MTT SCIENCE 14

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Doctoral Dissertation

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Molecular genetics of chicken egg quality

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Abstract

Faultless quality in eggs is important in all production steps, from chicken to packaging, transportation, storage, and finally to the consumer. The egg industry (specifically transportation and packing) is interested in robustness, the consumer in safety and taste, and the chicken itself in the reproductive performance of the egg. High quality is commercially profitable, and egg quality is currently one of the key traits in breeding goals. In conventional breeding schemes, the more traits that are included in a selection index, the slower the rate of genetic progress for all the traits will be. The unveiling of the genes underlying the traits, and subsequent utilization of this genomic information in practical breeding, would enhance the selection progress, especially with traits of low inheritance, gender-confined traits, or traits which are difficult to assess.

In this study, two experimental mapping populations were used to identify quantitative trait loci (QTL) of egg quality traits. A whole genome scan was conducted in both populations with different sets of microsatellite markers. Phenotypic observations of albumen quality, internal inclusions, egg taint, egg shell quality traits, and production traits during the entire production period were collected. To study the presence of QTL, a multiple marker linear regression was used. Polymorphisms found in candidate genes were used as SNP (single nucleotide polymorphism) markers to refine the map position of QTL by linkage and association. Furthermore, independent commercial egg layer lines were utilized to confirm some of the associations.

Albumen quality, the incidence of internal inclusions, and egg taint were first mapped with the whole genome scan and fine-mapped with subsequent analyses. In albumen quality, two distinct QTL areas were found on chromosome 2. *Vimentin,* a gene maintaining the mechanical integrity of the cells, was studied as a candidate gene. Neither sequencing nor subsequent analysis using SNP within the gene in the QTL analysis suggested that variation in this gene could explain the effect on albumen thinning. The same mapping approach was used to study the incidence of internal inclusions, specifically, blood and meat spots. Linkage analysis revealed one genome-wide significant region on chromosome Z. Fine-mapping exposed that the QTL overlapped with a tight junction protein gene *ZO-2,* and a microsatellite marker inside the gene. Sequencing of a fragment of the gene revealed several SNPs. Two novel SNPs were found to be located in a miRNA (*gga-mir-1556*) within the *ZO-2*. MicroRNA-SNP and an exonic synonymous SNP were genotyped in the populations and showed significant association to blood and meat spots. A good congruence between the experimen-

tal population and commercial breeds was achieved both in QTL locations and in association results. As a conclusion, *ZO-2* and *gga-mir-1556* remained candidates for having a role in susceptibility to blood and meat spot defects across populations. This is the first report of QTL affecting blood and meat spot frequency in chicken eggs, albeit the effect explained only 2 % of the phenotypic variance.

Fishy taint is a disorder, which is a characteristic of brown layer lines. Marker-trait association analyses of pooled samples indicated that egg-taint and the *FMO3* gene map to chicken chromosome 8 and that the variation found by sequencing in the chicken *FMO3* gene was associated with the TMA content of the egg. The missense mutation in the *FMO3* changes an evolutionary, highly conserved amino acid within the *FMO*-characteristic motif (FATGY).

In conclusion, several QTL regions affecting egg quality traits were successfully detected. Some of the QTL findings, such as albumen quality, remained at the level of wide chromosomal regions. For some QTL, a putative causative gene was indicated: miRNA *gga-mir-1556* and/or its host gene *ZO-2* might have a role in susceptibility to blood and meat spot defects across populations. Nonetheless, fishy taint in chicken eggs was found to be caused with a substitution within a conserved motif of the *FMO3* gene. This variation has been used in a breeding program to eliminate fishy-taint defects from commercial egg layer lines.

Objective

The objective of this thesis was to map loci affecting economically important egg quality traits in chickens and to increase knowledge of the molecular genetics of these complex traits. The aim was to find markers linked to the egg quality traits, and finally unravel the variation in the genes underlying the phenotypic variation of internal egg quality. QTL mapping methodology was used to identify chromosomal regions affecting various production and egg quality traits (I, III, IV). Three internal egg quality traits were selected for fine-mapping (II, III, IV). Some of the results were verified in independent mapping populations and present-day commercial lines (III, IV). The ultimate objective was to find markers to be applied in commercial selection programs.

Keywords:
chicken, egg quality, QTL, gene mapping, gene and marker assisted selection, albumen quality, blood and meat spots, fishy taint, FMO3

Kananmunan laatuun vaikuttavien geenien kartoitus

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Kananmunan laadun virheettömyys on tärkeää koko tuotantoketjulle aina kanalasta kuluttajalle asti. Kestäväkuorisuus on yksi tärkeimmistä pakkaamojen laatukriteereistä; kuluttaja arvostaa eniten tuotteen turvallisuutta ja makua. Lisääntymisbiologian näkökulmasta hyvä hedelmällisyys ja elinkykyiset poikaset ovat tärkeimmät laatuominaisuudet. Laatu merkitsee myös parempaa taloudellista kannattavuutta. Nykyään laadun parantaminen sisältyykin jalostusohjelmaan. Jalostuksen avulla saavutettava edistyminen hidastuu kuitenkin sitä mukaa, mitä enemmän ominaisuuksia sisällytetään perinteiseen valintaindeksiin. Siksi ominaisuuksia säätelevien geenien tunnistaminen ja 'täsmätiedon' sisällyttäminen jalostusvalintaan nopeuttaisi edistymistä. Suurin etu geenitiedosta saavutetaan alhaisen periytyvyysasteen, sukupuoleen sitoutuneiden ja vaikeasti arvosteltavien ominaisuuksien suhteen.

Tämän väitöskirjan tarkoituksena oli kartoittaa kananmunan laatuun vaikuttavia geenejä. Nämä ominaisuudet ovat luonteeltaan kvantitatiivisia, eli niiden säätelyyn vaikuttaa lukuisa joukko geenejä (quantitative trait loci, QTL), ympäristö ja näiden yhteisvaikutukset. Tilastollisten kartoitusmenetelmien (monen markkerin lineaarinen regressioanalyysi ja assosiaationanalyysi) avulla etsittiin yhteyksiä DNA-merkkien ja tutkittavien ominaisuuksien väliltä. Tutkimusmateriaalina käytettiin kahta F_2 -risteytysaineistoa, joissa molemmissa tehtiin koko genomin kartoitus. Tutkimuksessa keskityttiin valkuaisen kiinteyden, veri- ja lihapilkkuisuuden ja hajuvirheen kartoittamiseen. Kandidaattigeeneistä etsittiin sekvensoimalla muuntelevia kohtia (SNP, single nucleotide polymorphism), joilla mahdollisesti olisi toiminnallinen yhteys tutkittavien laatuominaisuuksien ilmentymiseen. Lisäksi niitä käytettiin edelleen DNA-merkkeinä QTL:ien hienokartoituksessa. Tulokset pyrittiin varmistamaan riippumattomasta aineistosta assosiaatioanalyysiä käyttäen.

Koko genomin kartoituksen tuloksena löydettiin valkuaisen laatuun, veripilkkuisuuteen ja kalanhajun ilmenemiseen vaikuttavia kromosomialueita, joita analysoitiin hienokartoitusmenetelmällä. Valkuaisen laatuun löytyi kaksi lähekkäistä aluetta kromosomista 2. *Vimentiini*, alkuperäisellä QTL-alueella sijaitseva geeni, jonka tehtäviin kuuluu mm. solun eheyden ylläpitäminen, valittiin valkuaisen laadun kandidaattigeeniksi. Se ei kuitenkaan selittänyt valkuaisen laadun vaihtelua. Samaa kartoitusmenetelmää käytettiin veri- ja lihapilkkuisuuden kartoittamisessa. Kandidaattigeeniksi valikoitui *ZO-2* –geeni, joka säätelee mm. solujen välisen tiiviin liitoksen muodostumista: geeni vaikuttaa solujen väliseen läpäisyesteeseen mm. verisuonissa. Geenin osittainen sekvensointi tuotti useita muuntelevia SNP-merkkejä, mm. kaksi uutta SNP:iä geenin sisällä olevasta säätelytekijästä, miRNA:sta (mikroRNA). Tiheämmän kartan ja SNP-geenimerkkien lisääminen QTL- ja assosiaatioanalyyseihin paransivat QTL-alueen paikantamista. QTL:n olemassaolo testattiin kahdessa riippumattomassa kaupalli-

sessa jalostusaineistossa. Tulokset näistä kahdesta kanalinjasta tukivat kokeellisten risteytysten tuloksia: joko itse *ZO-2* –geenillä tai miRNA:lla näyttäisi olevan vaikutusta veri- ja lihapilkkuisuuden ilmenemiseen eri kanalinjoissa.

Kananmunien kalanhaju on oireyhtymä, jota esiintyy pelkästään ruskeilla munijoilla. Sekä ominaisuus että kandidaattigeeni, *FMO3* kartoitettiin kromosomiin 8. Geenin sisäistä muuntelua selvitettiin sekvensoimalla koko koodaava alue. Geenimuuntelun yhteyttä testattiin kalanhajun aiheuttajaan (TMA-pitoisuus; trimetyyliamiini). Osoittautui, että mutaatio kanan *FMO3*-geenissä oli yhteydessä TMA-pitoisuuden kohoamiseen. Lisäksi selvisi, että mutaatio sijaitsi geenin hyvin konservoituneella alueella.

Kaiken kaikkiaan tutkimus tuotti kananmunan laatuun vaikuttavia kromosomialueita, kandidaatti- ja kausaaligeenejä. Valkuaisen laadun kartoittamisessa jäätiin vielä laajan kromosomialueen tasolle. Veri- ja lihapilkkuisuuden yksi todennäköisistä aiheuttajista löytyi kromosomissa Z, alueelta, jolla sijaitsee mm. ZO-2 geeni ja miRNA-säätelytekijä (gga-mir-1556). Lopullisen syyn selvittäminen jää jatkotutkimusten ratkaistavaksi. Tämä on ensimmäinen raportointi veri- ja lihapilkkujen geenikartoituksesta.

Kalanhajun aiheuttava geenimutaatio kartoitettiin onnistuneesti: osoitettiin, että se aiheutuu *FMO3*-geenin mutaation seurauksena. Tätä tulosta on hyödynnetty kansainvälisessä jalostusvalinnassa, etenkin oireyhtymän kantaja-diagnostiikassa. Lisäksi löydös on patentoitu.

Avainsanat:
kana, kananmunan laatu, QTL, geenikartoitus, geeni- ja DNA-merkkiavusteinen valinta, valkuaisen laatu, veri- ja lihapilkut, kananmunan kalanhaju, FMO3, TMA, trimetyyliamiini

Abbreviations

AIL(s)	Advanced intercross line(s)
ANOVA	Analysis of variance
ArkDB	Farm animal database ArkDB
BAC	Bacterial artificial chromosomes
BMS	Blood and meat spots
cDNA	Complementary DNA
CI	Confidence intervals
cM	centiMorgan
CNV	Copy number variation
CR1	Chicken repetitive 1 element
DNA	Deoxyribonucleic acid
EST	Expressed sequence tag
FATGY	Conserved protein motif of FMO3
FASGY	mutated protein motif of FMO3
FMO3	Flavin containing monooxygenase 3
GAS	Gene assisted selection
Gb	Gigabase
GS	Genomic selection
GWA	Genome-wide association
HU	Haugh-unit
kb	Kilobase
LD	Linkage disequilibrium
Mb	Megabase
MAS	Marker assisted selection
miRNA	MicroRNA
mRNA	Messenger RNA
PCR	polymerase chain reaction
qPCR	Quantitative PCR
QTL	Quantitative trait loci
QTN	Quantitative trait nucleotide
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphisms
RH	Radiation hybrid
RIR	Rhode Island Red
SD	Standard deviation
SG	Specific gravity
RNA	Ribonucleic acid
SNP	Single nucleotide polymorphism
sSNP	synonymous SNP
nSNP	nonsynonymous SNP
SSR(s)	Simple sequence repeat(s)
TMA	Trimethylamine
TMAU	Trimethylaminuria
TSHR	Thyroid stimulating hormone receptor
Vim	Vimentin
WGA	Whole genome association
WL	White Leghorn
WR	White Rock
ZO-2	Tight junction protein 2 alias Zonula occludens protein 2
3'-UTR	3'-untranslated region

List of papers

This thesis is based on the following papers, which will be referred to in the text by the Roman numerals assigned below.

I M. Tuiskula-Haavisto, M. Honkatukia, J. Vilkki, D.-J. de Koning, N. F. Schulman, and A. Mäki-Tanila. Mapping of Quantitative Trait Loci Affecting Quality and Production Traits in Egg Layers. Poultry Sci. 2002, 81, 7, 919-927.

II M. Honkatukia, M. Tuiskula-Haavisto, D.-J. de Koning, A. Virta, A. Mäki-Tanila, and J. Vilkki. A region on chicken chromosome 2 affects both egg white thinning and egg weight. Genet. Sel. Evol. 2005, 37, 563-577.

III M. Honkatukia, K. Reese, R. Preisinger, M. Tuiskula-Haavisto, S. Weigend, Roito, A. Mäki-Tanila, and J. Vilkki. Fishy taint in chicken eggs is associated with a substitution within a conserved motif of the FMO3 gene. Genomics. 2005, 86, 2, 225-232.

IV M. Honkatukia, M. Tuiskula-Haavisto, V. Ahola, M. Schmutz, R. Preisinger, D. Cavero, P. Vennerström, J. Arango, N. O'Sullivan, J. Fulton and J. Vilkki. Mapping of QTL affecting incidence of blood and meat inclusions in egg layers (manuscript).

