Gene hunting:
molecular analysis of the chicken genome

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Proefschrift

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Gene hunting: molecular analysis of the chicken genome

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Abstract

This dissertation describes the development of molecular tools to identify genes that are involved in production and health traits in poultry. To unravel the chicken genome, fluorescent molecular markers (microsatellite markers) were developed and optimized to perform high throughput screening of resource populations. The markers can be divided in markers located within chicken genes or ESTs (type I markers) and random markers (type II). The microsatellite markers (430) were subsequently used for the development of a highly informative comprehensive linkage map of the chicken genome. The type I markers provide the necessary links to create a comparative map between chicken and human. A further step in the analysis of the chicken genome is the construction of a physical map and the improvement of the chicken-human comparative map. Therefore a chicken Bacterial Artificial Chromosome (BAC) library was constructed with a 5.5x genome coverage and an average insert size of the BAC clones of 134 kb. Physical mapping was performed by building a BAC contig of chromosome 10 by chromosome walking. Using a bi-directional approach that utilizes the information from the chicken as well as the human genome, a detailed comparative map was obtained for chicken chromosome10 and human chromosome 15. This approach involved sample sequencing of BAC clones as well as FISH mapping. The STS markers developed for chromosome walking are currently used for the development of SNP markers, which will subsequently be used in the advanced intercross lines of the Wageningen resource population to narrow down the chromosomal regions containing the QTL. This information together with a very detailed comparative map will allow the identification of candidate genes for these particular QTL.

Stellingen

Behorende bij het proefschrift
"Gene hunting: molecular analysis of the chicken genome"

1. Het feit dat het genoom van de kip drie maal kleiner is dan die van andere landbouwhuisdieren is een groot voordeel bij de jacht op genen.

2. Microsatellietmerkers zijn tot op heden de beste merkers voor een analyse van het totale genoom

3. Het kloneren van kleine stukjes DNA is een aan te leren vaardigheid, van grote stukken een kunst.
(dit proefschrift)

4. Er zijn minimaal zes inversies en twee deleties nodig om de genvolgorde van kip chromosoom 10 overeen te laten komen met humaan chromosoom 15.
(dit proefschrift)

5. Het aantal autosomale DNA segmenten dat tussen kip en mens geconserveerd is bedraagt eerder 400 dan de door Burt *et al.* voorspelde 96.
(dit proefschrift, Burt et al., *Nature* 1999, 402:411-412)

6. In het kader van het behoud van biodiversiteit verdient het aanbeveling de oud-Hollandse hoenderrassen te bewaren.
(Crooijmans, *Zeldzaam Huisdier* 1998, 3:20-23)

7. Multicellulaire sferoiden van humane tumorcellijnen zijn een geschikt *in vitro*-model voor therapie van humane tumoren.
(Crooijmans *et al.*, *Anti Cancer Res.* 1991, 11:297-300)

8. Een linkage kaart is als een soort kapstok, waar je van alles aan kunt ophangen.
(dit proefschrift)

9. Het krijgen van kippenvel komt in een ander perspectief te staan als blijkt dat de kip meer op de mens lijkt dan we dachten.
(dit proefschrift)

10. De ene (kippen) bank is de andere niet.
(dit proefschrift)

11. Des te kleiner de rentabiliteit van een project des te groter het prestige.
(de Betuwelijn)

12. Is het niet merkwaardig dat een computersysteem al verouderd kan zijn voordat de helft van de gebruikers erachter is hoe je ermee moet werken.

13. Streven naar succes zonder hard te werken is trachten te oogsten waar je niet hebt gezaaid.

14. Wat ons land nodig heeft zijn minder mensen die in dezelfde straat wonen en meer goede buren.

31 oktober 2000,
Richard Crooijmans

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Richard

Contents

Chapter I

General introduction

1.1 The chicken Genome

The chicken genome consists of 38 pairs of autosomes and two sex chromosomes Z and W. The chromosomes (Figure 1) can be divided in two size groups, 9 cytogenetically distinguishable macrochromosomes and 30 cytogenetically indistinguishable microchromosomes. In chicken the female is the heterogametic sex (ZW) and the male is the homogametic sex (ZZ). The estimated haploid genome size of the chicken is approximately 1.2 x 10^9 bp (Stevens, 1986) which is small compared to the genome size in mammals (3 x 10^9 bp), whereas the amount of recombination is similar to that in mammals (Rodionov *et al.*, 1992). The smaller genome size is mainly due to a lower number of repeats and smaller intron sizes in chicken compared to mammals (Hughes and Hughes, 1995).

Figure 1. Chicken metaphase chromosomes.

1.2 Genome Mapping

The presence of a large number of highly polymorphic sites in the genome of vertebrates has made it possible to develop many highly polymorphic DNA markers that can be used to construct linkage maps in these species. The major goal for these maps in the livestock species is to identify genes that control the expression of economically important traits. The vast majority of these traits are quantitative traits, which are controlled by a relatively large number of loci (QTLs) as well as influenced by environmental factors. If a QTL for a particular trait is closely linked to a marker, different marker alleles will appear to be associated with different levels of performance for that trait. This association, which is likely to occur within families, can be detected by statistical techniques such as regression

analysis or maximum likelihood. When a complete genetic map is available and sufficient animals are analysed, any QTL with an appreciable effect on performance can be located between a pair of linked markers.

In chicken there are three reference populations used as mapping population for genetic markers (Table 1):

The Compton (C) reference population is a backcross (BC) of two partially inbred White Leghorn lines (line N and 15I). These lines differ in their susceptibility to a number of diseases, but in particular, line N is resistant to salmonellosis while line 15I is highly susceptible. A subset of 56 progeny was chosen as one of the three reference populations in an international effort to produce a linkage map of the chicken genome (Bumstead and Palyga, 1992).

The second population is the East Lansing (EL) reference population which was produced by backcrossing a partially inbred Red Jungle Fowl (JF) line to a highly inbred White Leghorn (WL) line. A subset of 52 progeny was used as the international reference population.

The third population is the Wageningen resource population (WAU). This experimental population contains 10 full sib families of a cross between two extreme commercial broiler lines of Nutreco BV. The G_0 generation consisted of two broiler dam lines originating from the White Plymounth Rock breed. Unrelated G_1 animals were mated to produce 10 full sib families with an average of 46 G_2 offspring per family. A subset of this population (4 families; 191 animals) was used as a reference population for mapping new markers.

Table 1. Characteristics of the three chicken reference populations.

population	type	# of animals	max. # of informative	reference
WAU	full sib	456 G_2	912	Groenen et al., 1998
C	backcross	56 BC1	56	Bumstead and Barrow, 1987
EL	backcross	52 BC1	52	Crittenden et al., 1993

1.3 Genetic DNA markers

Genetic DNA markers can be divided into two groups according to O'Brien and Graves (1991): the so-called type I loci (within or adjacent to known genes) and the type II loci (random DNA markers). For type I and II loci, different kind of markers have been developed over the years. Several of these markers have been used in genetic mapping of the chicken genome. Additional information about the chicken loci is available on the web sites in Wageningen, Roslin and East Lansing (address[1]). The most important types of markers are described below.

1.3.1 RFLPs

Restriction fragment length polymorphisms (RFLPs; Botstein *et al.*, 1980) are caused by DNA sequence variation at restriction sites and often only detect two alleles. Often, cloned cDNA sequences are used to detect RFLPs. Bumstead and Palyga (1992) reported the first preliminary linkage map of the chicken genome based on RFLP markers.

1.3.2 VNTR

Within the genome of vertebrates many different DNA elements are found to be repeated and dispersed throughout the whole genome. Two different types of tandem repeats can be distinguished:

1. **Minisatellites,** which are repeated sequences of 20 to 60 bp in length (Jeffreys *et al.*, 1985). At a given locus these elements occur as direct repeats and the number of repeats varies between different alleles. In human these markers appear not at random but tend to cluster at the telomeres. Isolation and characterisation of minisatellite markers in chicken has been described by Bruford and Burk (1994) and Bruford *et al.* (1994). In chicken the minisatellite markers are also not distributed at random, and several linkage groups consists primarily of minisatellite markers (e.g. E26C13) (Groenen *et al.*, 2000)
2. **Microsatellites** or simple sequence repeats are sequences that consist of a direct repeat of a mono, di, tri or tetra-nucleotides such as $(T)_n$, $(CA)_n$ or $(CAC)_n$ where n can vary from 8 to over 30. Microsatellites are very abundant in the genome of most (or all) vertebrates (Hamada *et al.*, 1982; Tautz and Renz, 1984). They are estimated

[1] http://www.zod.wau.nl/vf/research/chicken; http://www.ri.bbsrc.ac.uk/chickmap ; http://poultry.mph.msu.edu/

to appear at least once every 10^5 bps, which means that in the genome of most vertebrates over 10^4 and probably as many as 10^5 microsatellites are present. This is substantially more compared to chicken where the total number of repeats is estimated to be 10 times less (Crooijmans *et al.*, 1994; Primmer *et al.*, 1997). As with the larger minisatellites, the number of repeats varies between different alleles (Litt and Luty, 1989; Tautz, 1989; Weber and May, 1989). A big advantage of using microsatellites as markers, is that their total length, including the flanking DNA, is short enough (70-340 bp) to make them amenable to polymerase chain reaction (PCR) amplification (Weber and May, 1989). Polymorphism is detected by separating the PCR amplified fragments on high resolution polyacrylamide gels. This typing method is faster than the conventional RFLP analysis, requires only very small amounts of DNA and is suitable for automatisation (chapter 4). Other advantages of microsatellites are: their random distribution throughout the genome, there relative ease of isolation, and the high percentage that is polymorphic. For these reasons many groups have isolated a considerable number of microsatellite markers in chicken either randomly or within genes. Although microsatellites generally have a random distribution, the microchromosomes in chicken have a relatively low concentration of CA microsatellites (Primmer *et al.*, 1997). In total more than 800 polymorphic microsatellite markers have been mapped in chicken (Groenen *et al.*, 2000). The chicken microsatellite markers are outlined in more detail in chapters 2 and 3.

