 2013a; Sun *et al.*, 2014).

**Linkage analysis**

The genetic linkage map was constructed using 19 full-sib families (six males and 12 females from F0, five males and 20 females from F1, and 148 males and 152 females from F2). A total of 42,585 SNPs were analyzed with the improved version of CRI-MAP (2.503a, run in a 64-bit Unix system), which has been described in previous studies (Groenen *et al.*, 2009; Elferink *et al.*, 2010; Sun *et al.*, 2014). The total length of the sex-average map was 3040.8 cM and the recombination rate of the map was 2.9 cM/Mb (Sun *et al.*, 2014).

In order to reduce the effect of linkage disequilibrium (LD) on the results, 6518 independent SNPs were acquired in all autosomal chromosomes using the indep-pairwise option, with a window size of 25 SNPs, a step of five SNPs, and $r^2$ threshold of 0.2 (Sun *et al.*, 2013a). The independent SNPs were used for a QTL analysis of the physical appearance traits. A least-squares regression model was used for single-QTL analysis in the web-based software GridQTL (Allen *et al.*, 2012). This included the fixed effects of sex, hatch and family, along with additive and dominance coefficients for the putative QTL. Significance thresholds were calculated with a permutation test (Churchill and Doerge 1994). A total of 10000 permutations were computed to determine 5% and 1% chromosome-wide significance levels. The 5% and 1% genome-wide significance levels were calculated following the Bonferroni correction:

$$P_{\text{genome-wide}} = 1 - (1 - P_{\text{chromosome-wide}})^{Ga/Gc}$$

where Ga is the length of the genetic map of each chromosome and Gc is the length of the
genetic map of all autosomal chromosomes. Confidence intervals for QTL positions were estimated by bootstrapping, as presented by Visscher et al. (1996).

RESULTS

Traits statistics

We recorded and measured six physical appearance traits, which included three qualitative traits (feather-crested head, beard and feathered feet) and three quantitative traits (comb weight, wattles weight and length), in the F2 chicken population. Chi-square results for the qualitative traits and descriptive statistics for the quantitative traits are shown in Table 1 and 2, respectively. For the qualitative traits, the observed ratio between F2 chickens with traits and those without traits deviated significantly from the expected 3:1 ratio (P < 0.05) (Table 1). For the quantitative traits, the comb and wattles traits showed large phenotypic variation (the C.V. from 74-144%, Table 2).

Johnson transformations (Minitab 15, Minitab Inc., Quality Plaza, PA, USA) were implemented before analysis of the wattles length trait as the data deviated from normality (data not shown).

QTL mapping results

The linkage analysis results for the physical appearance traits are shown in Table 3. The physical positions of the QTLs were provided based on the SNPs markers physical positions in the genetic map (Sun et al., 2014). For six traits, 18 QTLs were detected at 5% or 1% significance at the chromosome-wide level or significance at the 1% genome-wide level.

Crest and comb

Three QTLs for feather-crested head were detected on chromosomes GGA2, GGA28 and linkage group LGE22. One QTL located at 5 cM/0.51 Mb on LGE22 was significant at the 1%
genome-wide level and explained 32.1% of the phenotypic variation. The other two QTLs (located at 4 cM/1.25 Mb on GGA2 and 48 cM/3.81 on GGA28, respectively) were significant at the 5% chromosome-wide level and explained from 2.76% to 4.71% of the phenotypic variation.

For the comb weight trait, four QTLs were detected on GGA3, GGA7, GGA25 and GGA27. These QTLs explained between 2.74% and 4.29% of the phenotypic variation. One QTL explained 3.66% of the phenotypic variation and was located at 2 cM/0.14 Mb on GGA25, with 1% chromosome-wide significance level. Three QTLs with 5% chromosome-wide significance level were located on GGA3 (74 cM), GGA7 (52 cM) and GGA27 (49 cM).

**Beard and wattles**

One QTL for the beard trait was detected at 4 cM/1.51 on GGA27, with a 1% genome-wide significance level. It explained 11.2% of the phenotypic variation.

For the wattles traits, a total of six QTLs were detected on five chromosomes and explained from 3.44% to 6.79% of the phenotypic variation. Two QTLs, with a 1% chromosome-wise significance level, were detected on GGA27. The QTL for wattles weight was detected at 2 cM/1.37 Mb, whilst the QTL for wattles length was detected at 1 cM/1.23. They explained 5-6 % of the phenotypic variation. The other four QTLs, with 5% chromosome-wide significance level were located at 210 cM/100.64 Mb on GGA2, 14 cM/3.80 on GGA9, 11 cM/4.21 Mb on GGA14 and 55 cM/1.67 Mb on GGA25. These QTLs explained approximately 3-5% of the phenotypic variation.