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Jokioinen, December 2010

Mervi Honkatukia

Contents

1 Introduction

1.1 History of the modern chicken

Since the chicken was domesticated around 8,000 years ago, it has served in various roles: at the beginning it had great cultural significance in religious purposes. Later on, breeding brought along new role as a rich food source (Crawford, 1990). The domestication of the chicken is still controversial and remains subject to debate. It has been speculated that the modern chicken originates solely from the red jungle fowl (Al-Nasser et al., 2007). However, recent studies suggest multiple origins of domestication and indicate that several wild progenitors have contributed to the current chicken genome (Eriksson et al., 2008; Liu et al., 2006). The geographical location of the chicken's domestication has also been questioned. At present, it is assumed that the chicken was tamed independently at several places in Asia (Kanginakudru et al., 2008). Interestingly, the wild progenitors, the jungle fowls, have remained alive to this day.

Breeding has extensively changed the physiology, production capacity and behavior of chickens. Despite the physiological discrepancies between the wild ancestors and current chicken breeds, it is still possible to cross–breed them(e.g. Kerje et al., 2003; Rubin et al., 2007). Very intensive breeding has led to very different types of chicken breeds, such as broilers and layers (Figure 1). The commercial egg layer lines have been formed from a narrow origin: all the white layers originate from the White Leghorn, and brown lines are founded on dual-purpose breeds such as the Rhode Island Red and the White Plymouth Rock (Muir et al., 2008).

Intensive poultry breeding started in the 1930's, when the poultry industry specialized either on meat or on eggs (Stevens, 1991). Today, commercial layer breeding is centralized and business is mainly in the hands of two multinational companies. The global consumption of chicken eggs is more than 55 million metric tons per year (Muir et al., 2008). In addition to being

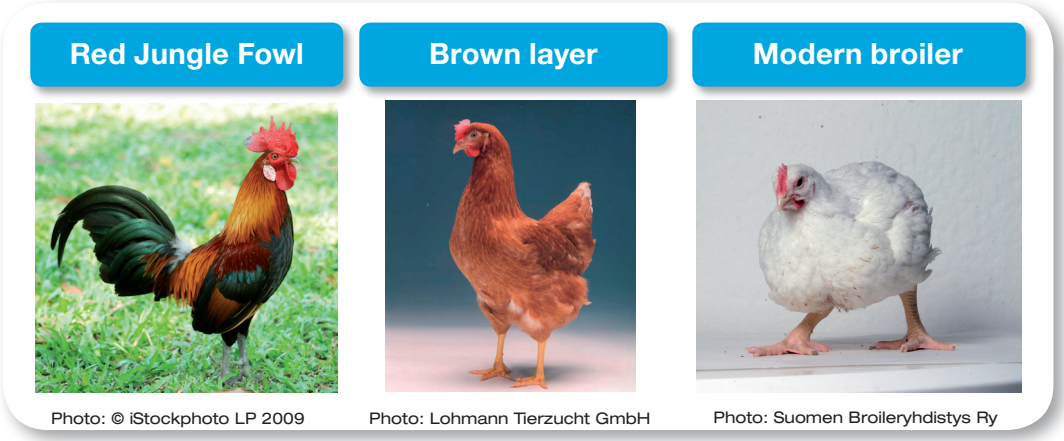

Figure 1. Examples of an intensive selection and breeding. The appearance of chicken has changed remarkable: on the left a red junglefowl, one of the ancestors of the modern chicken; in the middle a representative of layer line, a brown layer; a typical modern broiler on the right.

one of the main sources of protein supply for humans, the chicken is an ideal model species for various research areas, including genetic mapping and QTL analysis.

1.2 Egg quality

Egg quality is composed of hygienic, nutritional, sensory and technological properties. A first-class egg is a fresh and uniform product with a solid shell (Jacob et al., 2000; Roberts, 2004). Many factors affecting quality are hereditary, controlled by quantitative loci, i.e. many genes, the environment and their interactions are contributing to the trait.

The aspects of ideal egg quality vary depending on the perspective of industry, breeding companies, the consumer, or the hen itself. For the hen, the egg is a vehicle for reproduction, containing all the necessary components for a new life in its optimum proportions. Egg quality is important for reproductive performance relating to hatching and hatchability. For the consumer, the most important criteria are the egg's sensory characteristics, its microbiological safety, and its appearance. For the breeder and producer, the most important quality aspects are the durability of the egg shell and consumer acceptance.

Internal egg quality is composed of air cell size, albumen (commonly known as egg white) quality, yolk quality, and the presence of blood or meat spots (Jacob et al., 2000). For example, the albumen structure makes a strong contribution to the microbiological quality of the egg, which in turn affects egg safety. Egg white thinning, which happens when the portion of thick albumen is declining, is a phenomenon that affects the internal quality of the egg. A fresh and firm egg white restricts microbial growth because egg white proteins have antimicrobial properties, while a thin and runny egg white exposes the egg to microbial infections due to altered egg structure (yolk can get in contact with the egg shell). Also changed biochemical composition (egg white liquefaction) has been speculated to increase the microbiological penetration (Gurtler et al. 2009; Gast et al. 2005).

Occasionally, eggs contain internal inclusions such as blood and meat spots. This quality fault is included in selection programs, especially in brown chicken lines (Muir and Aggrey, 2003). In addition to being an aesthetic and ethical problem, there have been indications that blood or pieces of reproductive tissue inside the egg may increase the risk of infections (Smith and Musgrove, 2008). Other quality characteristics important to consumers are flavor and shell color.

Storage and handling of eggs are automated in the egg industry, thus setting special demands for egg size and shell strength. A study of egg appearance in markets showed that up to 45 % of all cartons examined had at least one cracked egg (reviewed by Gomez-Basauri, 2008). The presence of a weak or broken shell exposes eggs to pathogen contamination. Economic losses associated with egg shell defects are estimated to be in the order of 6-8% (Gomez-Basauri, 2008).

The priorities in breeding have evolved a lot during last decades. High quality egg has been recognized as an important asset for the breeding companies. Thus, in layer breeding, egg quality, as well as egg production, behavior, and feed management, will gain more emphasis in the future (Preisinger, 2010). Good egg quality benefits all production steps and eggs of high quality are commercially profitable.

1.2.1 The chicken genome

The chicken genome contains several unique characteristics when compared to other vertebrates. The karyotype consists of 39 pairs of chromosomes: 38 autosomes

and one pair of sex chromosomes (ZW). In contrast to mammals, female chickens are heterogametic (ZW) and males are homogametic (ZZ). There is a great variation in chromosomal sizes, and the chromosomes can be sorted into three subtypes: macrochromosomes (GGA1-5, Z) are between 50 and 200 Mb; intermediate chromosomes (GGA6-10) vary from 20 to 40 Mb; and microchromosomes (GGA11-28, W) are 12 Mb on average (Megens et al., 2009; Schmid et al., 2000). The microchromosomes are conserved in many avian species, but also in primitive amphibians and most reptiles (Nie et al., 2010). Besides the differences in physical sizes between the various subsets of chromosomes, there are also structural divergences. It has been illustrated that recombination frequency, gene density and the level of gene expression correlate with chromosome size - the microchromosomes have higher gene density, GC content, expression and recombination rate, as well as with smaller gene size (Groenen et al., 2009; Hillier et al., 2004; Nie et al., 2010). The higher recombination rates in microchromosomes are reflected in the ratio of the physical map with the linkage map length being 50-100 kb/cM, whereas it is approximately 300 kb/cM in the macrochromosomes (Wong et al., 2004; Elferink et al., 2010). This ratio is around 1cM/1Mb in mammals (Georges, 2007). The higher recombination rate in microchromosomes has been suggested to result from a higher density of cohesion binding sites (Megens et al., 2009). Studies suggest more regional transcription regulation, with co-regulated gene clusters. The lengths of exons and introns are also shorter in microchromosomes. This is suggested to be associated with transcription efficiency (Nie et al., 2010).

The chicken genome is compact in comparison to that of mammals. The size (1.2 Gb) is only one third of the size of a human's, but it is predicted to include a similar number of genes. This smaller size has been suggested to be a consequence of smaller interspersed repetitive elements (Wang et al., 2010).

The chicken Z chromosome is less gene-dense than the autosomes. It contains about 1000 genes, which is 11 genes per Mb - less than half of the chicken's autosomal average (25 genes /Mb) (Bellott et al., 2010). In contrast, the density of interspersed repeats is 60% higher in the Z chromosome than in autosomes. Although the Z chromosome and mammalian X chromosome share common features such as low gene density and increased interspersed repeat content, it has been proposed that Z and X chromosomes have evolved independently from different autosomes (Bellott et al., 2010). According to opposite speculations, Z and X might have originated from separate segments of a common proto-sex chromosome (reviewed by Bellott et al., 2010).

The chicken was the first bird, agricultural species, and descendant of the dinosaurs to have its genome sequenced (Hillier et al., 2004). The sequence was derived from a female red jungle fowl and the coverage was 6.6 x. The sequencing revealed some significant features of the chicken genome, including common intra-chromosomal rearrangements between humans and chickens, novel evolution of some non-coding RNA genes, and a novel class of clustered non-coding sequences. The current genome build version 2.1, WASHUC2 (May 2006) covers 29 autosomes and the sex chromosomes. However, some of the microchromosomes are still missing from the genome build (Groenen et al., 2009).

1.2.2 Linkage maps

Besides having the first genome sequence among livestock species, the chicken was also the first farm animal to have a linkage map. Hutt (Hutt, 1933) published a map of a linkage group with four loci. The mapped loci were phenotypic characters. A

revolution in the construction of linkage maps occurred nearly sixty years later with the use of molecular markers in a reference family. One of the first whole genome linkage maps, the Compton map (Bumstead and Palyga, 1992), was based on a cross between two inbred White Leghorn lines and 100 restriction fragment length polymorphisms (RFLP). A contemporary reference mapping population, East Lansing, was constructed from a single pair of founder animals (Levin et al., 1994). It was a backcross of an inbred Red Jungle Fowl male and an inbred White Leghorn female. This map included 19 linkage groups and was built from 98 various markers, such as blood groups, RFLP, random amplified polymorphic DNA (RAPD), and chicken CR1 repeat-element polymorphisms. The third reference map, based on a Wageningen mapping population, was published a few years later (Groenen et al., 1998). Implementation of neutral DNA markers such as simple sequence repeats (SSRs, microsatellites) substantially improved the resolution. This map consisted of 430 microsatellite markers on 27 autosomal linkage groups and a Z-chromosome-specific linkage group. The extent of the reference maps was improved by the construction of a consensus map. The consensus map combined all the marker data of the three mapping populations mentioned (Groenen et al., 2000). The resulting chicken genome map contained 1889 marker loci within 3800 cM. The updated version was published 2005 (Schmid et al., 2005). Thereafter, many research groups have published their own linkage maps. These maps were brought together in the ArkDB database, in which over 40 different chicken linkage maps are currently available (http://www.thearkdb.org/)(Hu et al. 2001). A very important milestone for linkage map development has been the development of SNP markers, which has provided an outstanding enhancement for the mapping resolution. A huge number of SNPs (2.8 million) were identified by comparing sequences derived from four species: the broiler, the layer, the Chinese silkie and the red junglefowl (Wong et al., 2004). The first high resolution linkage map was published in 2009 (Groenen et al., 2009). The map increased the marker density fivefold when compared to the former consensus map, and contained 8599 SNP markers in addition to 669 markers from the consensus map. Chromosomes are relatively well represented in this map, apart from five microchromosomes, which turned out to be challenging due to technical issues during sequencing (Groenen et al., 2009). The latest high-density linkage map of 13340 SNPs (with a total length of 3054 cM) does not cover the missing microchromosomes either (Elferink et al., 2010). This map is based on two broiler populations with a high number of individuals and markers. It has been used to improve the current genome build, for example, to correct mistakes in the order of sequences in the genome build.

1.2.3 Physical maps

A physical map is very important for anchoring loci or sequences to genetic maps. Validation is needed for loci between different linkage maps, but also in the genome build. Bacterial artificial chromosomes (BAC) can be used in large fragment cloning because BACs are capable of maintaining large inserts of foreign genomic DNA with high degree of stability. BAC libraries are used in the construction of high genome coverage contig maps (Crooijmans et al., 2000; Lee et al., 2003; Ren et al., 2003). BAC libraries and maps are a good source of DNA markers, and a tool for comparative mapping (Primrose, 1998). Some chicken microchromosomes are difficult to cover with genetic or BAC contig maps. Thus, radiation hybrid (RH) mapping has been used to improve the genome build (Morisson et al., 2007). RH mapping is a somatic cell technique that is used for ordering markers along chromosomes and estimating the physical distance between them. An RH map is not subject

to cloning biases like BAC clones and, unlike in the construction of a linkage map, it does not require polymorphic markers or pedigree. A chicken RH map for 20 chromosomes and linkage groups of four other chromosomes is available at (http://chickrh.toulouse.inra.fr/).

1.2.4 Examples of functional variation

It was not long ago that the focus was limited to the coding regions of the genome; others were ignored and classified as junk DNA. Increasing understanding of the genome is occasionally producing a novel class of regulatory elements underlying the functional variation of the genomes. Relatively new elements of this kind are microRNAs and copy number variations. MicroRNAs (miRNAs) are short, single stranded RNA molecules (~22 nt) (Berezikov et al., 2006; Georges et al., 2007). They are involved in gene regulation via binding to the mRNA of a target gene. Regulation is based on either triggering mRNA cleavage (called RNA interference), inhibiting of the translation, or up-regulating translation (Shao et al., 2008). Regulation takes place in the 3'-UTR (untranslated region) of the target mRNA. MicroRNA mediated regulation was first observed in plants (reviewed by Morisson et al., 2007). miRNAs are universally found in animals, plants, and even in some unicellular organisms (Shao et al., 2008). A majority of miRNAs are transcriptionally linked to their host gene (a microRNA may be located within genes, herein called a host gene) expression and processed from the same primary transcripts (Lutter et al., 2010).