1.3.3 RAPDs

The random amplified polymorphic DNA (RAPD) method (Williams *et al.*, 1990) uses short arbitrary primers, usually 10 bp long, one at a time to amplify random genomic fragments by PCR. The PCR products are separated on an agarose gel and the fragments are visualised with simple staining techniques. The result is a number of fragments with different lengths, and the polymorphisms are observed as the presence or absence of one of these fragments. Advantages of this method are: a large number of reactions can be conducted at one time, little input is needed for the development of the markers, and the polymorphism is easily detected. A disadvantage of RAPDs is that they are not typable as co-dominant markers because the heterozygous state is not detected. Furthermore, a problem when using this type of marker is the poor repeatability across different laboratories due to small differences in conditions used. Therefore, this method probably is most useful in crosses between two inbred lines such as in the chicken East Lansing

backcross population where 65 RAPD markers have been mapped (Cheng *et al.*, 1995; Levin *et al.*, 1993).

1.3.4 AFLPs

The Amplified Fragment Length Polymorphism (AFLP) technique (Vos *et al.*, 1995) is a DNA fingerprinting technique based on three steps. (i) restriction of the DNA with two restriction endonucleases and ligation of oligonucleotide adaptors, (ii) pre-selective and selective amplification of sets of restriction fragments with PCR primers that have corresponding adapter- and restriction-site-sequences and flanking nucleotide(s) as their target sites, and (iii) gel analysis of the amplified fragments. These markers are useful to rapidly increase the number of markers on a linkage map. In chicken, 552 AFLPs have been mapped which are described by Knorr *et al.* (1998) and Herbergs *et al.* (1999). The major disadvantage of these markers is the fact that only 2 alleles are detected and that AFLP's are multilocus markers. Moreover, dominant or co-dominant inheritance of the marker is not always clear. Because of these disadvantages this type of marker generally is not so useful in small outbred families.

1.3.5 CR1 repeat element polymorphisms

The chicken middle repetitive CR1 element is a member of a family of non-LTR retrotransposon-like repeats, whose copy number has been estimated at around 100.000 per haploid genome (Vandergon and Reitman, 1994). Because of the genome-wide distribution and the highly polymorphic character of CR1 elements, it seemed likely that single CR1 primers could be used to generate PCR DNA fragments. Such amplification products may arise from the fortuitous location of 2 CR1 elements nearby in the genome in the forward and reverse orientation. This method is analogous to that described for mammalian *Alu* and L1 repeats (Cox *et al.*, 1991; Zietkiewicz *et al.*, 1992). Chicken CR1 repeat-element polymorphisms (47 *MSU-* markers) were typed in the East Lansing backcross population (Levin *et al.*, 1994a, b).

1.3.6 SSCPs

Single strand conformational polymorphism (SSCP) is a PCR type marker in which the difference in electrophoretic mobility of single stranded DNA on nondenaturing gels depends not only on their chain lengths but also on their conformations (Sheffield *et al.*, 1993). This method is also used in chicken, mainly for mapping of genes or monomorphic

microsatellite markers (Morisson *et al.*, 1998; Pitel *et al.*, 1998; Burt *et al.*, 1999; Nanda *et al.*, 1999).

1.3.7 ASOs

The allele specific oligo (ASO) technique is based on the PCR amplification of parts of specific genes. Sequence analysis of the cloned products from the parents of the mapping population was initially conducted to determine whether base substitutions occurred in either parent. Once the sequence polymorphism was found, a PCR protocol was designed to enable the identification of a specific allele. This alternative approach to map anchor loci was predicated on the frequent occurrence of base substitution (Neel, 1984) and indications that introns are less conserved than exons (Perler *et al.*, 1980). Already 71 genes have been mapped in chicken by using this technique (Smith *et al.*, 1996, 1997; Dodgson, unpublished results).

1.3.8 SNPs

Recently, another type of marker, the single nucleotide polymorphism (SNP), has seen an increase in its popularity mainly because of the possibility to be used on DNA chips or other high throughput systems. The classic RFLPs are in fact a subclass of SNP markers in which the mutation results in the creation or destruction of a restriction recognition site. Although this type of marker is primarily bi-allelic, its high abundance makes it very powerful. The frequency of SNPs is rather high about 1 per kb in human (Wang *et al.*, 1998). In chicken a frequency as high as 1 per 100 bp has even been observed (Vignal *et al.*, 2000). Because of their abundancy these markers have a high potential for the detailed haplotype analysis, *e.g.*, association studies (Collins *et al.*, 1996).
In Chicken, some SNP markers located within genes have already been mapped in the East Lansing reference population (Ed Smith, unpublished results).

1.4 Mapping of markers and genes

1.4.1 Genetic mapping

Two genetic loci are linked if they are inherited together in pedigrees more often than would occur by chance. Linkage maps are based on recombination frequencies (range 0-0.5) between the two pairs of loci. The observed recombination frequency is a measure for the distance between the two loci, the smaller the recombination frequency the smaller the distance between the two loci. The distance between two loci is expressed in centimorgans

(cM), which is a function of the recombination frequency. The precise relation between the recombination frequency and distance is dependent on the mapping function used, but for small recombination frequency 1 cM generally represents a recombination frequency of 1%. The physical length that corresponds with 1 cM is highly dependent on the amount of recombination in the species involved (*e.g.*, in Arabidopsis 1cM on average is 140 kb, in human 1 cM on average is 1100 kb and in chicken 1 cM on average is approximately 340 kb). International collaborative efforts in genome mapping in chicken have resulted in a genetic map with around 1900 markers (Groenen *et al.*, 2000) and the total length of almost 4000 cM (Kosambi mapping function).

1.4.2 Physical mapping

In situ hybridisation is the most direct way to physically map genes or markers (Pardue, 1985; Korenberg *et al.*, 1992). A cloned DNA fragment is labelled and directly hybridised to metaphase chromosomes. After hybridisation, the gene or marker is physically mapped to a specific chromosome. In chicken, this is possible for the macrochromosomes but is a problem for the microchromosomes. Although probes can be mapped to microchromosomes from a certain size class, it is not possible to unequivocally identify a particular microchromosome. A possible solution is the development of a set of chromosome specific FISH markers that can be used in two colour FISH. In this case the marker to be mapped is labelled with a particular fluorescent dye and used together with another probe of unknown chromosomal location that is labelled with another fluorescent dye. However this still requires many different hybridisations to identify the specific microchromosome. A second method is the use of a radiation hybrid panel. This is a procedure where chromosome fragments generated by lethal irradiation of donor cells are rescued by fusion with suitable recipient cells (Walter and Goodfellow, 1993). A chicken radiation hybrid panel is currently under construction (A. Vignal and A. Ponce de Leon, personal communication)

Assigning genes and markers to specific chromosomes can also be performed by using FACS (fluorescence activated cell sorter) (Bartholdi *et al.*, 1987) sorted chromosomes or by the isolation of individual chromosomes by scraping them from metaphase chromosome spreads. Both permit sorting of individual chromosomes and the construction of chromosome-specific DNA libraries. In chicken flow sorting of the chromosomes is possible but the resolution is not sufficient for the separation of the individual microchromosomes. Chromosome specific libraries of the larger

macrochromosomes (1 to 4) have been made and used for the isolation of specific microsatellite markers from these chromosomes (A. Ponce de Leon unpublished).

Another way of physical mapping is contig building with large insert libraries such as Yeast Artificial Chromosome (YAC) libraries, Bacterial Artificial Chromosome (BAC) libraries and P1 derived Artificial Chromosome (PAC) libraries. In chicken, a YAC library has been described by Toye *et al.* (1997) and a BAC library by Crooijmans *et al.* (2000)(chapter 6). Screening of such libraries can either be performed by hybridisation of high-density filters or by PCR when DNA pools are available.

Genome-wise contig building can be performed with large insert clones by fingerprinting where restriction fragment comparison is used to obtain overlapping clones. This technique can also be performed on automated DNA sequencers (Gregory *et al.*, 1997). Chromosome specific contigs can be constructed by chromosome walking where large insert clones are identified from fixed starting points (mapped markers). Sequencing of the ends of the large insert clones will generate new probes for further rounds of screening. Chromosome walking in chicken is described in chapter 7.

1.4.3 Comparative Mapping

Although comparative mapping (Nadeau, 1989) is not particularly useful for mapping markers, it can give valuable information on the possible location of certain genes or candidate genes for mapped QTL. During evolution and divergence of vertebrate species, numerous recombinations and translocations have occurred. These events have lead to different number of chromosomes, and the dispersion of previously linked genes over different chromosomes, in different species. However, certain genes still are linked to one another on the same chromosome in different species. Many conserved synteny (segment homology) between chicken and man and between chicken and mouse have been observed (Burt *et al.*, 1999; Groenen *et al.*, 2000; chapter7).

1.5 The scope of this thesis

The aim of the work described in this thesis is the development of the essential tools for genome analysis in chicken needed to localise quantitative trait loci (QTL) for economically important traits and the subsequent identification of the genes underlying these QTL effects.

In order to investigate the chicken genome the development of molecular tools started with the isolation of polymorphic microsatellite markers, either random (chapter 2) or from genes/ESTs (chapter 3). Genetic mapping of a large number of microsatellite markers in a large population has to be performed efficiently. Therefore methods and techniques are optimised and standardised to perform high throughput genotyping (chapter 4). Linkage analysis of the genotyping data results in a genetic linkage map (chapter 5) which is essential for performing a QTL mapping experiment. The next step is finemapping of the QTL regions and the construction of a detailed gene map of those regions. A valuable tool towards this goal is a BAC library. The construction of a BAC library in chicken is described in chapter 6. A detailed gene map of a chicken chromosome is obtained by analysing BAC clones of chicken chromosome 10. Refining the human chromosome 15/ chicken chromosome 10 comparative map has resulted in the identification of many inter and intra chromosomal rearrangements (chapter 7).

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Chapter II

Microsatellite markers in poultry

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Crooijmans R.P.M.A., Dijkhof R.J.M., van der Poel J.J. and Groenen M.A.M. (1997). New microsatellite markers in chicken optimized for automated fluorescent Genotyping. *Animal Genetics* **28**: 427-437.