**Feathered feet**

For the feathered feet trait, four QTLs were detected on GGA8, GGA12, GGA13 and GGA15. Two QTLs explained more than 20% of the phenotypic variation and are located at 55 cM/1.56 Mb on GGA13 and 47 cM/12.22 Mb on GGA15 with a 1% genome-wide significance level. The other
two QTLs explained approximately 4% of the phenotypic variation and are located at 71 cM/22.82 on GGA8 and 4 cM/11.50 Mb on GGA12 with 5% chromosome-wide significance level.

**DISCUSSION**

In this study, the observed ratios of traits to non-traits for the feather-crested head, beard and feathered feet traits deviated significantly from the expected 3:1 ratio. These traits showed segregation in the F2 chicken population. The three quantitative traits, including comb weight, wattles weight and length traits, showed large phenotypic variations. The segregation and large variations in the F2 chicken population were used in the linkage analysis to identify the QTLs or genes.

**Crest and comb**

In chickens there are two feather crests phenotypes: the helmet-shaped, forward-inclining feather crests and the full crests (Bartels, 2003). Beijing-You chickens have full crests. Previous research has shown that homozygous crested chickens are distinguishable from heterozygous chickens by the lack of filoplumes in the crest. The full crest in the domestic chicken is encoded by an incompletely dominant autosomal gene (Bartels, 2003). Through further linkage analysis and GWAS, Wang *et al.* (2012) found that the crest gene is located in the LGE22. LGE22 of chicken corresponds to an orthologous region on human chromosome 12 (HSA12) near the 50 Mb region. The region between 45 Mb to 60 Mb on HSA12 shows patches of conserved synteny with other parts of the chicken genome including GGA1, GGA2, GGA7 and ChrUn_random, the latter consists of contigs that could not be localized to any specific chromosome. Expression analysis of tissues from crested and non-crested chickens showed that the crest is caused by acis-acting regulatory mutation
that affects the ectopic expression of HOXC8, which was in chicken genome ChrUn_random and in
HSA12. In this study, We used linkage analysis to identify three QTLs for the feather-crested head
trait, especially one QTL with 1% genome-wide significance, at 5 cM/0.51 Mb on LGE22 with 10
cM confidence interval, approximately 0.51 Mb (from 0.00 to 0.51 Mb) in physical map. There were
one microRNA and seven genes; MIR1668, LARP4, COX14, ACCN2, HDAC7, TWIST3, SLC48A1
and NEUROD4, in this region. The 0.51 MB region is likely very closely linked to the HOXC8. The
0.51 Mb (0.00-0.51 Mb) region could be important region for crest traits and should undergo further
studies. The QTLs at 4 cM/1.25 on GGA2 and 48 cM/3.81 on GGA28 have minor
chromosome-significant levels and without some confirming data, which are very likely to be false
positives.

In the domestic chicken there are three major variants of comb type; Rose-comb, Pea-comb and
Duplex-comb. The QTLs or genes responsible for the comb type and weight traits have being
gradually revealed (Wright et al., 2008; Wright et al., 2009; Sato et al., 2010; Johnsson et al., 2012;
Dorshorst et al., 2015). More information about comb weight has been provided through linkage
analysis in this study. One QTL for comb weight was detected at 74 cM/25.55 Mb on GGA3, which
partly overlaps with a region identified in a previous study (at 57.2 cM, from 46.5 to 75.5 cM;
Wright et al., 2008). An expressed sequence tag (EST) BU105297 identified by Boardman et al.
(2002) was located at this QTL peak. This QTL could be an important region and requires further
studies. Another three QTLs; at 52 cM/22.13 Mb on GGA7, 2 cM/0.14 Mb on GGA25 and 49
cM/4.39 on GGA27, were also identified in this study. These may represent new QTLs for comb
weight and also require further analysis.
Beard and wattles

Beards are feather modifications that appear in domestic chickens and are composed of elongated contour feathers that project downwards under the chin (Bartels, 2003). In this study, one QTL for the beard trait was detected in a 4 cM/1.51 Mb region (confidence interval, 0.0-11.0 cM/0.00-2.23) on GGA27. The location of this 11 cM QTL region was approximately 2.23 Mb (0.00-2.23 Mb) in the physical map, which partly overlaps with a region for wattles weight (0.00-4.07 Mb) and length traits (0.00-2.92 Mb) identified in this study. The 2.23 Mb region partly overlaps with a region for chicken body composition (Ankra-Badu et al., 2010). There were four microRNAs and 22 genes, especially two growth and development related genes, wingless-type MMTV integration site family, member 3 (WNT3) and growth hormone (GH) genes in this region. A previous study found that the size of the wattles was reduced by the presence of a beard (Bartels, 2003). These data indicate that the beard and wattles traits could have the same genetic basis. The 2.23 Mb region on GGA27 could be an important candidate region for beard and wattles traits in chicken.