Strategies to determine the biological function of miRNAs include, among others, bioinformatic predictions, reporter assays, overexpression and silencing. For example, 200 genes on average have been found to be controlled by a single miRNA by prediction search (Krützfeldt et al., 2006).

SNPs in miRNAs or their target sites may be an important source of variation underlying QTL effects and may contribute to disease susceptibility (Hu et al., 2009). A polymorphism in processed miRNA may affect the expression of several genes and have serious consequences, whereas a polymorphism in a target site may be more specific (Lutter et al., 2010). The molecular mechanism of muscularity in Texel sheep has been proved to be a mutation in the miRNA target site of the myostatin gene (Clop et al., 2006). Initially, the chromosomal area of the effect on muscle was detected by a whole genome QTL scan and was subsequently fine-mapped. The study undertaken by Yao et al. (Yao et al., 2008) suggested that microRNAs have a role in chicken's Mareks' virus pathogenesis.

Segmental copy number variation (CNV) involves submicroscopic chromosomal insertions, deletions, segmental duplications and other complex changes in size between 1 kb to several Mb. It is extensively studied in vertebrates (such as humans, chimpanzees, dogs, mice, rats and swine) reviewed by Wang et al. (Wang et al.,2010). Many inherited disorders in humans (including red-green color blindness, hemophilia A, Parkinson's disease and autism) have been shown to convey by copy number variation (Zhang et al., 2009). Analysis of the bovine genome indicated that segmental duplications were commonly found in gene families important to biological functions such as immunity, digestion, lactation and reproduction traits (Liu et al., 2009). Wang et al. (Wang et al., 2010) suggested that copy number variation is also a potential source of variation in agricultural or disease related traits in chickens. There are still only a few studies of copy number variation in birds available. An initial chicken copy number variation map of 96 loci was published recently (Wang et al., 2010). The map indicated that the amount of DNA located within the CNV areas was 1.34 % of the total genome. This is substantially lower than the CNV content of the human genome (the boldest 'guess' is up to 29.74% of the reference genome) (Zhang et al., 2009). However, this may be one factor

which contributes to differences in genome size between chicken and mammalian species. Considering copy number variation is needed not only for improvement of genome assembly but also as a new marker type in genome-wide association studies. SNP assays might have overlooked CNV regions due to variation in allele signal intensities.

1.3 Mapping of monogenic traits

A monogenic trait can be traced back to variation in a single gene. The pattern of the inheritance is simple, following the Mendelian laws of genetics. Monogenic traits can be divided to autosomal recessive, autosomal dominant and sex linked (Sudberry, 2002). Many diseases are caused by monogenic traits. The simplest way to map these traits is linkage or association analysis - testing the co-segregation of a genomic region (markers) and a binomial status of the trait (healthy/sick). However, other non-genetic factors may modify the simple pattern of inheritance, making the situation more complicated. For instance, the effect of an allele may depend on the environment, like the fishy taint disorder in chicken that is expressed only in certain feeding conditions. Without a proper feeding challenge the affected individuals will remain unrecogniced. Hence, many factors could complicate the success of gene detection.

1.4 Polygenic traits and QTL mapping

The phenotype of polygenic, or quantitative trait, depends on alleles at many different gene loci together with the environment and their interaction. The term quantitative trait loci (QTL) was first introduced to agricultural research (Geldermann, 1975) long before QTL mapping methodology was introduced (Knott and Haley, 1992; Lander and Botstein, 1989; Paterson et al., 1988). Relatively soon the terminology and mapping protocols were widely adopted, from agricultural studies to a wide range of studies in many different organisms from fruit flies to humans. A QTL is a region of genome harboring one or more genes (Falconer and Mackay, 1996). Quantitative traits have a complex inheritance; an unknown number of genes control the trait and gene effects are merged with background variation (Falconer and Mackay, 1996). This is causing a normal distribution of the phenotype. The complexity arises when, for instance, the same genotype induces a different phenotype due to interaction with other genes or environments. In contrast, different genotypes can result in the same phenotype (Lander and Schork, 1994). A well known fact is that most of the important traits in farm animals are quantitative, and therefore have a wide range of phenotypic variation (Falconer and Mackay, 1996).

Modern markers and sophisticated statistical methods have allowed studies aiming at dissection of the genetic architecture underlying quantitative variation (Goddard and Hayes, 2009). The principle of QTL analysis is to link phenotypes and genotypes in a way that genotypes are used to explain phenotypic data (Falconer and Mackay, 1996; Lynch and Walsh, 1998). QTL analysis requires a population where the genetic differences in a trait can be assessed jointly with polymorphic markers and statistical methods.

1.4.1 An overview for QTL mapping designs

There are various possibilities to design an experiment for QTL mapping. In the following, some of the most common approaches are reviewed. The most powerful design to map QTL is a cross between two inbred lines (Falconer and Mackay, 1996; Soller et al., 1976). Heterogeneity within lines is reduced and the lines are assumed to be fixed for alternative alleles in markers and QTL. This means that the F_1 generation is uniformly heterozygous and the F_2 is in turn segregating for both markers and QTL. Also the backcross model reduc-

es background genetic variation (Falconer and Mackay, 1996). The line cross model allows calculation of additive and dominance effects of putative QTL (de Koning et al., 1999). For most of the domestic animals it is not practical to produce inbred or highly selected lines, thus line cross approach was broadened to genetically divergent outbred lines (Beckmann and Soller, 1988; Haley et al., 1994). For instance, crosses between wild ancestors and modern breeding lines have been used in QTL mapping approaches, such as red junglefowl in chickens (e.g. Kerje et al., 2003) or wild boar in pigs (Andersson et al., 1994). However, the use of widely divergent lines has been criticized because the variation or QTL observed may not be relevant to current populations.

Experimental design needs to be optimized to increase the power of mapping QTL, but also to reduce the costs of data collection and genotyping. In livestock, data collection involves either outbred existing populations or experimental populations (Soller, 1991). Outbred populations have been used to identify QTL, in particular when motivated by an opportunity of marker assisted selection within that population, whereas experimental design has been used to identify candidate loci for a particular trait. Outbred populations have been widely used, especially in large farm animals like cattle, because the long generation intervals (among other things) hamper the utilization of experimental crosses. Again, artificial insemination among dairy cattle has provided a ready-to-use population structure of large half-sib families (Georges et al., 1995). In poultry and pigs, either full-sib or half-sib experimental populations have been used for mapping purposes.

1.4.2 Selective DNA pooling

Selective DNA pooling is used to significantly reduce the number of samples and markers to be genotyped (Darvasi and Soller, 1994; Lander and Botstein, 1989). For example, genotyping only 50 % of the population (the top and bottom 25 %) gives more than 90% of the information (Haley and Andersson, 1997). One pool includes samples from animals from the top of the phenotypic distribution and the other pool includes individuals from the lowest percentile. Pools can be formed within half-sib or full-sib families or over the whole population. The association between a marker and a phenotype can be inferred from differences in the allele frequencies between the opposing pools (Darvasi and Soller, 1994). DNA pooling is most efficient in large families or in large populations. However, selective pooling is restricted only to one trait of interest, thus not being a good option when several traits are to be analyzed simultaneously. Selective DNA pooling was used in mapping the fishy taint syndrome (III).

1.4.3 Statistical analysis

Statistical methods are needed not only to identify the most likely position and effects of QTL, but also to test the significance and reliability of the result. Mapping techniques for revealing QTL have evolved from single-marker mapping to various approaches: e.g. single QTL interval mapping, grid searching for two QTL, multiple trait mapping and search for epistasis among QTL.

The first method to identify presence of QTL was the single marker approach (Weller, 1986). It was based on a comparison of the phenotype averages between marker genotypes groups by t-test, ANOVA test or simple linear regression (Liu, 1998). Testing can be performed with many markers, but only one marker at a time. Interval mapping explores the intervals between pairs of flanking markers for a presence of a single QTL (Haley and Knott, 1992; Lander and Botstein, 1989; Martinez and Curnow, 1992). This approach provides a more accurate esti-

mate of QTL effect and position when compared to the single marker approach. The single marker approach is not capable of distinguishing between the effect of closely linked small QTL and the effect of distantly located large QTL (Falconer and Mackay, 1996; Knott and Haley, 2000). Several statistical approaches have been used to detect QTL, including least squares, maximum likelihood, and Bayesian methods. When compared to one another, most of these statistical approaches have fairly congruent outcomes (Liu, 1998). When the mere estimation of additive and dominance effects is inadequate, more sophisticated methods of analysis can be used. A trait by trait search has been commonly used in various mapping experiments. However, the studied traits are often correlated. The same QTL may have pleiotropic effects on two or more correlated traits. Using the information from traits simultaneously should improve the power and precision of the location estimates (Knott and Haley, 2000). Several approaches have been introduced to map multiple traits jointly: canonical transformation, maximum likelihood, multi-trait least squares, and likelihood-ratio tests as reviewed by Knott and Haley (Knott and Haley, 2000).

Epistasis seems to have an increasing role in unraveling the complexity of quantitative variation and QTL networks. Interaction between loci contributes to variation in complex traits (Carlborg and Haley, 2004). The effect of one locus might depend on the genotype of one or several other loci. Epistasis also implies that the phenotype of any genotype cannot be predicted by summing the effects of individual loci. QTL methods for detecting epistasis are simultaneously considering the mean effects of multilocus genotypes on the phenotype. There are several mapping applications including epistasis in the model. Recent versions are based on simultaneous scans and randomization tests (reviewed by Carlborg and Haley, 2004).

Permutation, an empirical test, can be used to determine significance threshold values for given QTL (Churchill and Doerge, 1994). The threshold values are obtained from the studied data set, thus permutation also serves as a quality control of the data. These thresholds are adjusted for the number of tests performed to get genome-wide thresholds. Usually, confidence intervals (CI) for the QTL location are also determined. A bootstrap method can be applied to estimate the confidence interval for the position of the QTL (Visscher et al., 1996).

1.4.4 Fine-mapping: towards the candidate gene

The typical confidence interval of a QTL includes tens or hundreds of genes (Georges, 2007). Finding the causative mutation or QTN, quantitative trait nucleotide (Lyman et al., 1999) from such a quantity of genes might be an overwhelming task. Thus, QTL mapping is solely a beginning to identify the genes underlying trait variation. Therefore, the chromosomal area needs to fine-mapped, and some additional strategies are required. The simplest way is to multiply the number of cross-overs at the QTL region by increasing the marker density and the number of genotyped individuals. Thanks to high-density SNP maps and complete genome sequences, alongside relatively low-cost and rapid genotyping methods, the genotyping task is not necessarily the limiting factor anymore. In poultry, additional generations (advanced intercross lines, AILs, F_3, F_4,...F_n or backcross animals) can be relatively easily produced in experimental pedigrees (Darvasi, 1998). However, it is sometimes difficult, time consuming and expensive to keep the animals until the phenotypes are scored and the decisions regarding parents for the next generation can be made.

Linkage disequilibrium (LD) mapping is a viable approach for fine-mapping (Farnir et al., 2000; Terwilliger and Weiss,

1998). Linkage disequilibrium is nonrandom association of alleles in a haplotype. LD mapping is based on tracking historical recombinations over multiple generations and tracing back shared chromosome segments (or founder mutation) inherited from a common ancestor (Sudberry, 2002). Recombinations are rapidly reducing LD in all but the closest linked markers in the following generations. This enables identification of markers that define QTL alleles (Terwilliger and Weiss, 1998) The power to detect loci of a small effect is better in LD methods than in linkage analysis (Pritchard and Przeworski, 2001). At present, linkage disequilibrium (LD) mapping and genome-wide association studies with high through-put genotyping platforms (up to hundreds of thousands or millions of SNPs at once) are widely exploited to identify and fine-map QTL in humans and farm animals. In a study of commercial chicken lines, appreciable LD was detected among markers located closer than 5 cM (Heifetz et al., 2005). LD was even found across the populations, but decreased rapidly along with increasing distance between the markers.

Dense SNP data also enables haplotype structure analysis, which can be used, for instance, in the study of evolution dynamics and domestication. An interesting detail was published in a study of domestication of chicken with SNP data. Rubin et al. (Rubin et al., 2010) re-sequenced the chicken genome and used over 7 million SNPs to detect signs of selective sweeps during chicken domestication. A mutation in the gene coding for *thyroid stimulating hormone receptor (TSHR)* was revealed to have been selected in all domestic chickens (including broilers). This allele was suggested to be related to the absence of the strict regulation of seasonal reproduction.

The LD is strongly affected by population structure and history (such as admixture, isolation, effective population size etc.), in addition to the density of the map used (Mackay, 2004). Thus the estimation of LD is not necessary comparable between different studies. However, some generic estimation of the extent of LD can be proposed: higher levels of LD in longer distances have been detected in livestock (cattle, pigs and sheep) than in the humans reviewed by Andreescu (Andreescu et al., 2007). The magnitude of LD in humans is between 10 -20 kb (Georges, 2007). In a study of dogs, estimated LD within a breed extended over 0.5-1.0 Mb, whereas across breeds it was only 10 kb (Wilbe et al., 2010). A study of LD in several chicken breeds and different genomic regions indicated that there was a large differences in patterns of LD between different genomic regions (Aerts et al., 2007; Megens et al., 2009). It was also shown that LD in a layer line extended longer than it did in a broiler line (4 cM and 1 cM respectively). Similar results were found in a study of broiler lines by Andreescu (Andreescu et al., 2007), where the LD extended over shorter distances than expected. The substantial LD observed in layers has implications for gene mapping. The occurrence of large LD blocks will hamper fine-mapping of monogenic traits or QTL by association approaches.