Abstract

Several research groups have been working on the development of microsatellite markers in poultry, which is outlined in this chapter. Starting with the preparation of chicken genomic libraries, microsatellite-containing clones have been isolated and sequenced. Primers were made flanking the microsatellite repeat and one of them was labelled with a fluorescent phosphoramidite either 6-FAM, HEX or TET. The PCR conditions in our laboratory have been standardised with only the annealing temperature as variable. Markers were tested for polymorphism on the parents of the East Lansing, Compton and Wageningen reference populations. A total of 372 polymorphic microsatellite markers (MCW) have been isolated in our laboratory. In addition primers were made of 91 sequences with a microsatellite repeat isolated by the group of Dr T. Burke (LEI-markers), which resulted in a further 89 polymorphic markers. Furthermore, 9 polymorphic markers isolated at the Hebrew University of Jeruzalem (HUJ-markers) have been optimised to be used under our standard conditions. The ADL markers (obtained from Dr H. Cheng), were also tested under our standard conditions which resulted in a further 174 polomorphic and 19 monomorphic markers. In total we describe 644 polymorphic microsatellite markers which resulted in an average allele number of 4.0 in our test panel. Of these 644 microsatellite markers 89% could be mapped in the Wageningen resource population, 66% in the East Lansing reference population and only 46% in the Compton reference population.

Introduction

Great variability in the number of repeats at most microsatellite loci makes them useful in genetic mapping, population genetics and in a variety of other applications. In the genomes of vertebrates over 10^4 and probably as many as 10^5 microsatellites are present (Litt and Luty, 1989; Love *et al.*, 1990; Tautz and Renz, 1984). In chicken, the number of microsatellite is 6 to 10 folds lower compared to mammals (Crooijmans *et al.*, 1993; Primmer *et al.*, 1997). The development of polymorphic DNA based markers has resulted in the development of linkage maps in farm animals over de last decade. Linkage maps for cattle (Barendse *et al.*, 1994; Bishop *et al.*, 1994; Kappes *et al.*, 1997), swine (Archibald *et al.*, 1995; Marklund *et al.*, 1996; Rohrer *et al.*, 1996), sheep (Crawford *et al.*, 1995) and chicken (Bumstead and Palyga, 1992; Cheng *et al.*, 1995; Groenen *et al.*, 1998) have been reported but compared to human these maps are still less well developed.

Given the fact that the size of the chicken genome is around 4000 cM, and based on the assumption that a polymorphic marker is needed every 10-20 cM to efficiently perform a total genome scan, at least 150-300 evenly spaced informative markers are needed for such a study. However, as not all markers will be equally informative in all populations more markers are required. For example, the heterozygosity of 17 microsatellite markers in a number of commercial broiler lines on average was 54% whereas within a number of commercial layer lines it was only 26% (Crooijmans *et al.*, 1996b). Furthermore, the next step in QTL mapping experiments is the fine mapping of the regions containing the QTL of interest, which requires even more dense maps. Although other types of markers such as restriction fragment length polymorphism (RFLP) (Bumstead and Palyga, 1992), chicken repeat 1 elements (CR1 PCR elements) (Levin *et al.*, 1994), random amplified polymorphic DNA (RAPD) (Levin *et al.*, 1993) and amplified fragment length polymorphism (AFLP) (Knorr *et al.*, 1999; Herbergs *et al.*, 1999) can be used to increase the marker density of the chicken linkage maps, microsatellites are still the markers of choice. Fluorescent labelling of the PCR products in combination with automated fluorescent DNA fragment analysers allows data to be recorded automatically for multiple markers (Ziegle *et al.*, 1992; Levitt *et al.*, 1994; Reed *et al.*, 1994) and subsequently allows easy data analysis, which is essential for large scale genotyping experiments.

In this chapter 644 polymorphic microsatellites are described, that have been optimised and used in sets of multiple microsatellites, for efficient large scale semi-automated genotyping on ABI automatic sequencers.

Materials and methods

Development of microsatellite markers. Microsatellite markers (Microsatellite Chicken Wageningen; MCW) developed in our laboratory are derived from both male and female chicken White Leghorn DNA. Chicken DNA was digested with *Sau*3A, partially filled in with dC and dT and ligated in the partially filled in (dG and dA) *Xho*I site of lambda Zap II vector (Stratagene, La Jolla, CA, USA). The libraries were screened with a radioactively labelled $(TG)_{13}$ oligonucleotide (Crooijmans *et al.*, 1993, 1994 and 1996a). Linear PCR sequencing of the positive clones was performed using either radioactive and fluorescent labelling methods. Primers, 20 to 24 nucleotides in length, were designed flanking the repeat. One primer in each pair was labelled with either one of the fluorescent phosphoramidites (6-FAM, HEX or TET), which enables the polymerase chain reaction (PCR) products to be analysed on an ABI automated sequencer (Perkin Elmer, ABI). If possible, long stretches of the same base within

the primers were avoided. We also tried to match A/T and G/C content of the primers and preferably choose a C or a G at the 3' end of the primer.

Microsatellite markers obtained from the Avian Disease and Oncology Laboratory, USA (ADL) were isolated from 4 enriched libraries (Cheng and Crittenden, 1994; Cheng *et al.* 1995). Libraries 1, 2 and 4 were screened with a labelled $(TG)_{10}$ oligonucleotide, and library 2 was screened with $(TG)_8$, $(CAA)_6$ and $(GGAT)_4$. Primers were made with the software program Oligo (NBI). Primer pairs were synthesised for each microsatellite with the primer having the lowest melting temperature labelled with either 6-FAM, HEX, or TET fluorescent dye (Perkin Elmer).

Microsatellites isolated in Leichester, UK (LEI-markers) were obtained from an enriched library as described by Gibbs *et al.* (1995 and 1997). The library was screened with a poly (TG) oligonucleotide probe. Primers were designed with the computer program Primer v0.5 (Gibbs *et al.*, 1995 and 1997), or manually as described by Crooijmans *et al.*, 1997. Markers were labelled with one of the fluorescent dyes (6-FAM, HEX, or TET) as described above.

Microsatellite markers obtained from the Hebrew University of Jerusalem, Israel (HUJ) were derived from three genomic libraries (Khatib *et al.*, 1993). These libraries were screened with a radioactive labelled $(TG)_{10}$ oligonucleotide.

PCR and gel electrophoresis. All markers obtained were first tested on the crossbred parents of the international reference populations (backcross populations Compton (C) and East Lansing (E)) and a pooled sample of the 20 parents of the Wageningen resource population (W). The PCR reactions were performed in a total volume of 12 µl containing 10-100 ng of genomic DNA, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris.HCl pH=8.3, 1 mM Tetramethylammoniumchloride (TMAC), 0.1% triton X-100, 0.01% gelatin, 200 µM dNTP, 0.25 Unit Goldstar polymerase (Eurogentec S.A., Belgium), 2.3 pmoles of each primer and covered with 10 µl of mineral oil (Sigma). The PCR was performed for 5 min at 95°C and 35 cycles of 30 s at 95°C, 45 s at annealing temperature and 90 s at 72°C, followed by a final elongation step of 10 min at 72°C. The annealing temperature varied from 45°C to 60°C. The PCR amplification of each marker was tested on an ABI automated sequencer. In case of FAM or TET labelled markers, 0.05 µl of the PCR amplification product and in case of markers labelled with HEX 0.1 µl, was used to resolve on a 6% denaturing polyacrylamide gel (Sequagel-6; National Diagnostics, Atlanta, USA). Before loading on the gel, samples were denatured for five minutes at 95°C in 3.2 µl loading buffer (which contained the GENESCAN-350 TAMRA internal standard (Perkin Elmer) and formamide (final

concentration of 80%)). Loading was performed on the ABI 373A (12 cm well-to-read; loading 4 µl) or on the ABI 377 (12 cm well-to-read; loading 1.5 µl). When performing high throughput genotyping, PCR products of different markers (up to 21 markers) from DNA of the same animal were pooled in such a way that each marker signal on the ABI automated sequencer has a peak height of about 1000. The fragment sizes were calculated relative to the GENESCAN-350 TAMRA with the GENESCAN fragment analysis software (Perkin Elmer, ABI).

Results and Discussion

In high throughput genotyping of microsatellites on ABI automated sequencers, it is essential to be able to use the whole range of the gel (from 75 -330 bp) for all three dyes (HEX, TET and FAM) as efficiently as possible. Therefore, the main objectives in our choice for the primers for the microsatellite markers, were the expected sizes of the PCR products, in combination with the choice for the fluorescent dye. Another important objective in a high throughput semi-automated genotyping set-up is to standardise the procedure as much as possible. By using simple rules of thumb for designing the primers as described in Materials and Methods, we made primer pairs that can be used under essentially the same PCR conditions. The only variation is the primer annealing temperature during the PCR reaction (see Table 1, 2, 3, and 4; Appendix 1). For microsatellites that did perform poorly under the standard conditions one, and occasionally two, new primers were developed. Microsatellites that continued to perform badly after two rounds were discarded (data not shown).

In addition to the MCW microsatellites which were isolated and characterised in our laboratory, we also designed new primers for microsatellite sequences isolated by the laboratory of Terry Burke (Leicester University, Department of Zoology, UK), which are present in the Genbank sequence database (Table 2; Appendix 1). Finally, for the HUJ microsatellites (Khatib *et al.*, 1993) which performed poorly under our standard conditions, new primers were developed (*HUJ0001*, *HUJ0002*, *HUJ0003* and *HUJ0010*; Table 3; appendix 1). The PCR conditions for 193 ADL markers were determined in our test panel and resulted in 174 polymorphic and 19 monomorphic markers. The number of alleles observed in our test panel and the range for the allele sizes, are shown in Table 1 - 3 (Appendix 1). Because of the small size of our test panel, the number of alleles indicated should be regarded as a minimum number of alleles known to occur for that particular marker. Also for this reason we have listed the 44 monomorphic markers isolated in our laboratory (WS markers; Table 4; Appendix 1) which might be polymorphic when testing more animals or which could

be used in the future as markers for radiation hybrid mapping. Nevertheless, the number of alleles gives a good indication for the generally more informative markers. The number of alleles in our test panel, for the 372 polymorphic MCW markers described, was on average 4.0. Of these markers 89% is polymorphic in the Wageningen resource population which is much higher than in the two international backcross populations (East Lansing, 57%; Compton 43%). Analysing the 644 polymorphic markers (MCW, LEI, ADL and HUJ), 89% can be mapped in the Wageningen population, 66% in the East Lansing population and finally 46% in the Compton population. The average number of alleles for all of these markers in our test panel is 4.0 again. The main reason for the difference between the percentage of markers informative in the three populations is the number of families used in the Wageningen resource population (10). Furthermore, the Wageningen resource population is a G_2 cross in which all 20 parents can be informative. This in contrast to both reference populations, which are back crosses between (partially) inbred lines and therefore only one of parent will be informative. The size distribution of all fluorescent chicken microsatellite markers, which work efficiently in our laboratory, is shown in Figure 1.