Feathered feet

In many breeds of domestic chicken, such as the Silky and Beijing-You chicken, a mutative transformation of scales to feathers on the tarsus, feet and toes, the so-called feathered feet trait (ptilopody), has become a breed characteristic (Bartels, 2003). In a previous study, two QTLs for the feathered feet trait were identified (Somes, 1992), but the physical positions of these two QTLs were not found. In this study, two QTL regions, 55 cM/1.56 Mb (48.0-57.0 cM, 2.80 Mb region from 13.40 to 16.20 Mb) on GGA13 and 47 cM/2.22 (41.0-53.0 cM, 1.45 Mb region from 11.37 to 12.82 Mb) on GGA15, for the feathered feet trait were identified, at the 1% genome-wide significance level.
A total of one microRNA and 33 genes were in the 2.80 Mb region on GGA13. Especially, the follistatin-like 4 (FSTL4) gene was located at the QTL peak. There were one microRNA MIR1464 and five genes (C15H12ORF49, TBX3, TBX5, RBM19 and SDSL) in the 1.45 Mb region on GGA15. Therefore, the 2.80 Mb region (13.40-16.20 Mb) and the 1.45 Mb region (11.37-12.82 Mb) could be important candidate regions for the feathered feet trait and require further investigation. Two QTLs, at 71 cM/22.82 Mb on GGA8 and 4 cM/11.50 Mb on GGA4, for the feathered feet trait, were also identified in this study. Because the two QTLs have minor chromosome-significant levels, they are very likely to be false positives and need further study.

In conclusion, the candidate regions and genes for the six physical appearance traits were identified through linkage analysis in chickens. The candidate regions and genes include a 10 cM/0.51 Mb region (0.0-10.0 cM/0.00-0.51 Mb) on LG22 for crest trait, the QTL at 74 cM/25.55 Mb on GGA3 for comb weight, the 11 cM/2.23 Mb region with WNT3 and GH on GGA27 for beard and wattles traits, and the 9 cM/2.80 Mb region (48.0-57.0 cM/13.40-16.20 Mb) on GGA13 and 12 cM/1.45Mb region (41.0-53.0 cM/11.37-12.82 Mb) on GGA15 for the feathered feet trait. These candidate regions and genes provide more genetic information on physical appearance traits in the chicken.

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associated with ectopic expression of HOXC8 in cranial skin. PloS One 7(4):e34012.
Table 1 Chi-square test for feather-crested head, beard and feathered feet at 3:1 expected ratio of traits to non-traits in F2 chickens

| Traits             | N   | No. of traits | No. of Non-traits | $\chi^2$ | P       | DF |
|--------------------|-----|---------------|-------------------|----------|---------|----|
| Feather-crested head | 326 | 226           | 100               | 5.30     | < 0.05  | 1  |
| Beard              | 326 | 134           | 192               | 197.96   | < 0.05  | 1  |
| Feathered feet     | 326 | 188           | 138               | 51.30    | < 0.05  | 1  |

Table 2 Descriptive statistics for comb and wattles traits in F2 chickens

| Traits                | N   | Mean | SD   | C.V.  | Min  | Max  |
|-----------------------|-----|------|------|-------|------|------|
| Comb weight(g)        | 314 | 1.34 | 1.61 | 120.11| 0.03 | 9.07 |
| Wattles weight (g)    | 296 | 0.77 | 1.11 | 144.20| 0.02 | 6.28 |
| Wattles length (mm)   | 259 | 13.58| 10.06| 74.10 | 0.76 | 40.60|
Table 3 Physical appearance trait QTLs mapped in the chicken CAAS F2 resource population