Alternatively, the more traditional approach of candidate gene analysis is a good option when there is prior knowledge of the approximate location and function (biology and pathways) of putative candidate genes for the observed phenotypic variation. Fine-mapping can reduce the number of candidate genes in the QTL region to a reasonable number. Before a more detailed analysis of the genes is undertaken, it is important to predict which genes are relevant for the phenotypic effect. It is also essential to carefully select the correct representative phenotype measurements (e.g. individual, tissue, developmental stage) to be used in the analysis.

The search for variation in the candidate genes can be done by sequencing the entire

gene (or, with the new sequencing methodologies, the entire QTL region) from a few phenotypically extreme individuals. Alternatively, individuals with known QTL genotype (e.g. a known heterozygous parent) can be sequenced. The effects of the polymorphisms are then tested in groups of individuals (phenotypic extremes). Optional fragments of the gene can be sequenced for the detection of SNPs, which are thereafter used as genetic marker screenings for many individuals subsequently used in association analysis.

A majority of the QTL effects are speculated to be regulatory (Mackay, 2004). A powerful option for global evaluation of gene expression and the factors controlling transcription regulation are microarray platforms, such as cDNA and oligonucleotide arrays, launched since microarray technology emerged in 1990's (Woo, Y. 2004). Global expression analysis stands for simultaneous measurements of the expression of thousands of genes in a high-throughput fashion. Well-controlled experiments are needed to ensure that the effect of interest will not be confounded with other effects. Subsequent validation of expression differences of specific genes are commonly done with the quantitative-PCR (qPCR) method (Livak and Schmittgen, 2001).

The use of microarrays for gene expression studies can be combined with linkage analysis, called genetical genomics (De Koning et al., 2007; Jansen and Nap, 2001). In this approach, gene expression levels are treated as phenotypes. Marker data is collected as usual and the mRNA level of each gene is treated as a separate quantitative phenotype. The association between marker genotypes and the mRNA is then determined by conventional QTL mapping.

1.4.5 Modern approaches

The technologies used in the genomic research have developed greatly in past ten years. The capacity of the next and third generation sequencing instruments fit well for sequencing whole transcriptomes or whole genomes at once. This evolution has also influenced to the strategies used in the mapping studies. Some of the traditional mapping approaches described in the thesis have more or less been compensated by more efficient methods such as large whole genome association studies (WGAS) implemented by SNP arrays, RNA applications such as genome-wide profiling (by arrays or sequencing) of mRNAs, siRNAs, miRNAs, or global exon arrays (Cookson et al., 2009; Hu et al. 2009). Also, the combination of GWA studies and eQTL analysis offers effective means to unravel candidate genes and pathways.

Most of the QTN are regulatory and affecting through gene networks. This is causing a special challenge to bioinformatics in linking variations to effects, especially when the associations are found in non-coding regions (Mackay, 2004; Mackay et al. 2009). Another challenge is the handling and storage of the data flood from massive high-throughput methodologies (Cookson et al, 2009).

1.5 Chicken QTL database and identified QTL

In general, QTL mapping in chickens has been very successful. Currently (November 2010) the total number of QTL in the chickenQTLdb (http://www.animalgenome.org/cgi-bin/QTLdb/GG/browse) is 2284. The number of different traits included is 229. The traits are divided to four categories: exterior, health, physiology and production. The most abundant QTL category is production, accounting for over 80% of the detected QTL. From single production trait, body weight is predominant, with 395 reported QTL. Other well-studied traits are abdominal fat (208 QTL) and Marek's-disease-related traits (115 QTL). There are 208 quality-related QTL. However, the quality also includes

bone traits, whose proportion of the QTL results is the highest.

Very few QTL have been reported for albumen quality, 15 altogether (Figure 2A). Furthermore, the QTL cover different phenotypes (albumen height, albumen weight and Haugh-units, HU), which complicates the comparison of the QTL. In brief, three genomic locations are common to more than one QTL: a QTL area for albumen height and Haugh-units is found on chromosome 1 (Hansen et al., 2005), for early and late HU on chromosome 2 (Honkatukia et al., 2007; Tuiskula-Haavisto et al., 2002), and for albumen weight and Haugh-units on chromosome 4 (Wardecka et al., 2002). Regardless of fine-mapping efforts, no marker-based selection tool for improving the albumen quality is yet available.

Two olfactory traits have been mapped: egg aftertaste (Wright et al., 2006) and taint (Honkatukia et al., 2005); both were mapped to chromosome 8, but apart from each other. The tainting (trimethylaminuria, Phene ID 2764, Group 001360 in OMIA) belongs to those few quality traits whose gene has been successfully mapped and the causative mutation revealed.

At present, there are 32 egg shell quality QTL in the QTL database (Figure 2B) (http://www.animalgenome.org/cgi-bin/QTLdb/GG/index). There are two main clusters of egg shell QTL. The first is found on chromosome 1. The center of the region is located at approximately 111 Mb (co-locating with MCW200) and contains QTL for shell weight, thickness and strength, as reported by Sasaki et al. (Sasaki et al., 2004). A separate QTL for specific gravity locates further down the chromosome, at marker position MCW145 (162 Mb) (Wardecka et al., 2002). In addition to these two areas, a novel QTL for egg shell deformation was found between MCW36 and MCW23 (129-153 Mb) (Tuiskula-Haavisto et al. 2010). Additional support for this QTL cluster is in the study conducted by Dunn et al. (Dunn et al., 2007), where a QTL for bone quality was detected in the same chromosomal area (92.3-120.8 Mb). Bone quality and shell characters are related to calcium metabolism. Three adjacent regions are pinpointed on chromosome 1 by different

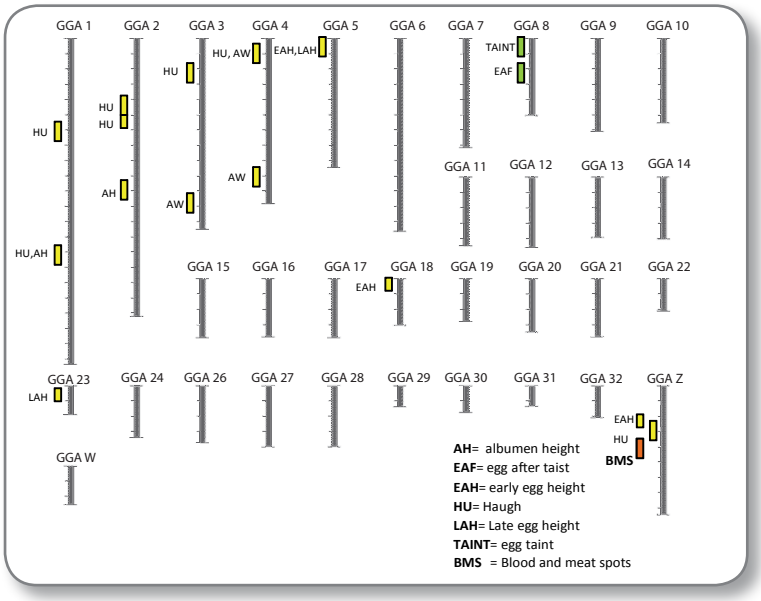

Figure 2. Egg quality QTL reported in Chicken QTLdb (August 2010). Bars are indicating approximations of the central positions without any scale. (A) Albumen quality QTL are shown in yellow, tainting and egg after taste in green, blood and meat spots in orange. (B) Egg shell quality QTL are shown in pink.

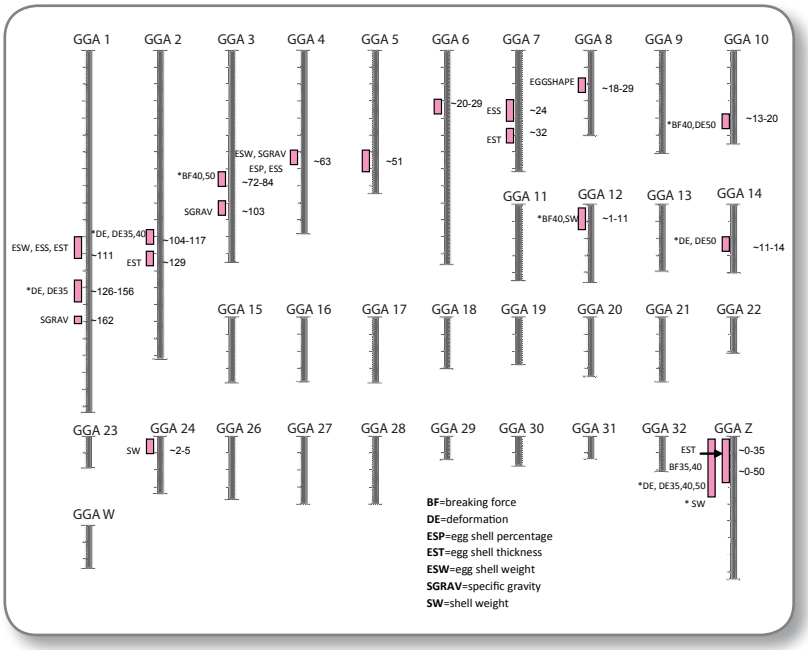

Figure 2B.

studies. Nonetheless, it is difficult to judge whether or not these regions are truly separate, because all of the studies have implemented low to medium density microsatellite marker maps whose resolution are not the best possible. Also the QTL positions have relatively long confidence intervals.

The other cluster includes shell QTL for strength, shell weight, shell percentage, and specific gravity (Sasaki et al., 2004; Schreiweis et al., 2006; Wardecka et al., 2002) on chromosome 4. It overlaps with the well established weight QTL area on chromosome 4 (e.g. Ambo et al., 2009; Honkatukia et al., 2007; Kerje et al., 2003; Nadaf et al., 2007; Tuiskula-Haavisto et al., 2002; Van Kaam et al., 1998).

A recently published gene expression profiling study provided a list of 54 proteins expressed in the shell gland during egg shell formation. Most of the genes were related to ion transport proteins, which provided eggshell mineral precursors (Jonchere et al., 2010). Some of the proteins listed in Jonchere et al.'s study have genes located in the egg shell QTL area found by Tuiskula-Haavisto (Tuiskula-Haavisto et al. 2010). For example, on chromosome 2, the *desmoglein-2* gene is found within the QTL area affecting deformation and breaking force.

High-throughput analysis methods for sequencing and genotyping have also already provided an enormous amount of empirical data in poultry genomics. In one of the first high marker density studies in chickens, Abasht et al. reported LD and association analysis of 3000 SNPs for production and quality traits in commercial layer lines (Abasht et al., 2009). For instance, two novel QTL for albumen height on chromosomes 5 and 18 were detected. Significant association of albumen height was also found on chromosome Z, which overlaps the QTL region found by Honkatukia et al. (Honkatukia et al., 2010b). Many previously detected QTL were also verified, including the egg shell strength QTL on chromosome 7 previously found by Sasaki et al. (Sasaki et al., 2004). Abasht et al. detected a high degree of LD at short

distances across the lines (Abasht et al., 2009). Hence they proposed that SNP detected in one line may also be useful in another. Although such loci are basically feasible for selection, the pleiotropic effects on other traits need to be estimated. The allele effects may also have different consequences in lines with different genetic backgrounds.

1.6 Future prospects in layer breeding

One of the main motivations for QTL mapping in agricultural species is that the results may be used in marker-assisted-selection (MAS). Once the locus linked to the phenotypic variation is identified, the information can be used in selection. The most straightforward and already used selection method for practical poultry breeding is gene assisted selection (GAS) (Dekkers, 2004), a subtype of MAS. It is targeted, based on direct relation between causative mutation in the gene and the selected trait. It is valid across various populations, thus it may be used directly in breeding programs to introduce favorable (or eliminate unfavorable) QTL alleles. For instance, it is very useful for screening putative carriers of an autosomal recessive disorder.

Genomic selection (GS) is a genome-wide version of marker assisted selection. It exploits dense and random SNP data to predict genomic breeding values instead of identified functional mutations (Goddard and Hayes, 2007). Once the effects of each SNP have been estimated from a large reference population, genomic selection can provide estimates of breeding values without having to measure the trait itself. MAS or genomic selection are not tied to any time point or specific sex; for example, traits related to egg laying can be estimated from males as early as needed. Genomic selection can increase the rate of genetic gain by shortening the generation interval (Meuwissen et al., 2001). However, validation is required, especially if the population is different from that in which the effect was estimated (Goddard and Hayes, 2007).

Usually any QTL accounts only for a small fraction of the phenotypic variance, typically so small that even large human association studies lack the power to find them (Goddard and Hayes, 2009). Although the genetic improvement may remain small in one generation, it is permanent and cumulative (Falconer and Mackay, 1996). In traditional breeding, the selection of the most economically important traits is straightforward. However, the addition of ancillary traits to the selection index lowers the rate of genetic progress for all of the traits. The use of genome information (MAS, GAS, GS) is advantageous, especially for traits with low heritability or for those which are difficult and/or expensive to measure (Goddard and Hayes, 2007). In poultry breeding it is particularly relevant in selecting superior individuals among full brothers, which all have identical breeding predictions, when evaluation is based in sisters' phenotype observations (Muir and Aggrey, 2003; Preisinger, 2010).