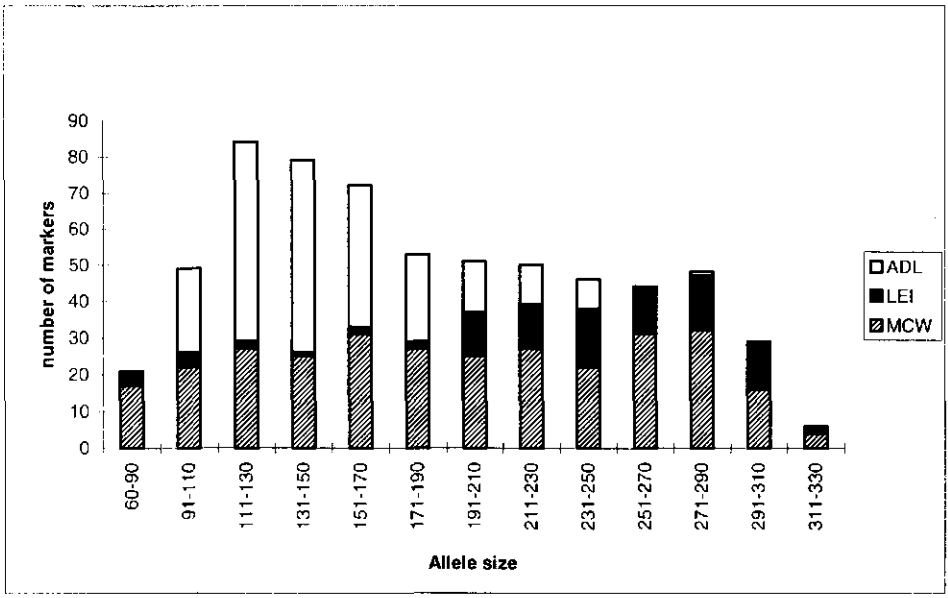

Figure 1. Size distribution (according to the sequenced strand) of the 3 main groups of fluorescent chicken microsatellite markers (MCW, ADL and LEI) performing well in our laboratory.

The distribution of the MCW markers is uniform over the whole 75 to 330 bp range, whereas the ADL markers (Cheng and Crittenden, 1994; Cheng *et al.*, 1995) are more designed in the

90 to 200 bp range. To correct for the uneven distribution of the 200 to 330 bp size range, as compared to the range of 100 to 200 bp, we designed the primers for the LEI markers mostly in the range of 200 to 330 bp. The distribution of the fluorescent dye, used within each size range for the three groups of markers, is about equal.

In chicken the size range of alleles detected for a particular marker generally is smaller than the size range observed in mammals. This smaller allele range per marker makes it possible to create larger sets of markers (in chicken up to 21 markers per set) which can be run simultaneously on the automated sequencers. In conclusion, the development and optimisation of chicken microsatellie markers is performed in such a way, that they can be used efficiently in a semi-automated set up in a total genome scan.

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Chapter III

Microsatellite marker development in chicken genes and ESTs

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Groenen M.A.M., Crooijmans R.P.M.A., Dijkhof R.J.M., Acar R., and van der Poel J.J., (1999). Extending the chicken-human comparative map by placing 15 genes on the chicken linkage map. *Animal Genetics* **30**:418-422.

Abstract

To increase the number of type I loci on the chicken linkage map, chicken genes containing microsatellite sequences based on mononucleotide repeats [$(N)_n$, where $n > 18$], dinucleotide repeats [$(NN)_n$, where $n > 8$] and trinucleotide repeats [$(NNN)_n$, where $n > 7$] were selected from the nucleotide sequence database and primers were developed to amplify the repeats. Another way of increasing the number of type I loci on the chicken map is by screening cDNA libraries for repeat containing clones. These expressed sequence tags (ESTs) are in most cases anonymous genes until homology is found with a known gene. In our laboratory we established 97 markers of which 51 are located within known genes and 46 are located within ESTs. In total 67 microsatellites representing a type I locus were mapped in one of the mapping populations. In addition, 2 genes were added to the chicken map by fluorescent in situ hybridisation. As the map position of the human homologues of the genes is known, these markers extend the comparative map between chicken and man.

Introduction

One of the main reasons for the development of genetic maps in farm animals is to locate and identify genes underlying diseases and economically important traits. The marker density of the chicken linkage map has seen a tremendous increase, and currently contains approximately 1900 loci (Groenen *et al.*, 2000). There are two types of markers. These are the type I markers which represent genes and ESTs and the type II markers which are anonymous (O'Brien, 1991). In chicken the type II markers are primarily microsatellites and AFLPs. So far, more than 300 type I loci (genes) have been mapped on the chicken linkage map which already made it possible to identify many regions of conserved synteny between chicken and mammals (Klein *et al.*, 1996; Burt *et al.*, 1995; Hu *et al.*, 1995). However, a large proportion of these type I loci are expressed sequence tags (ESTs) derived from unknown genes whose human homologues have not yet been identified. To be able to more precisely identify the conserved regions between the chicken genome and the genomes of mammalian species (man, mouse) many more type I loci are needed on the chicken map. To work towards this objective microsatellite markers have been developed known to reside within known genes whose sequence was already deposited in the public nucleotide database (Crooijmans *et al.*, 1995; Groenen *et al.*, 1999) and by isolating and sequencing microsatellite containing ESTs (Ruyter-Spira *et al.*, 1996 and 1998a).

Materials and methods

Development of microsatellite markers. Database searches were performed for sequences of mononucleotide repeats (poly A), for dinucleotide repeats (poly TA, CA or TC) and for trinucleotide repeats (poly GCT, CGG, GAG, CTT, GCG, CTT or GCA) (Crooijmans *et al.*, 1995; Groenen *et al.*, 1999). Primers were designed on both sites adjacent to the repeat for the mononucleotide repeats larger than 18, for dinucleotide repeats larger than 8 and for trinucleotide repeats larger than 5.
A chicken brain cDNA library (Clontech) and a chicken embryonic cDNA library (Stratagene) were screened for TG repeat containing clones as described by Ruyter-Spira *et al.* (1996 and 1998a) and positive clones were sequenced. In all cases primers were made flanking the repeat, and the markers were tested for polymorphism as described according to Crooijmans *et al.* (1997).
All microsatellite markers were tested as described in chapter 2.

Reference families and linkage analysis. Linkage analysis was performed on the Wageningen population as described by Groenen *et al.* (1998). Markers that were not informative in this population (*MCW0041, MCW0047, MCW0050, MCW0075, MCW0143, MCW0163, MCW0346, MCW0348,* and *MCW0353*) were genotyped in the East Lansing reference or Compton reference population. Markers *MCW0054, MCW0070,* and *MCW0072* were not polymorphic in the 4 families of the Wageningen mapping population but polymorphic in one or more families (of the six additional families) of the Wageningen resource population. Marker *MCW0074, MCW0144, MCW0203, MCW0344, MCW0356,* and *MCW0371* although polymorphic, were not informative in any of the three chicken reference populations. The linkage analyses were performed with the CRIMAP version 2.4 (Green *et al.*, 1990) linkage program or in case of the East Lansing data, with MAPMANAGER version 2.6 (Manly, 1993) (Groenen *et al.*, 1998).

Fluorescent in situ hybridization. Two-colour in situ hybridisation on metaphase spreads of chicken embryo fibroblasts was performed as described previously (Ruyter-Spira *et al.*, 1998b). Chicken bacterial artificial chromosome (BAC) clones (Crooijmans *et al.*, 2000) isolated from *WS0036* (*AGC1*) and *MCW0356* (*TPM1*), were labelled with biotin-16-dUTP and a BAC clone containing marker *ADL0038* (mapped to linkage group E29C09W09) was labelled with digoxigenin-11-dUTP. The DNA was counter stained with DAPI.

Results and discussion

All markers isolated either from the database sequences or from cDNA libraries, that gave a reproducible clear amplification product, are listed in Table 1. When the marker is mapped in one of the reference populations, the map location in chicken and when known the map location of the human homologous gene is given. Characteristics of the markers developed as primer sequence, fluorescent dye and PCR annealing temperature are given in Table 1 and 4 (Appendix 1). The monomorphic markers (*WS* markers; Table 4, appendix 1) might be polymorphic in other populations and could be used in the future as markers for radiation hybrid mapping.

Table 1. Microsatellites within identified gene.

Marker	Gene	Accession Number[a]	Chicken Chrom[b]	Human[c] Chrom.
MCW0041	Y-gene chicken ovalbumin family (*OVY*)	V00439	Chrom. 2	-
MCW0042	B-cell lymphoma 2 (*BCL2*)	D11381	Chrom. 2	18q21.3
MCW0043	14 k beta-galactoside-binding lectin gene (*BGBL4*)	D00311	Chrom. 1	-
MCW0044	Duplicated genes for histone H2A, H4 and H3 genes	X02218	Chrom. 1	-
MCW0045	Embryonic myosin heavy chain gene (*MYHE*)	M20006	E21E31C25W12	17pter-p11
MCW0046	Alpha-A-crystallin gene (*CRYAA*)	M17627	Chrom. 1	21q22.3
MCW0047	High mobility gene-14A (*HMG14A*)	X63083	Chrom. 4	21q22.3
MCW0048	N-myc protein gene (*MYCN*)	D90071	Chrom. 3	2q24.3
MCW0049	Lysosomal associated membrane protein 1 (*LAMP1*)	M59361	Chrom. 1	13q34
MCW0050	cDNA proto-oncogene C-SRC (*SRC*)	S43620	E not linked	-
MCW0051	Vitamin-D-induced calbinding 4 28K gene (*CALB1*)	M33143	Chrom. 2	8p12
MCW0052	Immunoglobulin V26 and V6 gene (*IGVPS*)	D13439	E18C15W15	-
MCW0054	NF-kappa B p100 (*NFkB*)	U00111	-	-
MCW0059	Phospholamban gene (*PLN*)	M59037	Chrom. 3	6q22.1
MCW0070	Chicken lipoprotein lipase gene (*LPL*)	X60547	-	8q22
MCW0071	Engrailed protein gene (*EN2*)	L12696	Chrom. 2	7q36
MCW0072	USIL-1 DNA	X54093	-	-
MCW0073	Heat chock factor 3 (*HSF1*)	L06098	E46C08W18	21pter-qter
MCW0074	Chox-4d gene for homeodomain protein (*HOXD10*)	D10287	-	2
MCW0075	C-ets mRNA for p54 protein (*ETSB*)	X13026	E not linked	11q23.3
MCW0076	Type 1 collagen alpha-1 chain mRNA (*COL1A1*)	M17607	E59C35W20	17q21.3
MCW0079	MAX-protein (*MAX*)	L12469	Chrom. 4	14q23
MCW0106	EST	L48902	Chrom. 1	-
MCW0107	EST	L48906	Chrom. 1	-
MCW0108	EST homolog GTP binding protein (*RAB6*)	L48903	Chrom. 1	2q14-21
MCW0109	EST	L48904	Chrom. 1	-