| N  | Traits               | GGA | Position (cM/Mb) | Confidence interval (cM/Mb) | F-ratio<sup>2</sup> | AE<sup>3</sup>            | DE    | PV<sup>4</sup> |
|----|----------------------|-----|------------------|-----------------------------|----------------------|---------------------------|-------|--------------|
| 1  | Feather-crested head | 2   | 4/1.25           | 0.0-282.0/0.00-135.46       | 8.05*                | -0.02 ± 0.04              | 0.24 ± 0.06 | 4.71         |
| 2  | Feather-crested head | 28  | 48/3.81          | 0.0-48.0/0.00-3.81          | 5.06*                | 0.03 ± 0.04               | 0.19 ± 0.06 | 2.76         |
| 3  | Feather-crested head | LGE22<sup>1</sup> | 5/0.51         | 0.0-10.0/0.00-0.51          | 68.84****            | 0.43 ± 0.04               | 0.30 ± 0.07 | 32.1         |
| 4  | Comb weight          | 3   | 74/25.55         | 14.0-223.0/4.60-106.58      | 7.26*                | -0.33 ± 0.12              | -0.40 ± 0.16 | 4.29         |
| 5  | Comb weight          | 7   | 52/22.13         | 12.0-101.0/3.52-36.52       | 6.36*                | -0.25 ± 0.11              | -0.44 ± 0.16 | 3.71         |
| 6  | Comb weight          | 25  | 2/0.14           | 0.0-58.0/0.00-1.78          | 6.29**               | 0.41 ± 0.12               | 0.06 ± 0.19 | 3.66         |
| 7  | Comb weight          | 27  | 49/4.39          | 7.0-49.0/1.87-4.39          | 4.93*                | -0.0 ± 0.11               | -0.53 ± 0.17 | 2.74         |
| 8  | Beard                | 27  | 4/1.51           | 0.0-11.0/0.00-2.23          | 19.05****            | 0.22 ± 0.04               | 0.08 ± 0.06 | 11.2         |
| 9  | Wattles weight       | 2   | 210/100.64       | 72.0-307.0/28.62-153.31     | 8.9*                 | 0.19 ± 0.10               | 0.53 ± 0.14 | 5.63         |
| 10 | Wattles weight       | 9   | 14/3.80          | 12.5-92.0/3.62-24.46        | 5.74*                | -0.14 ± 0.10              | 0.46 ± 0.15 | 3.46         |
| 11 | Wattles weight       | 25  | 55/1.67          | 0.0-56.0/0.00-1.69          | 5.71*                | 0.04 ± 0.09               | 0.49 ± 0.15 | 3.44         |
| 12 | Wattles weight       | 27  | 2/1.37           | 0.0-48.0/0.00-4.07          | 8.44**               | -0.37 ± 0.10              | -0.11 ± 0.15 | 5.32         |
|   | Traits             | Value1 | Value2 | Value3   | Value4   | Value5   | Value6   | Value7   | Value8   | Value9   | Value10 |
|---|--------------------|--------|--------|----------|----------|----------|----------|----------|----------|----------|----------|
| 13| Wattles length    | 14     | 11/4.21| 0.0-62.0/0.00-14.79 | 6.10*    | -0.48 ± 0.85 | -4.65 ± 1.38 | 4.18     |
| 14| Wattles length    | 27     | 1/1.23 | 0.0-21.0/0.00-2.92 | 9.53**   | -3.96 ±0.92  | 0.64 ± 1.36 | 6.79     |
| 15| Feathered feet    | 8      | 71/22.83| 14.0-90.0/4.19-29.44 | 6.98*    | -0.17 ± 0.05 | -0.00 ± 0.07 | 3.99     |
| 16| Feathered feet    | 12     | 4/1.50 | 0.0-75.0/0.00-2.01 | 7.03*    | -0.02 ± 0.04 | 0.24 ± 0.07 | 4.04     |
| 17| Feathered feet    | 13     | 55/1.56| 48.0-57.0/13.40-16.20 | 53.94****| 0.37 ± 0.04 | -0.17 ± 0.06 | 26.9     |
| 18| Feathered feet    | 15     | 47/12.22| 41.0-53.0/11.37-12.82 | 40.06****| 0.33 ± 0.04 | 0.15 ± 0.06 | 21.4     |

Notes: 
*1LGE22, LGE22C19W28_E50C23 linkage group; 2*5% chromosome-wide significance; **1% chromosome-wide significance; ****1% genome-wide significance; A, Additive; D, Dominance; PV = Percent of phenotypic variance explained by the QTL. PV% = [(MSR - MSF)/MSR] × 100 where MSR-residual mean square in the reduced model, MSF-residual mean square in the full model.