Some issues need to be solved before genomic selection will become an economically profitable option in commercial poultry breeding. Industrial poultry breeding is based on the hybridization of selected pure breeding lines (Albers et al., 2006; Preisinger, 2010). Commercial lines are usually hybrids of four grandparental lines. If all these lines need to be tested, the cost of establishing genome selection will increase substantially. Besides, breeding companies might have several production lines with distinct genetic backgrounds. This multiplies to launching costs. Even so, it seems that the breeding companies have recognized the possibilities of applying the genomic information in selection and have taken up the challenge by investing in future research.

2 Materials and methods

2.1 Mapping populations

2.1.1 RIR x WL

The initial mapping population was a reciprocal intercross between two egg layer lines, Rhode Island Red (RIR) and a (synthetic) White Leghorn (WL). The lines were chosen because of the difference between the egg quality traits found in the two lines. Albumen height, an indicator of albumen quality, was notably poorer in the RIR line than in the WL line, 53 and 91.0 HU, respectively (SD 7.1). The specific gravity, representative for egg shell durability, was 1.082 in RIR and 1.059 in WL (SD 0.005). The mapping population was founded on eight animals in the parental generation, two males and two females per line. The F_2 of 305 individuals was achieved by mating eight F_1 males with 32 F_1 females.

2.1.2 WR x RIR

A larger mapping population was created between the two commercial egg layer lines (Lohmann Tierzucht, Cuxhaven). White Rock (WR) and Rhode Island Red (RIR) were chosen as the grandparental lines to create an F_2 population by reciprocal mating. A half-sib mapping population was created by an optimized plan. In the F_1 generation, RIR x WR animals (from mating of RIR males to line WR females) included 122 males and 590 females; the reciprocal WR x RIR included 32 males and 163 females. The F_2 generation was generated from the most informative matings of F_1 individuals (via genotyping for 11 microsatellite markers associated with seven known QTL in layers) to maximize the power for linkage analysis. Twenty F_1 males were used (10 from each reciprocal line), each mated to three different full sister pairs. The progeny from four hatches included between 90 and 120 female offspring in each half-sib family. In the study, 17 half-sib families were included, including total of 1783 individuals: 30 grandparental males and 47 females, 17 F_1 parental males, 90 F_1 females, and 1599 F_2 individuals. See Table 1 for the summary of the experimental populations.

2.1.3 Commercial lines

Commercial lines were used for the confirmation studies. Confirmation of egg taint disorder (III) was carried out with an F_2 line cross design in Germany (herein called German F_2). 21 Rhode Island Red

Table 1. Summations of the experimental populations.

	RIR x WL	WR x RIR
created (year)	1994	1998
number of F_2 hens	305	1599
number of tested markers	117	192
number of mapped markers	99	162
studied chromosomes	14	27
mapped area in cM	2311	2585
average marker spacing	23.3	16
number of studied quality traits	6	12
genome-wide QTLs	6	26

hens known to produce tainted eggs were crossed with four White Leghorn cocks (no tainting) to produce an F_2 generation with 450 hens. Confirmation of internal inclusions (IV) was conducted in two independent commercial populations: Lohmann Brown and a Hy-Line brown sire line. Descriptions of these populations are provided in (III, IV).

2.2 Phenotypes

2.2.1 Albumen quality

Egg white quality was assessed with Haugh-unit, which indicates the height of the thick albumen. Estimates of the Haugh-unit are based on measurements of the egg weight and the height of thick albumen and calculated as log10 of weight corrected (Haugh, 1937). In the RIR x WL F2-mapping population, albumen quality was measured at 36-40 (HU40) and 57-60 (HU60) weeks of age. Also, the egg albumen thinning was monitored during the storage as albumen height (stored HU). The length of the storage was relative to the sales period on the market, i.e. 28 days. In the WR x RIR population the egg quality traits were measured at 35, 40 and 50 weeks of age.

2.2.2 Blood and meat inclusions

The blood and meat spot phenotypes were collected from WR x RIR population. The data collection was done at 35, 40 and 50 weeks of age. In addition both types of the spots were treated as single trait, blood and meat spots (BMS), solely as an indication of the fault. The blood and meat spots trait was studied in more detail in the commercial lines. Phenotypes were collected between ages of 36-42 weeks. Five phenotype variables were measured in Lohmann Brown lines, including the size and type of the spots (IV). In the Hy-Line population, phenotypes were expressed as sire-daughter averages of meat inclusions and blood inclusions. Some illustrations of internal inclusions are shown in Figure 3.

2.2.3 Shell quality

Egg shell quality was recorded in RIR x WL as egg shell strength and specific gravity at early and late production periods (36-40 and 56-60 weeks of age, respectively). The egg shell strength was measured with a Canadian Egg Shell Tester as compressive fracture force (i.e. breaking force); that is the power needed to crack the shell. The specific gravity is assessed by Archimedes' principle. Specific gravity measurement reflects the amount of cal-

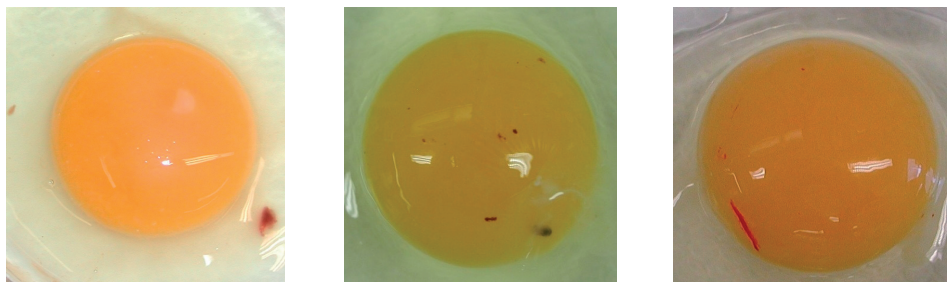

Photo: Mervi Honkatukia

Figure 3. Appearance of different size of blood and meat inclusions in eggs.

cium carbonate deposited per egg. In the WR x RIR population, egg quality traits were measured at 35, 40 and 50 weeks of age. This time the breaking force was analyzed together with deformation with the Canadian Egg Shell tester. Deformation illustrates the elasticity or stiffness of the egg shell. In addition, egg shell weight was measured once at 42 weeks of age. Detailed description of egg shell quality traits together with production traits in the RIR x WL is provided in (I) and in the WR x RIR in Tuiskula-Haavisto et al. (Tuiskula-Haavisto et al. 2010).

2.2.4 Organoleptic observation of taint

To get organoleptic observations of egg taint, a challenge test was needed. The WR x RIR F_2 hens were fed with feed containing 10% rapeseed meal for 2 weeks at 50-54 weeks of age. The taint was scored by olfactory tests for 3 consecutive days. See the phenotyping details in (III). In the confirmation study, egg tainting was also scored by chemically analyzing the TMA-N content in egg yolks (Kretzschmar et al., 2007). A summary of quality traits scored across the populations is presented in Table 2.

A comprehensive description of all production traits recorded in the RIR x WL F_2 population is provided in [I]. Detailed information on shell quality traits in the WR x RIR F_2 population is in Tuiskula-Haavisto et al. (Tuiskula-Haavisto et al., 2010). Recording and transforming the data for internal inclusions in various populations is explained in [IV].

2.3 DNA extraction

Genomic DNA was purified from whole blood by phenol-chloroform extraction followed by ethanol precipitation. To map the egg taint disorder, DNA pools were used to identify differences in allele frequencies between the phenotype groups. The pools were formed within five segre-

Table 2. Egg quality traits evaluated in various populations. Time of evaluation is indicated as weeks of age (in the experimental populations). Other populations used to study the traits are shown also.

Trait	RIR x WL	WR x RIR	Other populations	Published
Albumen quality	36-40	40		I, II,Honkatukia et al. 2010b
	56-60			
Blood and meats spots (BMS)		35, 40, 50[1]	Hy-Line, Lohmann Brown	IV
Egg taint/organoleptic	54	50,59	German F_2[2]	III, Kretzschmar et al. 2007
Egg shell strength	36-40	35,40, 50	Hy-Line	I, Tuiskula-Haavisto et al. 2010
	56-60			
Specific gravity	36-40	NA		I
	56-60			
Deformation	NA	35,40,50,70		Tuiskula-Haavisto 2010 et al.
Shell weight (SW)	NA	42		Tuiskula-Haavisto 2010 et al.

[1]Blood and meat spots as one trait (BMS)
[2]TMA content determined chemically

gating families. Two pools were formed within sire families, each containing 20 hens from the phenotypic extremes. The pooling was carried out by adding 20 µl of whole blood from each hen. Thereafter, the DNA from pooled blood samples was extracted with the same procedure as from individual samples.

2.4 RNA analysis

For analysis of *FMO3* gene expression, liver samples were collected from three hens of each of the three genotype groups of the T329S mutation. Samples were stored in an RNA Later buffer in -20°C. Total RNA was extracted and RNA preparations were stored at -80°C. RT–PCR amplification was carried out using a specific kit (ImPromII Reverse Transcription System kit, Promega). Gene expression levels were examined to detect possible differences between the three genotypes and analyzed by quantitative PCR (qPCR) using the relative quantification method (Livak and Schmittgen, 2001) with ABI Prism 7000 Sequence Detection System (Applied Biosystems). A specific summary of RNA analyses is given in (III).

2.5 Genomic tool kit

2.5.1 Microsatellite marker maps

Microsatellite markers were chosen from published chicken maps (Compton, East Lansing and Wageningen, or later from the consensus chicken map http://www.thearkdb.org/arkdb/). Genotyping was done using standard protocols based on PCR fragment length separation in gel matrix (A.L.F. and A.L.F Express) or capillary electrophoresis, MegaBace 1000 (GE Healthcare). Allele size determination was performed with Fragment profiler software. Genotyping and map construction is described in detail in [I] and [II].

2.5.2 Screening polymorphisms in candidate genes

Three candidate genes, chosen based on their location and possible role in the phenotypic variation in different aspects of internal egg quality, were studied at the sequence level. The sequenced genes were *vimentin* for albumen quality (II), *FMO3* for the egg-taint defect (III), and *ZO-2* (IV) for blood and meat spots. The screening was done both to identify sequence variations that could explain the observed QTL effect and to find potential genetic markers to fine map the QTL area. Primers corresponding to candidate genes were designed based on available sequences in existing databases (GenBank™/EMBL, http://www.ncbi.nlm.nih.gov/genbank/, http://www.ensembl.org/Gallus_gallus/Info/Index). Primers were designed with Primer3 (http://frodo.wi.mit.edu/primer3/). Amplified PCR fragments were sequenced either with gel or capillary electrophoresis. DNA sequence analyses were performed with Sequencher software (Gene Code Corporation).

2.5.3 Scoring SNPs

Variations detected by sequencing were used as markers in linkage analyses. The SNPs in *vimentin* (II), *FMO3* (III) and *ZO-2* (IV) were scored using minisequencing genotyping (SNuPE,GE Healtcare). In this approach, the SNP region of interest is amplified within a PCR fragment, which is in turn used as a template in a thermally cycled minisequencing reaction with a sequence specific primer ending one base before the polymorphic base. This 'Snupe' protocol is explained in (II).

SNPs were also analyzed in a multiplex of 384 loci with the Illumina BeadXpress platform (IV). Genotyping was performed with BeadStudio software (Illumina). Only handful of SNPs in the OPA set were from the chromosome Z and thus included in this study. Two novel varia-

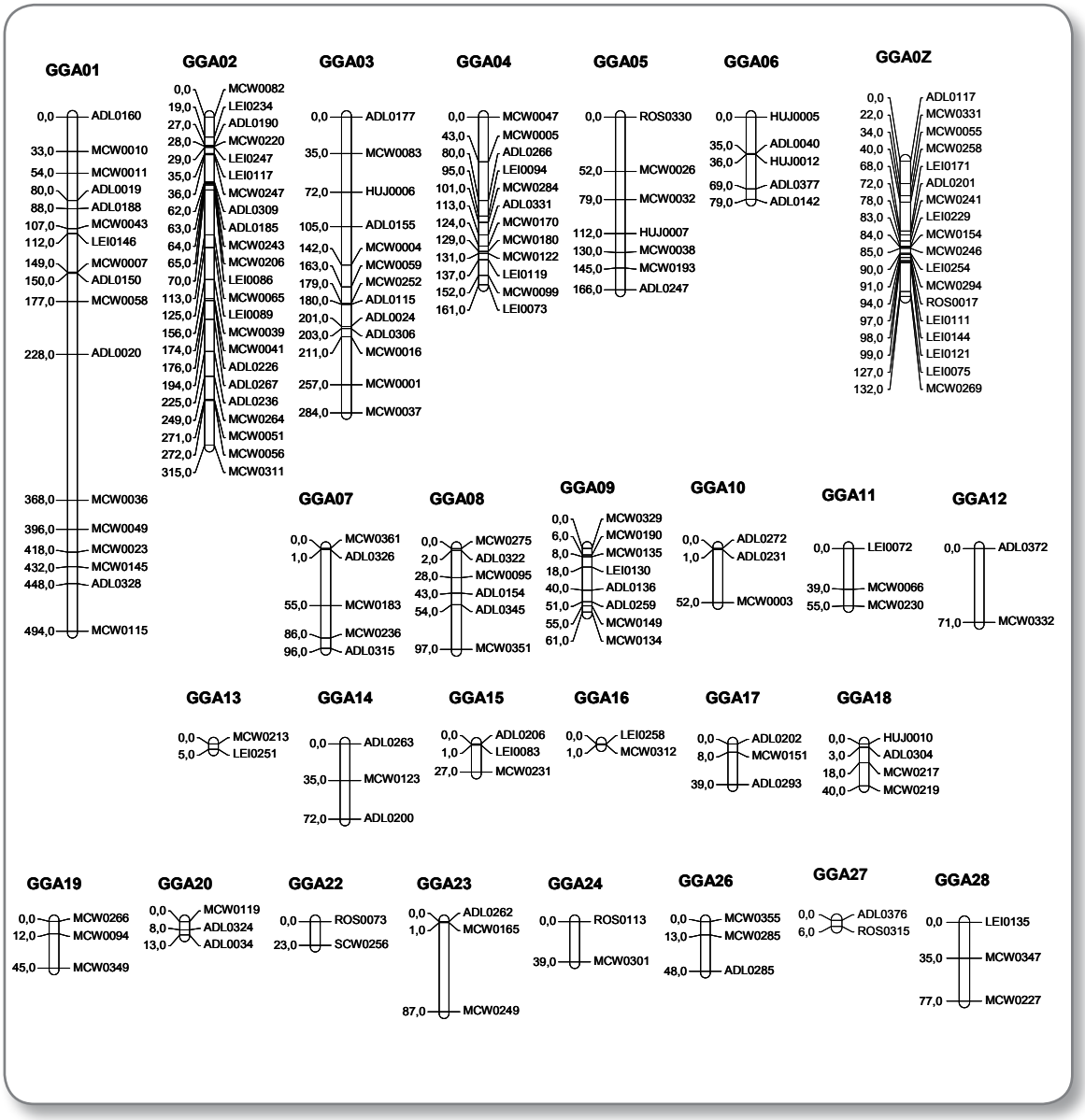

Figure 4. Genetic linkage maps of WR x RIR population. The positions are in cM based on Haldane's mapping function.