MCW0110	EST	L48908	E48C28W13W17	-
MCW0111	EST	L48909	Chrom. 1	-
MCW0113	EST	L48905	Chrom. 5	-
MCW0141	EST	L48883	Chrom. 3	-
MCW0142	EST	L38882	Chrom. 2	-
MCW0143	EST	L48880	C33	-
MCW0144	EST	L48877	-	-
MCW0149	EST	L48895	E36C06W08	-
MCW0153	EST	L48885	Chrom. 2	-
MCW0155	EST	L48886	-	-
MCW0162	EST	L48891	Chrom. 3	-
MCW0163	EST	L48890	Chrom. 2	-
MCW0186	EST human Zincfinger ZFX or ZFY (*ZFX/Y*)	L48892	Chrom. 1	Xp22.1
MCW0187	EST	L48899	Chrom.3	-
MCW0188	EST	L48897	Chrom. 1	-
MCW0189	EST	L48893	E46C08W18	-
MCW0190	EST	L48881	E36C06W08	-
MCW0191	EST	L48878	Chrom. 4	-
MCW0197	EST	L48901	E48C28W13W27	-
MCW0203	EST	AF030581	-	-
MCW0204	EST	AF030578	E53C34W16	-
MCW0206	EST	AF030579	Chrom. 2	-
MCW0216	EST	AF030586	E48C28W13W27	-
MCW0221	EST	G54427	-	-
MCW0225	Netrin-2 mRNA (*NTN2*)	L34550	E35C18W14	16p13.3
MCW0271	EST	AF030577	Chrom. 8	-
MCW0272	EST	AF030580	Chrom. 2	-
MCW0273	EST	AF030582	Chrom. 1	-
MCW0274	EST	AF030587	Chrom. 2	-
MCW0275	EST	AF030584	Chrom. 8	-
MCW0276	EST	AF030585	Chrom. 4	-
MCW0299	EST	AF030583	-	-
MCW0338	Zinc finger 5 protein mRNA (*ZFP161*)	U51641	Chrom. 2[d]	18
MCW0341	Activin receptor IIB mRNA (*ACVR2*)	U31223	Chrom.2	2
MCW0344	bZIP nuclear protein MafB	D28600	-	-
MCW0346	GATA-3 gene (*GATA3*)	S78786	Chrom. 1	10p15
MCW0347	Anti-mullerian hormone (*AMH*)	X89248	E53C34W16[d]	19p13.3
MCW0348	Activin beta B mRNA (*INHBB*)	Z71594	Chrom. 7	2cen-2q13
MCW0349	Pineal opsin gene (*PNO*)	U87449	E52W19	-
MCW0350	T-cell receptor alpha mRNA (*TCRA*)	U04611	E59C35W20	14q11.2
MCW0351	Chicken EST; CLFEST63	D26339	Chrom. 8	-
MCW0353	bZIP nuclear protein MafF	D16184	Chrom. 1	-
MCW0354	Adenylate cyclase activating polypeptide 1 (*ADCYAP1*)	U71183	Chrom. 2	18p11
MCW0355	Neurofascin gene (*NRF*)	Y14347	E60E04W23	-

MCW0356	Alpha tropomyosin gene (*TPM1*)	X57991	**E29C09W09**	15q22
MCW0357	Aromatase gene (*CYP19*)	D50335	E29C09W09	15q21
MCW0359	c-Maf proto-oncogen (*CMAF2*)	D28598	E30C14W10	16
MCW0361	Homeobox protein gene (*GBX2*)	AF022151	Chrom. 7	2q37
MCW0362	Retinoic acid receptor beta (*RARB*)	X57339	Chrom. 2	3p24
MCW0366	Insulin growth factor I receptor (*IGF1R*)	S40818	E29C09W09	15q26.1
MCW0382	EST	AJ397960	-	-
MCW0383	EST	AJ394144	-	-
MCW0384	EST	AJ393384	-	-
MCW0385	EST	AJ393912	-	-
MCW0386	EST	AJ397995	Chrom. 1	-
WS0001	c-KIT	D13225	Chrom. 4	4q12
WS0002	EST	G32088	-	-
WS0003	EST	G32089	-	-
WS0004	EST	G32090	-	-
WS0005	EST	G32091	-	-
WS0006	EST	G31922	-	-
WS0007	EST	G32092	-	-
WS0008	gdretm RNA	Z49898	-	-
WS0029	EST	G32108	-	-
WS0036	Aggrecan gene (*AGC1*)	U83593	**E29C09W09**[c]	15q26
WS0037	Homeobox protein Chox-z mRNA	X17612	-	-
WS0039	Alpha-1 collagen type III gene (*COL3A1*)	M36662	Chrom. 7[f]	2q31-q32
WS0040	GABA-A receptor gamma-2 subunit mRNA	X54944	-	5q34
WS0041	Erythroid transcription factor gene (*GATA1*)	M59937	-	-
WS0042	Hox1.4	X52669	-	-
WS0043	MyoD gene	L34006	-	-
WS0044	Scaffolding protein II	X80792	-	-

[a] Nomenclature of the linkage groups is according to Groenen *et al.*, (2000) and refers to the original linkage groups in the East Lansing (E), Compton (C) and Wageningen (W) linkage maps. The loci for which the chromosomes are indicated in bold have been mapped using fluorescent in situ hybridisation.

[b] Map location refers to the comprehensive linkage map of the chicken genome of Groenen *et al.*, (1998).

[c] The location of the human genes has been derived from GDB except for those in bold which are derived from the human radiation hybrid map (Gene Map '98).

[d] Previously mapped on East Lansing map by Smith and Cheng (1998).

[e] Previously mapped on East Lansing map by Jones *et al.* (1997).

[f] Mapped on the Compton map by Girard-Santosuosso *et al.* (1997).

We were able to map 67 of the markers on at least one of the three reference populations (Table 1), which recently have been integrated into a single consensus linkage map (Groenen *et al.*, 2000). In addition, two markers (*MCW0356* and *WS0036*) were used for the isolation of chicken BAC clones, which subsequently were used to add the *TPM1* and *AGC1* genes to linkage group E29C09W9 by fluorescent in situ hybridisation (Table 1; indicated in bold). For 35 genes the map location of their human and/or mouse homologue is known. Map locations of 31 of these genes (Figure 1; indicated in bold) identified new conserved regions or confirmed previously identified regions that are conserved between chicken and man. Three genes (*LPL*, *HOXD10* and *GABA-A*) were not mapped in chicken and one gene (*ETSB*) was mapped in the East Lansing population but was not linked to another marker. From the 31 genes, 10 genes point towards new syntenic regions between chicken and man. The GATA-3 gene (*GATA3*; HSA10p15), the alpha-A-crystallin gene (*CRYAA*; HSA21q22), and zincfinger X/Y gene (*ZNX/Y*; Xp22) were mapped in chicken to chromosome 1. The genes, activin receptor IIB (*ACVR2*; HSA2), and retinoic acid receptor beta (*RARB*; HSA3p24) were both mapped to chicken chromosome 4. The high mobility gene (*HMG14A*) is mapped in chicken to chromosome 4 and in man to HSA21q22. The gene c-Maf proto oncogen (*CMAF2*) did map in chicken to E30C14W10 and in man to HSA16. The anti-mullerian hormone gene (*AMH*) did map in chicken to E53C34W16 and in man to HSA19p13. The heat chock factor 3 gene (*HSF1*) did map in chicken to E46C08W018 and in man to HSA 21, and finally the T-cell receptor alpha gene (*TCRA*) did map in chicken to E59C35W20 and in man to HSA14q11. The GTP binding protein (*RAB6*) which mapped to chicken chromosome 1 is conserved in the mouse to a chromosome 9 segment (with genes *Pgr* and *Fut4*) but not conserved in man. The genes *PGR* and *FUT4* are located in man on chromosome 11q and *RAB6* on 2q14-21. The gene *ETSB* (C-ets mRNA for p54 protein) is mapped in the East Lansing population but not linked and this gene is mapped in man to chromosome 11q23.3 (Table 1). The gene proto-oncogene C-SRC (*SRC*) is also mapped in the East Lansing population and not linked to another marker but not mapped in man.

It has been estimated (D. W. Burt, personal communication) that at least 2000 different orthologous genes need to be mapped in chicken to be able to find at least 90% of the conserved segments between chicken and man. Currently, close to 300 genes have been mapped on the chicken linkage map (Groenen *et al.*, 2000) and more than 100 different genes have been mapped on the physical map as well, primarily by FISH (D. W. Burt, personal communication).

Chromosome 1

SMOH	7q31-q32	-
▶ GATA3	10p15	2
NRCAM	7q31.1-31.2	12
LYZ	12	10
MGF	12q22	10
IGF1	12q22-q23	10
GNRH	8p21-p11.2	14
NAGA	22q13-qter	15
ADSL	22q13.1	15
H5	22q13.1	15
LGALS4	22	7
MGP	12p	6
LDHB	12p12.2-q12.1	6
GAPD	12p13	6
TCRB	7q35	6
HSD3B	1p13.1	1
G6PD	Xq28	-
▶ CRYAA	21q22.3	17
▶ ZFX-Y	Xp22.1	X
▶ LAMP	13q34	8
P2Y5	13q14.3	-
RB1	13q14.12-q14.3	14
▶ RAB6	2q14-21	8
PGR	11q22.1-22.3	6
FUT4	11q21	9
WNT11	11q13.5	7
HBB	11p15.4	7
NFYB	12q22-24.1	-

Chromosome 2

▶ ACVR2	3p22-p21.3	
SHH	7q36	5
▶ EN2	7q36	5
VIM	10p13	2
MRC1	10p13	2
NPY	7p15.1	6
▶ RARB	3p24.3-p24.2	14
CP49	3q21-q25	-
TGFBR1	9q33-q34.1	4
BMP6	6pter-qter	13
▶ BCL2	18q21.33	1
YES1	18p11.31-p11.22	5
MYL	18p11.3	5
▶ ZFP161	18pter-p11.21	17
▶ ADCYAP1	18p11	-
PENK	8q11.23-q12	4
▶ CALB1	8p12-q11.2	4
CA2	8q22	4
TRHR	8q23	15
▶ MYC	8q24.12-q24.13	15

Chromosome 4

▶ HMG14A	21q22.3	16
VEGA	Xq24-q25	X
PGK1	Xq13.3	X
FMR1	Xq27.3	X
MADH1	4q28	-
OC	4q12-q13	5
ALB	4q11-q13	5
SPP1	4q21-q25	5
ANX5	4q26-q28	3
NFKB1	4q24	3
BNC1	4	5
MSX1	4p16.3-p16.1	5
TGFBR2	[illegible]	
[illegible]	[illegible]	5
TPP2	[illegible]	4

Chromosome 3

BMP2	20p12	2
ADPRT	1q42	1
TGFB2	1q41	1
ACTN2	1q42-q43	13
ARHGDIG	16p13.3	-
HMX1	4p16.1	-
MPR1	6q25.3	17
TCP1	6q25.3-q26	17
ESR	6q25.1	10
VIP	6q24-27	10
MYB	6q23.3-24	10
▶ PLN1	6q22.1	10
FYN	6q21	10
GSTA2	6p12	9
ME1	6q12	9
BMP5	6p12-13	9
EEF1A	6q14	4
RPL18A	19q13.2-q13.3	-
ODC1	2p25	12
▶ MYCN	2p24.3	12

Chromosome 5

INS	11p15.5	7
TH	11p15.5	7
CAPN1	11q13	19
RYR3	15q14	2
PTAFR	1p35-p34.3	4
HTR1D	1p36.3-p34.3	4
BRF1	14q24-24	-
TGFB3	14q24	12
HSPCAL4	14q32.3	12
CKB	14q32.3	12
DNECL	14q32.3-qter	14
BMP4	14q22-23	14

Chromosome 6

PSAP	10q22.1	10
PDE6C	10q24	7
ACTA2	10q22-q24	7
SCD1	10q23	19

Chromosome 7

▶ COL3A1	2q31-q32.3	1
FN1	2q34	1
▶ GBX2	2q37	-
NDUSF1	2q33-34	1
EEF1B	2	1
CD28	2q33	1
VIL	2q35	1
NRAMP1	2q35	1
RPL37A	2q33-37	-
▶ INHBB	2cen-q13	1
MCM6	2q14-q21	2

Chromosome 8

GLUL	1q25	-
PLA2G2A	1q23-qter	-
PTGS2	1q25.2-25.3	1
VTG2	9p21	4
RPL5	1p33-p32	-
GGTB1	1p33-q34	4
JAK1	1p32.3-p31.3	4
DDIT	1p31.2-31.1	3

Chromosome Z

PRLR	5p14-p13	15
GHR	5p14-p12	15
CTSL	9p22.1-q22.2	3
PTCH	9q22.3	13
CHD1	5q15-q21	17
CHRNB3	8p11.2	8
LPL	8p22	8
ALDOB	9q21.3-q31	4
XPA	9q22.3	4
GGTB2	9p21-p13	4
ACO1	9pter-qter	4

Chromosome 16

MHC	6p21.3	17
TAP2	6p21.3	17
BAT8	6p21.3	17
RNF1	13p12	-

E36C06W08

TFRC	3q26.2-qter	16
SNON	3q25-27	3
NCL	2q12-qter	1

E29C09W09

B2M	15q21-q22.2	2
▶ CYP19	15q21	9
▶ TPM1	15q22	9
POLG	15q26.1	-
▶ AGC1	15q26.1	7
IGF1R	15q26.1-qter	7
RPL4	15q	-
GNRHR	4q21.2	5

E30C14W10

▶ CMAF2	16	8
H2AZ	6p21.3	-
CCNE	19q12-q13	-

E21E31C25W12

▶ MYH@	17pter-p11	11
H3F3B	17q25	11
FASN	17q25	11
RAC3	17q24-qter	11
NME2	17q21	11
HLF	17q22	11

E48C28W13W27

MSX2	5q34-q35	13
SPARC	5q31-33	-
POU4F3	5q31	18
SPOCK	5q31	18
CDX1	5q31-33	18
CAML	5q23	13

E35C18W14

HBA	16p13.3	11
▶ NTN2	16p13.3	-

E18C15W15

CRYBB1	22q11.2	5
IGLC1	22q11.2	-
MIFL2	22q11.2	10

E53C34W16

CAMK4	5q21-q23	16
▶ AMH	19p13.3	10

E41W17

Ring3L	9q34	-
ABL1	9q34	2
RPL7A	9q33-q34	2
AK1	9q34.1	2
CD30L1	9q34	2
AMBP	9q32-q33	4

E46C08W18

▶ HSF1	8q24.3	15
LIMK2	22q12	1

E52W19

ACACA	17q21	-
CRK	17p13	11
CASP1	11q22.2-q22.3	9

E59C35W20

▶ TCRA	14q11.2	14
GH	17q22-24	11
▶ COL1A1	17q21.3-q22	11
SLC4A1	17q12-q21	11

E49C28W21

ETS1	11q23.3-q24	9
OPCML	11q25-qter	9
RPS25	11q23.3	9
POU2F3	11	9
APOA1	11q23.3	9

E16C17W22

ARHGDIA	17q25.3	-
LUCA9	3p21.3	-
ARP1	3p21.2-21.1	-

E60E04W23

PGA@	11q13	-
TAX1	1q32	-
TNNT2	1q32	-

E22C19W28

GLI	12q13.2-13.3	10

E15C31

TGFB1	19q13.2	7
RYR1	19q13.1	7

E26C13

MCL1	1q21	3

E32

BMP7	20pter-qter	2
HCK	20q11-q12	2

E54

CDC2L1	1p36	4
AGRN	1p36.3-p32	4
ENO1	1p36ter-p36.13	4
PLOD	1p36.3-p36.2	4
SLC2A1	1p35-pP31.3	4

E57

TP53	17p13.1	11

C24

HSP5A	9q33-34.1	-

Figure 1. Comparative mapping results among chicken, man and mouse. The order of the loci is according to the linkage map described by Groenen *et al.* (2000). The second column in each linkage group shows the location of the loci on the human genetic map according to Genome Data Base (hhtp://www.gdb.org). The third column shows the map location in the mouse. Blocks of conserved synteny between chicken and man and between chicken and mouse are shaded. The genes described in this paper are indicated with arrows.

The recent localisation of QTLs for a number of different traits (Hu *et al.*, 1997; Vallejo *et al.*, 1998; Van Kaam *et al*, 1998 and 1999a, b) on several chicken linkage groups as well as the development of chicken YAC (Toye *et al.*, 1997) and BAC (Crooijmans *et al.*, 2000) libraries has boosted the mapping of genes on many of the linkage groups in chicken. To improve the efficiency of positional cloning in these QTL studies, high resolution comparative maps are clearly needed. Adding new genes to the chicken map and thereby identifying new regions of homology between chicken and man is a first step in obtaining this goal, and can be used as an anchor point for the mapping of additional genes to selected regions of interest in chicken.

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Chapter IV

High throughput mapping of chicken microsatellite markers by automated fluorescent genotyping

Abstract

Optimisation and standardisation are essential for efficient high throughput genotyping of many microsatellite markers in large populations. PCR programs and protocols, therefore, were standardised for every marker with only the annealing temperature varying. Every new marker is tested in a pooled sample of the parents of the Wageningen resource population for the amount of PCR amplification product obtained, possible background and allele size range. If a marker performs badly, adjustments are only made in the PCR annealing temperature. The marker information is subsequently used to make sets of microsatellite markers that are analysed simultaneously on an automated sequencer. Every marker set is tested on the individual parents of our resource population again to check for the amount of PCR amplification product and possible overlapping alleles of different markers with the same fluorescent dye. The amount of amplification product loaded on the automated sequencers is aimed to give a signal around 1000 to prevent read through in the other dyes. To obtain equal amounts of amplification product for the different samples DNA of high quality and equal concentrations are essential. Up till now more than 550 different microsatellite markers have been genotyped in a subset of our resource population (4 families; 196 animals also referred to as the Wageningen mapping population). A subset of these markers (286) has been genotyped in the complete Wageningen resource population (10 families; 486 animals). The number of markers mapped simultaneously in sets, varied from 8 to 21 with an average of 15. The computer programs Genescan and Genotyper were used to analyse the raw data, and finally the genotypings were checked twice for the right allele calling before entering into the database.

Introduction

The development of microsatellite markers, and marker maps in most livestock species has increased the scope of genetic mapping dramatically, making complete genome scans for the dissection of complex traits a feasible option. Microsatellite markers, because of the use of PCR in combination with the fluorescent-based automatic DNA fragment sizing technology (Applied Biosystems), are particularly well suited for the characterisation of genes involved in the more complex, economically important quantitative traits (Lander and Schork, 1994; Haley, 1995). The size of such a project and therefore the number of genotypes that is needed, made optimisation and standardisation of the techniques used essential.

Materials and methods

Mapping population. A three-generation population has been created for mapping both production and health traits in chicken. The G_1 and G_2 animals were genotyped while phenotypes were collected on the G_3 animals. The population consisted of 10 full-sib families with a total of 476 individuals (G_1 and G_2), and an G_3 generation consisting of over 18,000 animals. A subset of 4 families was used for mapping new markers to the Wageningen linkage map.