tions were detected in the miRNA *gga-mir-1556* when sequencing the candidate gene *ZO-2*. One of these two SNPs was included in linkage analysis as a marker. The miRNA genotypes were scored by sequencing a PCR fragment and analyzed with Sequencher software (Gene Code Corporation).

2.5.4 Feather lysis protocol

A fast gene test for identifying the genotype at the mutation site responsible for tainting was developed. The method was designed for analysis from single feather. In this protocol, a short pre-lysis treatment without DNA extraction was followed by minisequencing. The single feather lysis

method was used later with other genotyping applications to replace time consuming DNA extraction. In short, a single shaft of 1-2 mm is incubated 60 minutes in 55°C and 10 min 98°C in lysis mixture of Mg^{2+}-free PCR-buffer, Proteinase K and H_20. This simple lysis treatment provides enough DNA to low scale DNA application, ideal for screening a few loci per individual. Nonetheless, feather lysis was also used in SNP scoring with BeadXpress (OPAset) (Illumina) with reasonable success (approximately 50%).

2.5.5 Map construction

Linkage analysis of both microsatellite and SNP markers was performed using the CRI-MAP software, version 2.4 (Green et al., 1990) described in (I and II). First, all pairwise combinations of markers were checked (TWOPOINT). Then the order of markers within the linkage group was determined (BUILD). For uncovering possible optional orders, current order of adjacent markers was compared to optional order (FLIPS). Multiple recombination events were examined to detect possible genotyping errors (CHROMPIC). In general, the linkage maps constructed were consistent with the map order of the consensus map (Groenen et al., 2000), with a few exceptions. The 99 microsatellite markers in the RIR x WL population covered 13 linkage groups with a total length of 2311 cM. The mapped chromosomes were 1-11, 13 and Z. In the WR x RIR population, the 162 microsatellite markers covered 27 chromosomes and 2585 cM. The mapped chromosomes were 1-20, 22-24, 26-28, Z. A linkage map of the WR x RIR F_2 is shown in Figure 4.

2.6 Statistical analysis

2.6.1 QTL linkage analysis

QTL analyses were performed using linear regression with the line cross model (I, II, IV) (Haley et al., 1994). The whole-genome scan in the RIR x WL was the first genome scan done in layers. The analysis was carried out with a custom made regression program (I). The marker alleles of F_2 individuals were traced back to the grandparental origin of Rhode Island Red or White Leghorn. A general assumption of the line cross model is that the founder lines are fixed for the alternative alleles of the QTL. With this approach the approximation of QTL position and effects, both additive and dominant, could be estimated.

Fine-mapping of the two F_2 populations in (II) and (IV) was performed with a web-based linear regression application, GridQTL (formerly QTL Express) (http://www.gridqtl.org.uk/) (Seaton et al., 2002). For the two QTL model, an F-test was used to determine whether the best QTL pair explained significantly more of the variation than the best single QTL from the pair.

A custom-made program (multimarker regression) analysis of the sex chromosome in the line cross model by Knott (Knott et al., 1998) was used to analyze the additive effects of the QTL in the sex chromosome Z and to bootstrap the confidence intervals for the QTL (Visscher et al., 1996).

Significance thresholds for QTL were obtained empirically through permutation tests (Churchill and Doerge, 1994; Lander and Kruglyak, 1995). The impact of multiple testing was taken account by following de Koning's (de Koning et al., 2000) application of Bonferroni's correction in the significance levels: $P_{genome\text{-}wide}=1-(1-P_{chromosome\text{-}wise})^{1/r}$, where r is chromosome length/ genome length.

2.6.2 Segregation analysis

The QTL analysis was nested within half-sib families, whereas the segregation and association analyses were conducted across

the populations. The segregation analysis approach was used to map the egg taint defect in five segregating families of the WR x RIR F2 population (III) at genome-wide level. Two pools of DNA from phenotypic extremes within sire families were used to estimate the sire allele frequency distributions. The pools were genotyped for 119 microsatellite loci covering 25 linkage groups. A skewed allele distribution was taken as an indication of an association to be further analysis. All five families were genotyped for four microsatellites locating in the area. The linkage analysis was performed with FASTLINK, version 4.1P, under the assumption that the defect was autosomal recessive (with incomplete penetrance) (http://www.ncbi.nlm.nih.gov/CBBresearch/Schaffer/fastlink.html) (Cottingham et al., 1993; Schaffer et al., 1994). Options used for linkage analysis were MLINK for two-point analysis and ILINK for multipoint analysis. The disease status of all males and individuals without phenotype observations were coded as unknown.

2.6.3 Association analysis

For the purpose of confirming markers associated with internal inclusions, association analysis was used in two independent commercial lines (IV). Analyses were conducted separately for each marker and were focused on one microsatellite marker and different sets of SNP markers, depending on the information content and the population. Depending on the trait tested, either T-test, Wilcoxon Rank Sum or Fisher's exact test was implemented to find the association between phenotypic traits and markers. Analyses were carried out with SAS.

3 Results and discussion

3.1 Egg white quality (I, II)

Two experimental F_2 mapping populations RIR x WL and WR x RIR were established to identify quantitative trait loci (QTL) affecting egg quality. Both populations were examined across the genome with different sets of microsatellite markers (99 and 162, respectively). The marker coverage was not optimal for either of the marker sets: many large gaps remained and also several microchromosomes were uncovered. Several QTL regions affecting egg quality were identified by the genome scans. In the RIR x WL population, a total of 14 genome-wide significant QTL areas were detected, six of which were associated with egg quality traits: early albumen quality (HU40) on chromosomes 2, 4 and 8, and late albumen quality (HU60) on chromosome 2 (shown in Table 3); genome-wide significant QTL for egg shell quality were found on chromosome 5 for early specific gravity (SG40) and on chromosome Z for early egg shell strength (ES40) (I).

The focus of the first mapping project was on egg quality. The most interesting area affecting albumen quality was on chromosome 2, which was subsequently fine-mapped (II). The resolution of the area was improved with a denser microsatellite marker map. The average marker spacing in the initial scan was 27 cM. Adding markers decreased the overall marker spacing to 19 cM and to 15 cM in the QTL area. The intention was to cover the area with a denser map, but many of the tested markers had to be discarded due to uninformativity. Because the chicken genome was not yet available, there were not many genes known in that area. The candidate gene to study within the QTL region was selected on the basis of its possible role in maintaining the firm structure of albumen. Albumen consists of a protein complex: most of the proteins are in soluble form, except for ovomucin, which has a fibrous structure. Also, the differences between protein composition of thin and thick albumen is primary in their ovomucin content: thick albumen contains four times more ovomucin (Li-Chan et al., 1995). The *vimentin* belongs to a gene family coding for intermediate filaments, which function to control the cell shape and integrity of the cytoplasm (Zehner et al., 1987). The candidate gene was partially sequenced from a few hens with extreme Haugh phenotypes. More than 20 variations were detected in the sequenced regions of vimentin (details given in II). One of these SNPs, an intronic variation, was mapped to the linkage map and used as a marker in QTL analysis.

The possible existence of multiple QTL within chromosome 2 was studied in subsequent analyses. Fitting two QTL simultaneously indicated two distinct QTL for the early albumen quality (HU40) within the marker brackets MCW82-MCW220 and MCW206-MCW65. In addition, a novel QTL effect on late albumen quality (HU60) was detected co-locating with early albumen QTL between markers MCW206-MCW65. In the previous genome scan no strong evidence for a QTL affecting the egg white thinning in the later laying period (HU60) was found. Because both early and late albumen quality showed association at the same place (MCW206-MCW65), this area seems to contribute to the albumen quality throughout the laying period. The QTL explained a moderate portion (up to 6.7 %) of the phenotypic variance. The RIR allele effects were negative, degrading the height of albumen. The result was coherent with known line characteristics. Also, the RIR allele was found to negative-

Table 3. Genome-wide suggestive (10 %) QTL for egg quality traits found in both mapping populations.

Chr.	Trait	F-ratio	Flanking markers	Mapping population
1	Internal inclusions	6,18	MCW23-MCW145	WR x RIR
1	Deformation (35wk)	6,91	MCW23-MCW145	WR x RIR
2	Internal inclusions	5,32	MCW82	WR x RIR
2	Early albumen quality	10,66	MCW247-ADL217	WL x RIR
2	Late albumen quality	8,16	MCW247-ADL217	WL x RIR
2	Shell weight	9,48	ADL267-ADL236	WR x RIR
2	Deformation (35wk)	13,67	ADL236-MCW264	WR x RIR
2	Deformation (40 wk)	8,18	ADL236-MCW264	WR x RIR
2	Deformation (50 wk)	6,77	ADL236-MCW264	WR x RIR
3	Deformation (50 wk)	6,84	ADL24-ADL306	WR x RIR
3	Breaking force (70 wk)	4,98	ADL115-ADL24	WR x RIR
3	Breaking force (50 wk)	5,41	ADL306-MCW16	WR x RIR
4	Internal Inclusions	5,66	ADL331	WR x RIR
4	Early albumen quality	6,57	MCW180-MCW129	WL x RIR
4	Early albumen quality	5,49	MCW122	WR x RIR
5	Early specific gravity	6,20	MCW32-HUJ7	WL x RIR
5	Egg taint	5,16	MCW32-HUJ7	WR x RIR
6	Deformation (40 wk)	8,22	HUJ12-ADL377	WR X RIR
7	Early albumen quality	8,07	MCW183-MCW236	WR X RIR
8	Egg taint	14,35	ADL322	WR X RIR
8	Early albumen quality	5,90	ADL322-MCW95	WL x RIR
10	Deformation (50 wk)	5,12	ADL272	WR x RIR
12	Shell weight	6,59	ADL372-MCW332	WR x RIR
12	Deformation (50 wk)	6,8	ADL372-MCW332	WR x RIR
12	Breaking force (70 wk)	4,91	ADL372-MCW332	WR x RIR
14	Breaking force (70 wk)	4,97	ADL2263-MCW123	WR x RIR
14	Breaking force (50 wk)	5,0	MCW123-ADL200	WR x RIR
15	Deformation (40 wk)	5,69	LEI83-MCW231	WR x RIR
20	Early albumen quality	7,04	ADL34	WR x RIR
24	Shell weight	7,22	ROS113-MCW301	WR x RIR
28	Early albumen quality	6,09	LEI135-MCW347	WR x RIR
Z	Early egg shell strength	14,33	MCW246-MCW128	WL x RIR
Z	Early albumen quality	47,9	MCW258-LEI171	WR x RIR
Z	Internal inclusions	18,59	MCW258-MCW241	WR x RIR
Z	Shell weight	14,36	LEI144	WR x RIR

ly affect the egg weight QTL at the same chromosomal area, which was against the presuppositions.

The dominance effect on egg white thinning may also indicate mixed effects of two (closely) linked QTL instead of a sin-

gle over-dominant effect. However, dominance has been reported to have a significant role in egg white quality (Poggenpoel, 1986). Thus the genetic mechanism underlying the detected QTL is unsolved.

The effect of storage on egg albumen is well-known (reviewed by Roberts, 2010). Albumen height decreases quicker at higher temperatures. One reason for albumen deterioration is an increase in the pH. Storage may also have a negative impact on the quality of the vitelline membrane, predisposing the yolk susceptible to rupture (Li-Chan and Shuryo, 1989). One goal of this study was to identify loci affecting differences in egg quality during long time storage of eggs. However, no QTL affecting albumen quality during storage was found.