Microsatellite markers. Microsatellite markers used are described in chapter 2 and 3. Thus far, the total number of microsatellite markers developed and optimised for large scale automated fluorescent genotyping is more than 600. The majority of these markers are optimised, to be used efficiently with the automatic ABI sequencers. The PCR reactions were performed in a total volume of 12 µl containing 10 to 60 ng genomic DNA, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris.HCl pH=8.3, 1 mM tetramethylammoniumchloride (TMAC), 0.1% Triton X-100, 0.01% gelatin, 200 µM dNTP, 0.25 Unit Goldstar polymerase (Eurogentec) and 2.3 pmoles of each primer. The PCR reaction was covered with 10 µl of mineral oil to prevent evaporation. The PCR program currently used is: 2 min at 95°C and 35 cycles of 30 s at 95°C, 30 s at optimal annealing temperature (60° to 45°C) and 30 s at 72°C, followed by a final elongation step of 3 min at 72°C. Primer development was performed manually. Where the primers exist of an equal G/C to A/T ratio, long stretches of the same base were avoided and the 3' end of the primer is either a G or a C. Microsatellites are developed in such a way that the full potential of the fluorescence-based ABI system can be used (Khatib *et al.*, 1993; Cheng *et al.*, 1995; Crooijmans *et al.*, 1993, 1996 and 1997). It is essential that the size range of the microsatellites is evenly spaced over the complete range between 80 and 320 bp, for each of the three dyes (FAM, TET and HEX). Every microsatellite marker is tested on the possible heterozygote parent of the East Lansing Reference population (24000), the possible heterozygote parent of the Compton reference population (B50) and a pooled sample of the parents (20 animals) of the Wageningen resource population. The amplification products applied on the ABI gels when testing new markers was for a FAM or TET labelled marker 0.05 µl and for the HEX labelled marker 0.1 µl. The amount of amplification product is adjusted for each marker to get a signal on the ABI of around 1000. The allele size range of every marker obtained with the pooled sample is used to make the microsatellite sets.

Microsatellite set development. Combinations of markers with the same dye are selected without overlap of alleles according the test results of each marker on the pooled DNA sample. Overlap of alleles of different markers with a different fluorescent dye is possible. In

most cases at least a 10 bp difference between the largest allele of a marker and the smallest allele of the next marker within the same dye was used. Already known map position of markers is taken into account when selecting markers for genotyping in sets. Every microsatellite set is first tested on the 20 individual parents of the mapping population before genotyping the complete families (flow chart; Figure 1). This test is performed to adjust the amount of amplification products of each marker again to a signal of around 1000 on the ABI automated sequencers and to check that there is no overlap in size between adjacent markers of the same dye.

Fragment analysis. An internal standard (TAMRA 350) is added to every lane for size determination of the unknown fragments within the computer program Genescan (Perkin Elmer ABI). We were able to develop sets with up to 21 microsatellites that can be analysed simultaneously in a single lane of an ABI automatic sequencer. The amount of amplification product pooled from every marker is chosen in such a way that the final signal on the ABI sequencer for every marker is around 1000. A mixture of 1 to 1.5 µl of pooled amplification products and 3 µl loading mix (75% deionised Formamide, 10% loading dye and 12.5% Internal standard TAMRA 350 and 2.5% TAMRA labelled 70 bp PCR product) was made and denatured for 5 min at 95°C. This mixture was finally resolved on a 6% denaturing polyacrylamide gel, Sequagel-6 (National Diagnostics), using the ABI DNA sequencer.

Genotyper analysis. After sizing within Genescan the Genotyper software (Perkin Elmer, ABI) is used to define the loci included in the study. An algorithm is used for filtering out stutter peaks from the allele peaks. The software analyses all the peaks in a result file and genotypes each individual (Figure 1). Within the program, Mendelian inheritance and errors are checked twice and allele assignments are edited if required. Genotypes are then used for linkage and QTL analysis.

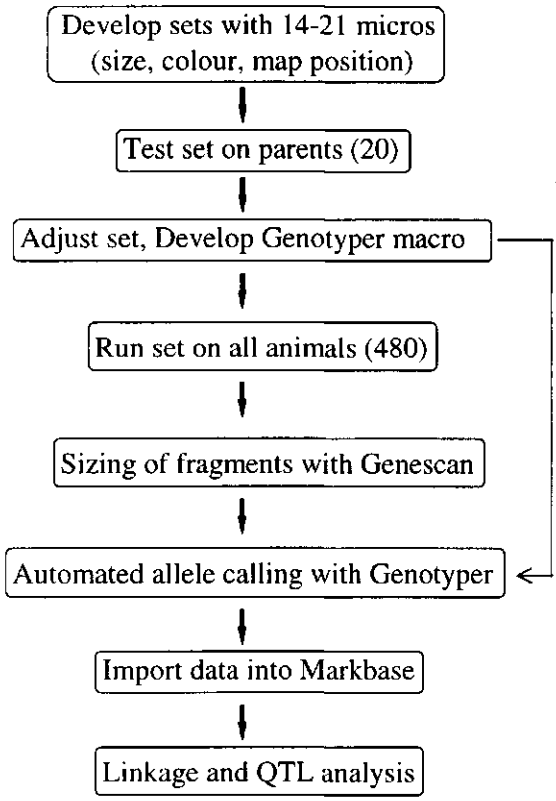

Figure 1. Flow chart showing the development and use of the microsatellite sets for large scale automated fluorescent genotyping in chicken. The arrow at the right side indicates the route when testing the parents.

Results and Conclusions

An experimental population was developed for the characterisation of genes involved in 6 different traits: growth, feed conversion, meat quality, malabsorption syndrome, ascites and susceptibility to salmonella. In total, over 50 different characteristics were measured for these traits. The population was produced by crossing 14 males with 14 females of two commercial broiler dam lines originating from the White Plymouth Rock breed. From the G_1 offspring, 10 males and 10 females were selected to produce the G_2 generation. In total, 456 G_2 offspring were produced. The 18,000 G_3 animals were produced in 5 or 6 batches per experiment in such a way that every male was mated with 6 females, and every female

was mated with 5 males. Furthermore, full-sib mating was avoided as much as possible. The animals were divided in 6 different groups, consisting of 2,000 or 4,000 animals respectively. Each group of animals was used for measuring one of the six different traits studied (Van Kaam *et al.*, 1998 and 1999a, b).

So far, 284 markers have been typed in the complete population (10 families: 478 animals). Because 4 of the 10 families are also being used as a mapping population for new microsatellite markers, an additional 266 markers were typed on these 191 animals, bringing the total number of markers used to 550. The markers are distributed over sets, where the number of markers per set varied from 10 to 21, with an average of 15 (example in Table 1A). These sets are specific for the population used and therefore might not be optimal for other populations because of different allele ranges in these populations. For every set the flowchart described in Figure 1 is used to develop and optimise the set. To diminish the number of PCRs initially, multiplex PCR was performed (Table 1B). A disadvantage of multiplex PCR is the time investment to obtain combination of markers working well together. Therefore we stopped the development of multiplex PCRs after set 7. An example of an ABI 373A gel with a set consisting of 19 microsatellite markers is shown in Figure 2. Marker information is given in Table 1A and 1B where for each marker the fluorescent dye, the allele range for the Wageningen resource population and the amount of amplification product loaded on the gel is given.

Table 1A. Microsatellite marker set 1 consisting of 19 markers grouped per fluorescent dye. The allele range per marker in bp is given for the Wageningen resource panel.

Marker with FAM dye	allele range (bp)	marker with TET dye	allele range (bp)	marker with HEX dye	allele range (bp)
MCW0092	071-077	MCW0100	089-095	MCW0083	088-092
ADL0112	128-134	MCW0110	100-110	MCW0061	118-130
MCW0036	168-176	MCW0059	157-175	MCW0078	139-143
MCW0020	183-185	ADL0040	208-214	MCW0068	172-194
MCW0018	221-235	MCW0035	230-236	MCW0052	235-255
MCW0093	255-265	MCW0103	269-273	MCW0087	269-287
MCW0096	284-298				

Table 1B. Microsatellite marker set 1. Set 1 consists of 19 markers that are amplified in 7 multiplex and 5 individual PCR reactions. The amounts of amplification reaction pooled and loaded on the gel are indicated. Finally 1.1 µl of the pooled amplification product is put on gel of the ABI automated sequencers together with the internal standards.

Marker (s)	amount on gel (µl)	amount pooled (µl)
MCW0083+ MCW0100	0.1	4
ADL0040 + MCW0103	0.04	1.5
MCW0061 + MCW0068	0.1	4
MCW0078 + MCW0052	0.29	12
MCW0093 + MCW0087	0.2	8
ADL0112 + MCW0036	0.04	1.5
MCW0018 + MCW0020	0.05	2
MCW0092	0.1	4
MCW0096	0.06	2.5
MCW0110	0.05	2
MCW0059	0.06	2.5
MCW0035	0.03	1
Total	1.12	45

After genotyping and automated allele calling with the Genotyper software v2.0 (Applied Biosystems), the genotypes were transferred to Markbase, an Oracle based database specifically designed to handle all the genotyping and trait data. From the genotype data set a genetic linkage map was constructed using the Cri-Map linkage package (Chapter 5).

The development of microsatellite sets for simultaneous automated fluorescent genotyping, has proven to be a fast and reliable method for the handling and analysis of several hundred thousands genotypings, necessary for these types of analysis. Important in such a study was the optimisation of every step and standardisation of the whole procedure. The first step starts with the choice of the size and dye of the marker to be able to use the full potential of the fluorescence based ABI system. The second step for every marker is to obtain a nice and clear PCR amplification product without background. Important in the whole set up is good quality genomic DNA with equal concentration to obtain equal signals, stored in an easy 96-well storage system. A single PCR program is performed for standardisation where only the annealing temperature is varied.