For traditional breeding, selection of albumen quality using phenotypic observations by Haugh-evaluation has been a viable solution. The trait has relative high heritability (0.23) and genetic progress up to 0.8 Haugh units in less than ten years has been gained (Nys, 2009). But because it is a function of two parameters of the egg – albumen height and egg weight – it is not the ideal phenotype for QTL mapping. The model used for calculating Haugh-value might pose some bias, especially for heavy eggs of old hens. Some of the QTL affecting egg white quality were found to co-locate with egg weight QTL (chromosomes 2, 4). In the WR x RIR F_2 no QTL for albumen quality or egg weight were found in the herein described area of chromosome 2. Instead, suggestive egg weight and shell weight QTL were located elsewhere on chromosome 2, at the positions 188 and 199 cM (respectively).

3.2 Internal inclusions (IV)

The WR x RIR population was also used to study internal egg quality traits, including the occurrence of blood and meat spots in eggs. Phenotypic observations of blood and meat spots were collected at 35, 40 and 50 weeks of age. In brief, conventional linkage analysis of 27 chromosomes with 162 microsatellite markers in a subpopulation of 668 F_2 hens revealed one genome-wide significant region affecting blood spot and meat spot frequency. The most likely position for the QTL was found on the Z chromosome (F=18.59, between markers MCW258 and MCW241). Other suggestive (at 10% genome-wide significance) QTL were found on chromosomes 1, 2 and 4. For fine-mapping of chromosome Z, 1599 F_2 hens were screened with a set of microsatellite markers and SNPs in the QTL area. By fine-mapping the significance of the QTL increased (F=32.9), and the location was pinpointed to the marker position MCW241. In fact, the microsatellite marker MCW241 was found to be located within an intron of the *tight junction protein 2* gene *TJP2* – also known as *ZO-2* (Figure 5). *ZO-2,* that controls epithelial cell adhesion, was partially sequenced as a potential candidate gene. Nine single nucleotide polymorphisms were detected. Three of the variations were located in the exon 18. Among the variations, two novel SNPs were detected in a microRNA sequence, gga-mir-1556, located within the ZO-2 gene. One of the miRNA-SNPs was used in the association study jointly with one exonic SNP. Both of them showed significant association to both blood and meat spots.

To further verify the existence of the QTL observed in the genome scan, association analyses were carried out in two independent commercial breeding lines with the microsatellite marker MCW241 and surrounding SNPs. The markers covered an area of 9.14 Mb (between 31.955.874-41.098.862 Mb). The association varied according to the studied population located most probably in a 3 Mb wide zone between 33.508.907 and 36.533.455 Mb. The list of genes in the QTL area is provided in appendix I. There was thus good

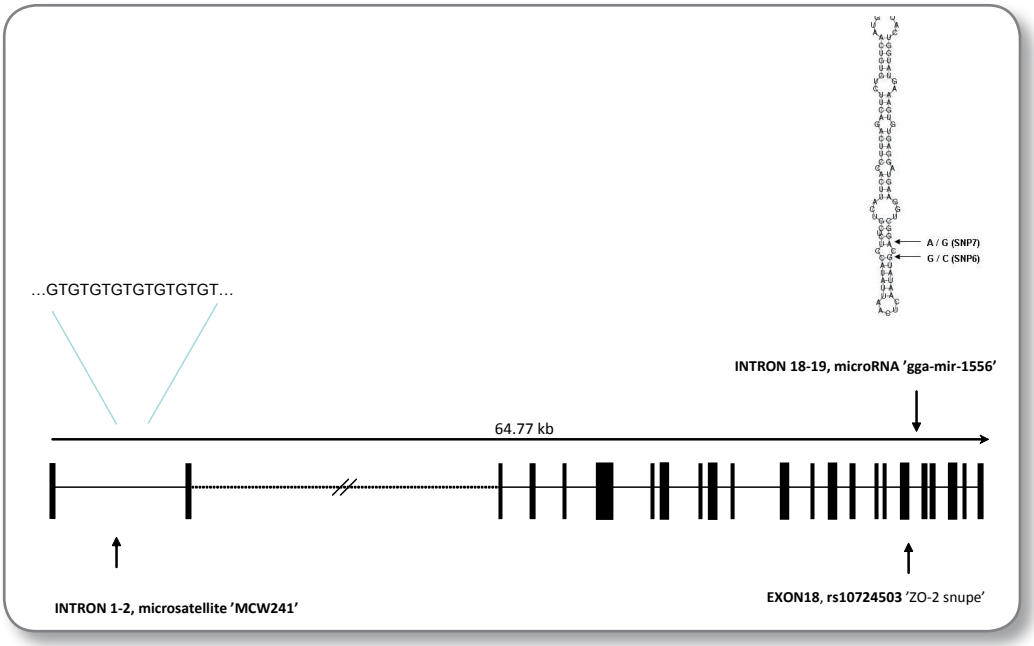

Figure 5. Schematic presentation of the ZO-2 gene in chicken. Locations of microsatellite MCW241, 'ZO-2 Snupe' and gga-mir-1556 are represented.

agreement between the location of the QTL region on chromosome Z and the association results in the commercial breeds analyzed. The effect was relatively small, explaining 2 % of the phenotypic variance.

A variation in the tight junction gene *ZO-2* or microRNA gga-mir-1556 may have an influence on susceptibility to internal inclusions. It remains unknown whether there is a relationship between the host gene *ZO-2* and the miRNA. Some reports in the literature are suggesting that a majority of miRNAs are transcriptionally linked to their host gene expression (Lutter et al., 2010). Functional roles of both the *ZO-2* gene and the miRNA are interesting. *ZO-2* is known to be involved in a human genetic defect (familial hypercholanemia, FHAC, OMIM 607748), the symptoms of which are (blood) clotting and bleeding (Carlton et al., 2003). The disease causes imbalance among the fat-soluble vitamins (A, D, E, K) and turns the metabolism system off the rails with possible severe consequences.

A bioinformatics tool, miRDB, was used to search miRNA target genes from databases (http://mirdb.org/). The search gave 80 target genes for gga-mir-1556. Among these genes are some interesting genes and genomic regions. For instance, one hit was in chromosome 1, nearby the area where one suggestive blood and meat spot QTL was located. Another exciting hit was in the *AGT* gene on chromosome 3. *AGT, angiotensinogen*, is a precursor of *angiotensin*, which increases blood pressure. It has been demonstrated earlier by Fry (Fry et al., 1968) that blood pressure is a factor in susceptibility to blood spot incidence.

The incidence of internal inclusions is less than 1 % in all eggs. However, the frequency in brown eggs could be as much as 18 %, or even higher. Different types of factors, including nutritional, environmental and hereditary factors, catalyze the

incidence of spots. Heritability estimates vary between 0.07 and 0.6 (Becker and Bearse, 1973). Nutritional aspects – alongside the vulnerability of certain genotypes – are very important factors affecting the quantity of the defect. Brown breeds are known to be susceptible: the frequency of spots has remained high compared to white breeds because of difficulties in detecting inclusions by mass candling due to dark shell color and subsequent failure to select it away.

The phenotype determination has been quite challenging with internal inclusion: evaluation was not similar between populations, although it was sufficient to be able to unveil the fine-mapped chromosome region across populations. On average, the detected associations are more or less shared by both types of the spots, with a few exceptions.

This was a first report of QTL affecting incidence of blood and meat spots in chicken eggs. The result was validated in two independent commercial lines – the association of markers to the trait supported the location of the QTL. Whilst the actual causative variation for the blood and meat spots remains uncertain, the LD between the trait and associated markers might be at an acceptable level across populations for marker assisted selection. Before use in selection, possible pleiotropic effects of the loci/associated markers to the other important traits should be studied. Actually, it seems that the same chromosome region has overlapping QTL for albumen quality (Honkatukia et al., 2010b).

3.3 Detecting the cause for fishy taint (III)

In humans, the earliest references of fish malodor can be found in anecdotal history, from Indian epics (1000 B.C.E) or in Shakespeares' texts (Mitchell et al., 1996; Mitchell SC, 2001). It was not until the 1990's that the condition was unveiled to be an autosomal recessive disorder, trimethylaminuria TMAU (Mitchell, 1996) (OMIM 602079) caused by mutation in the *flavin containing monooxygenase 3* gene. In poultry, eggs with a fishy odor have also been known to exist for a relatively long time. The relationship between an excess of trimethylamine (TMA) and fishy taint in eggs was recognized in the 1970's (Hobson-Frohock et al., 1973); the condition was assumed to be hereditary (Pearson et al., 1979). Furthermore, off-flavor in milk in cows was identified to be caused by a mutation in the *FMO3* gene (Lunden et al., 2002a; Lunden et al., 2002b). It has since been reported that *FMO3* polymorphisms affect pork pH and possibly also off-flavor in pigs (Glenn et al., 2007).

The egg tainting may be observed only under certain feeding regimes, which overload the hen with precursors of TMA (for example choline, lecithin or carnitine). Rapeseed meal may induce the problem also by a direct effect on the FMO3 enzyme. In order to induce the egg taint, the WR x RIR F_2 population was fed rapeseed meal for a period of two weeks. Five F_2 sire families were found to segregate for taint after the rapeseed challenge. Pooled DNA analyses across the whole genome (within the sire families) pinpointed few chromosomal areas as a candidate sites (chromosomes 4, 7, 8 and 26). Only the chromosome 8 region showed consisted results for adjacent markers within families. All five segregating families were genotyped for four microsatellite markers (MCW275, ADL322, LEI179, MCW305) and two polymorphisms in the *FMO3* gene on chromosome 8. Linkage analysis showed that *FMO3* maps to the distal end of chromosome 8.

3.3.1 Polymorphisms in *FMO3*

The entire coding sequence and partial intron sequences of the *FMO3* were sequenced from several individuals (tainting and non-tainting). In all, 17 polymorphic

DNA positions were found between tainting and non-tainting hens (Table 4). Nine of these variations were found in the coding regions; only one polymorphism led to an amino acid substitution (T329S). This identified amino acid change occurred in a very conserved amino acid motif containing the *FMO*-characteristic pentapeptide 'FATGY' in mammals and 'TG' in all lineages since archaeal divergence, (Ambrosi et al., 2000; Lattard et al., 2001; Stehr et al., 1998) (see Figure 6.). The functional property of this region has been proposed to be a substrate recognition pocket, common to mammalian flavin containing monooxygenases. Relevance of the evolutionary conservation and the postulated function of the motif suggested that the amino acid change itself may lead to dysfunction in the chicken's *FMO3* gene, thus leading to accumulation of TMA in the egg yolk in individuals homozygous for the mutations.

Table 4. Polymorphic sites found in the chicken FMO3 gene (Q8QH01_CHICK) by sequencing from 4 to 10 indivuals of the half-sib mapping population.The SNPs # 18-21 are additional variations found by sequencing 48 individuals of three commercial brown egg layer populations (gene region exon 5-exon 8). The sequences and SNPs are derived from the forward strand.

#SNP	Genomic position	Location	Flanking sequence of the SNP (forward strand)	Wild-type chromosome	"Tainting" chromosome
1	4,959,936	97 nt of exon 2	GCCCACCTG[Y]TTTGAGCAGAG	C	T
2	4,958,085	93 nt of exon 3	TCCCCTTCCC[Y]GATGACTACC	C	T
18	4,956,602	86 of exon 5	TGCTGGTGGT[Y]GGCTTGGGCA	T or C	T
3	4,956,590	98 nt of exon 5	GCTTGGGCAA[Y]TCYGGCTGTG	T or C	C
4	4,956,587	101 nt of exon 5	TGGGCAAYTC[Y]GGCTGTGACA	T or C	C
19	4,956,451	12 nt of exon 6	TTTACCTGAG[Y]TCCCGAAGAG	T or C	C
5	4,956,439	24 nt of exon 6	CCCGAAGAGG[Y]TCCTGGGTGA	C	T
20	4,956,292	171 nt of exon 6	AGTACAAACA[Y]GAGGATTTTG	T or C	C
212	4,956,133	19 nt of exon 7	GCAGGGAACC[R]GTGTTGAATG	G or A	G
6	4,956,073	79 nt of exon 7	TAAAGCCAAA[Y]GTGAAGGAAT	C	T
71	4,955,994	158 nt of exon 7	TATCTTTGCC[W]CTGGTTACTC	A	T
8	4,954,260	59 nt of exon 9	TGAGCTGACC[Y]CAGCCATCGG	T	C
9	4,954,170	149 nt of exon 9	CTGCACCCCC[Y]ACCAGTTCCG	T	C
10	4,957,482	Intron 4	TGATTCAGCC[R]TCACACAGCC	G	A
11	4,956,504	Intron 5	GCTGATGGCC[R]CCACRASGGT	A or G	G
12	4,956,499	Intron 5	TGGCCRCCAC[R]ASGGTGGCTC	A or G	G
13	4,956,497	Intron 5	GCCRCCACRA[S]GGTGGCTCAA	G	C
14	4,956,202	Intron 6	GGGTGGTATC[R]AGCCTGTACA	G or A	A
15	4,956,178	Intron 6	CCAGCAGCAG[S]AGCCAACTTA	G or C	C
16	4,955,311	Intron 8	GTAGGTGCAG[Y]TCATGGGACG	T	C
17	4,955,119	Intron 8	ACCCAGCTTC[Y]TTGGTGTCAG	C	T

[1]SNP7 in exon 7 amino acid T ->S
[2]SNP21 corresponds rs15901207

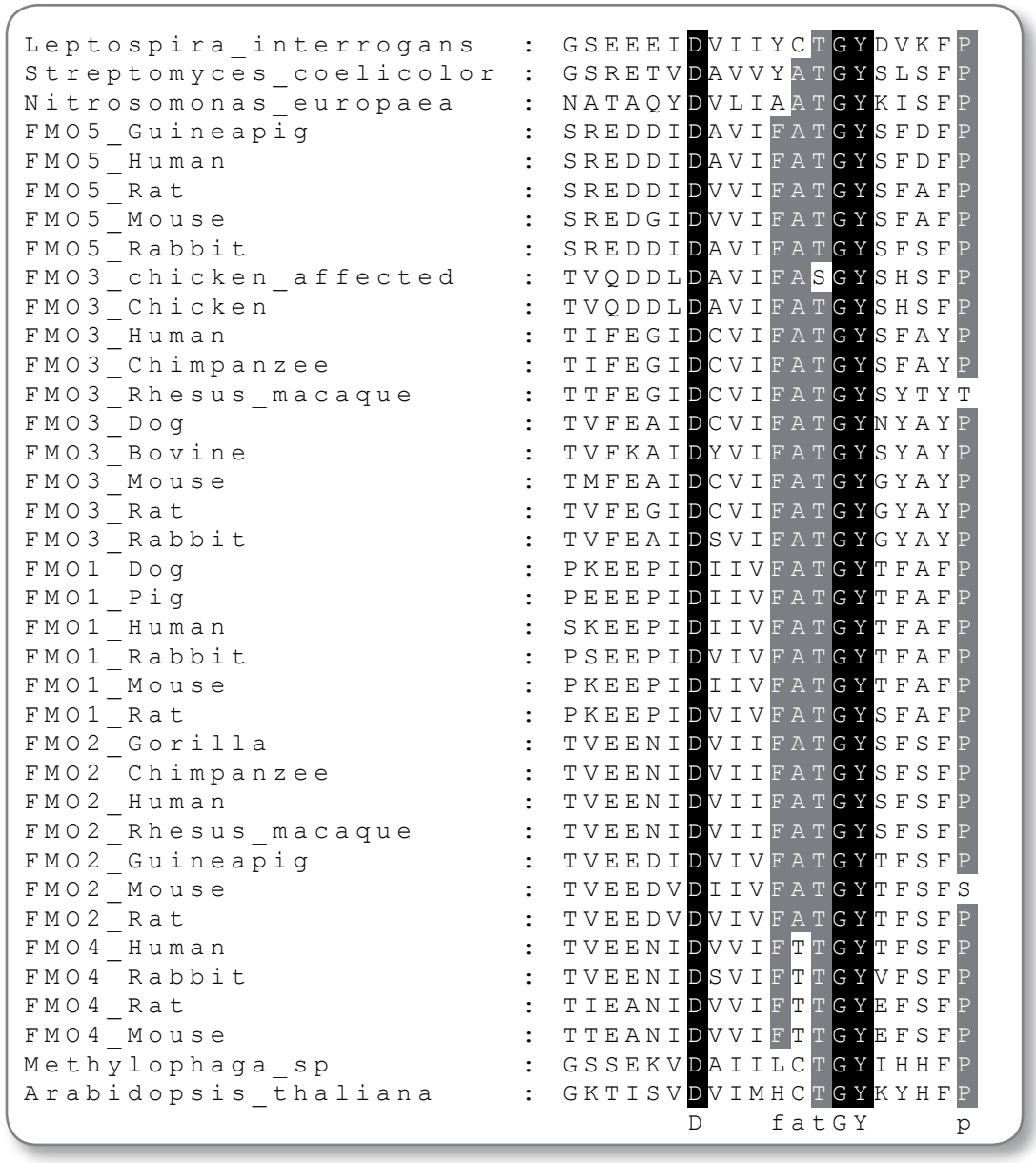

Figure 6. Multiple alignment of protein sequences related to the chicken FMO3 gene (Q8QH01_CHICK) for the region of the T329S mutation in the 'FATGY' domain. The chicken sequence containing the mutation T329S in indicated as 'affected' (Figure from III).

3.3.2 Confirming the association of *FMO3* genotype with TMA content

In order to confirm the tainting phenotype, trimethylamine content was chemically determined from a German F_2 (Kretzschmar et al., 2007). A group of 169 F_2 individuals were challenged with choline included in the feed. TMA was extracted from egg yolk and measured pho-

tometrically. Two groups were formed according to the determined TMA-content: hens in the low-group had less than 2.1 µg TMA-N/g of yolk, while hens in the high-group had more than 5.6 µg TMA-N/g. Before the choline challenge all hens produced eggs with low TMA content. Genotyping the SNP revealed that all SS-homozygotes belonged to the high-group, while heterozygotes TS and homozygotes TT expressed low TMA-N content in egg yolk. Three commercial brown layer lines with different genetic backgrounds were also tested; the same association was found between the elevated TMA-levels and *FMO3* genotypes (Figure 7.).

3.3.3 Origin of fishy taint in brown egg layers

Some additional studies on the origin of fishy taint were done. A fragment of 2690 bp extending in both directions from the mutation site was sequenced from 9 chicken breeds or lines. The tainting mutation was found to be common in various brown breeds; it was found in the ISA Brown, the TETRA, the Lohmann Brown, the Transylvanian naked-neck, the Green-legged partridge, and the Marans (Table 5). A phylogeny of the most common haplotypes shows grouping of all the haplotypes containing the amino acid S variant (Figure 8). A similar haplotype of *FMO3* was identified in all three commercial brown lines, indicating a common origin for the taint in present-day commercial brown layers. The haplotype phylogeny also suggests multiple origins for domestic chickens, which is supported by the recent publications of chicken domestication (Eriksson et al., 2008). Possible pleiotropic effects of tainting mutation were studied by the breeding company. No associations were found to any important production traits in the breeding lines (Lohmann Tierzucht, personal communication).

3.3.4 A gene test and marker assisted selection

A simple and fast method for identifying carriers and possible tainters from feather samples was developed. This method provides a selection tool for unveiling the heterozygous carriers (and homozygotes) with the SNP marker without a feeding challenge or an organoleptic evaluation. This gene test has been used to eradicate the defect from one of the parental lines

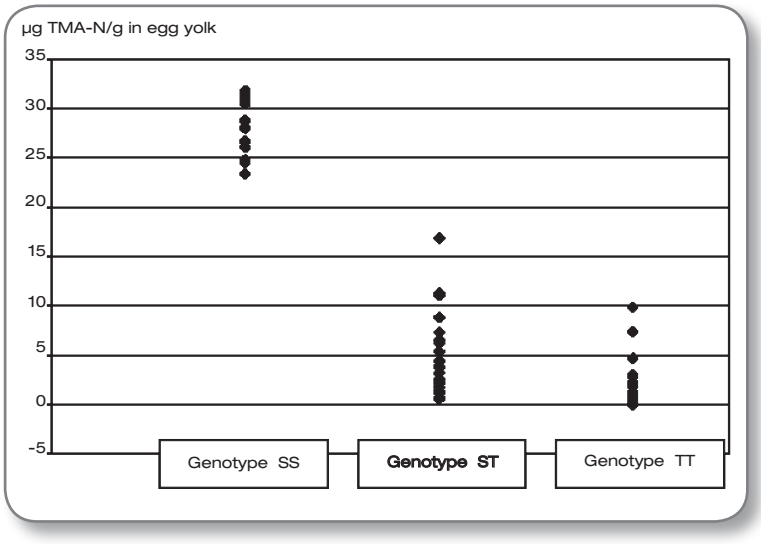

Figure 7. TMA levels in egg yolk (µg TMA-N/g). The T329S allelic state is associated with TMA-levels in commercial brown breed with different genetic backgrounds (from three different breeding companies). Genotypes are representing the tainting status: TT =normal, TS=heterozygote carrier, SS= tainting homozygote.

Table 5. Frequency of tainting variant (S) in various brown breeds. The variant S is common in various brown breeds. N=number of studied individuals. T=normal, non-tainting.

Breed	N	T	S
Gallus gallus gallus	10	1.00	0.00
Fayomi	10	1.00	0.00
Green-legged partridge	8	0.81	0.19
Iceland landrace	10	1.00	0.00
Isa Brown	71	0.58	0.42
Lohmann Brown	67	0.62	0.38
Marans	111	0.96	0.04
TETRA	70	0.61	0.39
Transsylvanian nakedneck	8	0.69	0.31

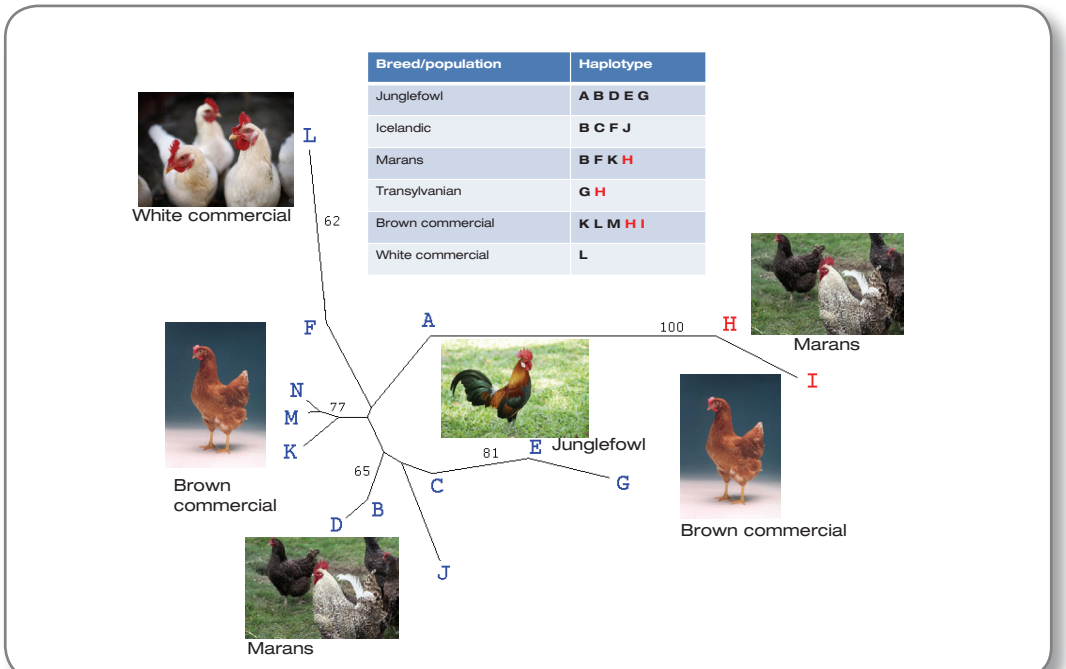

Figure 8. Tainting haplotypes (H and I; marked in red) in different chicken populations. 66 individuals from 10 populations (AVIANDIV, commercial brown and WR x RIR population) were included in the analysis. Haplotypes were determinate with PHASE.
Photos: White commercial, Marans and Junglefowl: iStockphoto LP 2009. Brown layer: Lohmann Tierzuch GmbH.

of the Lohmann Brown breeding program, namely, to eliminate the occurrence of homozygous tainters in the commercial hybrid. A patent for the application has been allocated by The European Patent Office (Marker assisted selection of chicken against fishy taint, European Patent EP1518936).

4 Conclusions

Large scale analyses to identify genomic regions associated with different internal egg quality traits were conducted. The populations used in this study were based on commercial lines. Thus the QTL found are relevant in practice. This was nicely proven with the case of blood and meat spots, where the association could be confirmed successfully in two independent commercial lines. In addition, the extensive size of the WR x RIR F_2 and the wide spectrum of the phenotypes collected from the population have provided a good foundation for detecting QTL.

Several QTL regions were detected. The confidence interval of the albumen quality QTL remained wide, spanning tens of centiMorgans. To date, none of the 15 QTL detected from various studies have contrived to identify genes affecting to albumen quality. In fact, the current understanding of the genetic architecture of the complex traits is more or less converging with the infinitesimal model, which assumes that very large (infinite) numbers of loci with small effects are underlying the complex traits (Goddard and Hayes, 2009).

For blood and meat inclusions a putative candidate gene was detected: miRNA ggamir-1556 and/or its host gene *ZO-2* might have a role in susceptibility to blood and meat spots across populations. Further studies to confirm the role of these genes are needed, although the confirmation study showed some evidence of possible LD, which might be sufficient to select against the incidence of internal inclusions.

Quite often the fine-mapping of wide QTL regions have failed to detect the actual gene or variation underlying the phenotype. In many cases, despite the very best attempts, there are not enough recombinations which would narrow down the area. Sometimes it might be difficult to choose any putative candidate genes in the QTL area. Regulatory mutations are more likely to cause the phenotypic variations. So, even if the gene could be considered as a candidate, it might be difficult to predict the effect of putative regulatory mutations to the phenotype (Georges, 2007; Wray, 2007).

In one case, mapping successfully led to the identification of causal mutation. Fishy taint in chicken was found to be caused by a substitution within a conserved motif of the *FMO3* gene. A brief screening of other chicken lines indicated that the tainting mutation was quite common and relatively old among the brown breeds.

One of the important motives for QTL mapping in agricultural species is that the results may be exploited in practice. Thus, a highlight of this study has been to be able to fulfill the ultimate objective such as finding markers that could be utilized in commercial selection programs. The mapping result was successfully used in gene assisted selection against fishy taint in a commercial breeding program. This has provided benefits to the breeding company, as well as to producers and consumers all around the world.

In recent years the progress in the field of genomics has been outstandingly fast. It has been only six years since the chicken genome was published. Today, complete genomes are (re)sequenced with second generation sequencing platforms (Shendure and Ji, 2008). The third generation platform has also evolved (Hayden, 2009). Targeted sequencing of large genomic regions and whole transcriptome analyses are commonly used in research, and have replaced the traditional mapping methods. Even though the most optimistic visions of the gene mapping have not been fulfilled during the past few decades, the genomic research now has excellent tools to rapidly expand the understanding of complex traits in the future.

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