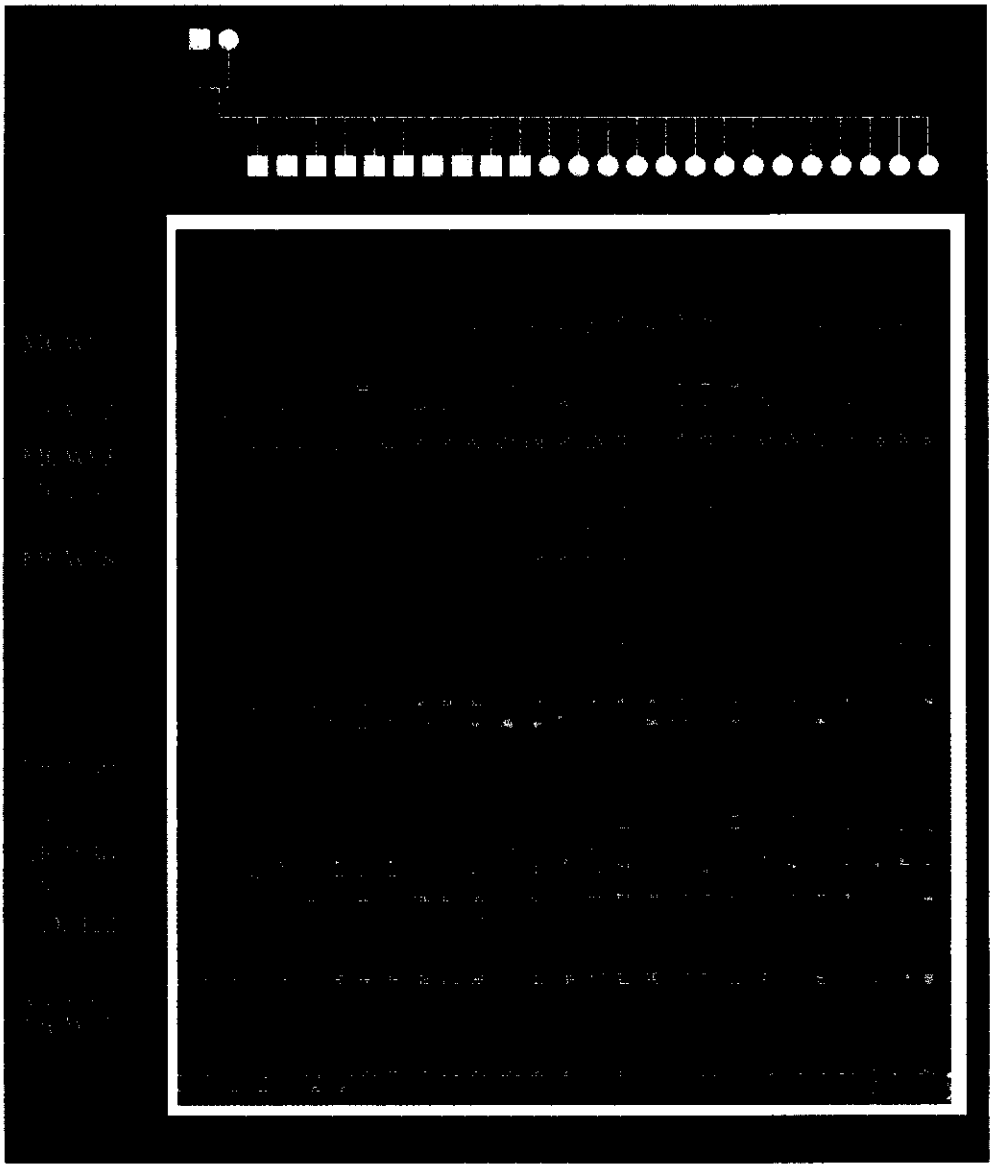

Figure 2. Image of chicken micro set 2 (19 different microsatellite markers) as detected by the ABI 373A automated sequencer. Family structure is indicated at the top where □= male and o = female. Marker names are indicated at the left side. Internal standard markers markers are indicated in red.

Using a single PCR buffer with a fixed amount of magnesium chloride (1.5 mM) and an enhancer (Tetramethylammonium chloride) further standardisation could be established. Finally, to perform these huge amounts of genotypes (almost 200.000) the right equipment is required. The capacity we have in our laboratory (3 ABI automated sequencers in combination with nine 96-well PCR machines) allows us to generate more than 3100 genotypes a day.

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Chapter V

A comprehensive microsatellite linkage map of the chicken genome

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Abstract

A comprehensive linkage map of the chicken genome has been developed by segregation analysis of 430 microsatellite markers within a cross between two extreme broiler lines. The population used to construct the linkage map consists of 10 families with a total of 458 F2 individuals. The number of informative meioses per marker varied from 100 to 900 with an average of 400. The markers were placed into 27 autosomal linkage groups and a Z-chromosome specific linkage group. In addition, 6 markers were unlinked, one of which was Z chromosome specific. The coverage within linkage groups is 3062 cM. Although, as in other species, the genetic map of the heterogametic sex (female) is shorter than the genetic map of the homogametic sex (male), the overall difference in length is small (1.15%).
Forty-five of the markers represent identified genes or ESTs. Database homology searches with the anonymous markers resulted in the identification of a further nine genes, bringing the total number of genes/ESTs on the current map to 54. The mapping of these genes led to the identification of two new regions of conserved synteny between human and chicken and confirmed other previously identified regions of conserved synteny between human and chicken. The linkage map has 210 markers in common with the linkage maps based on the East Lansing and Compton reference populations, and most of the corresponding linkage groups in the different maps can be readily aligned.

Introduction

The developments in molecular genetics in the past decade, particularly the development of microsatellite markers, has boosted the generation of genetic maps in livestock species in recent years (for a review, see Georges and Andersson, 1996). The major goal for these maps in the livestock species is to identify genes that control the expression of economically important traits. The vast majority of these traits are typical quantitative traits, which are controlled by a relatively large number of loci (QTLs) as well as being influenced by environmental factors. Several aspects regarding chickens make this species extremely well suited for experiments aimed at the localisation of QTLs, such as a short generation interval, the ability to generate large full sib pedigrees, and the ease of obtaining large quantities of DNA from the nucleated red blood cells. Furthermore, the size of the chicken genome is small (1.2×10^9 bp; Bloom *et al.*, 1993) compared to that in mammals (3×10^9 bp), whereas the amount of recombination is similar to that in mammals (Rodionov *et al.*, 1992; Burt *et al.*, 1995; this paper). Therefore, once a QTL has been mapped to a certain chromosomal region,

the actual size in basepairs that has to be examined to identify the gene itself is, on average, three fold smaller than in mammals. On the other hand, because of the large number of chicken chromosomes (2n=78) and the small size of the majority of these chromosomes (referred to as microchromosomes), it is more difficult to assign these small linkage groups to specific chromosomes, particularly because of the absence of a clear banding pattern on these microchromosomes.

In chicken, the first genetic map based completely on DNA markers, was published by Bumstead and Palyga (1992). This map, based on the Compton (C) reference population, however, consisted solely of restriction fragment length polymorphisms (RFLP) markers which are not well suited for performance of total genome scans in large populations. The second genetic map to be published (Levin *et al.*, 1993 and 1994) was based on the East Lansing (EL) reference population and consisted primarily of RFLPs, random amplified polymorphic DNA markers and chicken repeat element 1 markers. Since then, both populations have been used to map a considerable number of microsatellite markers as well (Khatib *et al.*, 1993; Crooijmans *et al.*, 1994, 1995 and 1996; Cheng and Crittenden, 1994; Cheng *et al.*, 1995; Gibbs *et al.*, 1995; Ruyter-Spira *et al.*, 1996). Nevertheless, the coverage obtained by these microsatellites is still far from complete. Also, because the sizes of both international mapping populations used to generate the linkage map are rather small (around 50 offspring each), and because both populations are back cross populations, the number of informative meioses for the markers is about 50 per population, which limits the mapping resolution of closely spaced markers.

Anonymous highly polymorphic DNA markers, also referred to as Type II markers (O'Brien, 1991), although ideal for the development of genetic linkage maps, often have the disadvantage of being species specific. To identify the corresponding chromosomal regions between different species and subsequently to be able to use the information available in the other "map-rich" species (comparative mapping), large numbers of genes (type I markers) are needed on the map as well. Currently, over 130 genes have been mapped on the East Lansing map and over 75 genes have been mapped on the Compton map.

Recently, we completed a total genome scan for the dissection of a number of different performance traits in a broiler x broiler cross (Groenen *et al.*, 1997; Van Kaam *et al.*, 1998). In total, 476 animals were typed for 284 microsatellite markers which provided the framework for a highly informative microsatellite linkage map. In addition, 191 animals were typed for an extra 146 microsatellites. Of the 430 microsatellite markers, 54 are adjacent to or within functional genes.

Materials and Methods

Wageningen resource population. In collaboration with the breeding company Euribrid B.V., an experimental population was created containing 10 full sib families of a cross between two extreme commercial broiler lines. The G_0 generation consisted of two broiler dam lines originating from the White Plymouth Rock breed. Unrelated G_1 animals were mated to produce 10 full sib families with an average of 46 G_2 offspring per family.

Analyses of microsatellite markers. The microsatellites used in the present study have been described previously (Crooijmans *et al,* 1993, 1994, 1995, 1996 and 1997; Cheng and Crittenden, 1994; Cheng *et al.*, 1995; Khatib *et al.*, 1993; Gibbs *et al.*, 1995 and 1997).
PCR amplifications were carried out in 12 µl reactions containing 25-50 ng genomic DNA, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH=8.3), 1 mM tetra-methylammonium chloride, 0.1% Triton X-100, 0.01% gelatin, 0.2 mM each dNTP, 0.25 U Goldstar polymerase (Eurogentec) and 2.3 pmol of each primer, one of which was labeled with a fluorescent dye at the 5' end. The amplification reactions were as follows: 5 min at 95°C followed by 35 cycles of 30 s 94°C, 45 s at 55°C, and 90 s at 72°C, followed by a final elongation step of 10 min at 72°C; occasionally, annealing temperatures of 45, 50 or 60°C were used. PCR amplification products for several markers were combined and analyzed simultaneously on a 6% denaturing polyacrylamide gel (sequagel-6: National Diagnostics) on an automatic sequencer (ABI, Perkin-Elmer). Electrophoresis was performed for 3 hours on 12 cm gels, and the results were analyzed using the Genescan and Genotyper software (ABI, Perkin-Elmer).

Linkage analysis. All genotyping data were generated using an ABI automatic sequencer and analyzed with the Genescan and Genotyper software. The number of microsatellites analyzed simultaneously within one lane of the sequencer varied from 14 to 21. Binning of alleles, was performed within the Genotyper program before export to Excel. Although the genotyping and binning are performed automatically by the Genotyper software, all individual genotypes were checked by manually twice (by two different persons). In addition, (potential) typing errors detected with the CRIMAP program were rechecked within Genotyper and corrected where necessary. The data was extracted from Excel worksheets and put into the correct format for the CRIMAP linkage analysis program. Linkage analysis was performed using CRIMAP version 2.4 (Green *et al.*, 1990). Initially, a two-point linkage in which all 430

markers were analyzed against each other analysis was performed. Based upon the results from the two-point analysis, data from markers clearly belonging to the same linkage group were assembled into separate linkage-group-specific files and analyzed using the CRIMAP build option. Finally, the order of the different loci was checked using the CRIMAP flips5 function. Loci whose order relative to one another is well supported (*i.e.* any change in order reduces the lod score by 3 or more) are considered framework loci (Keats *et al.*, 1991). Subsequently, these files were used in the multipoint linkage analyses.

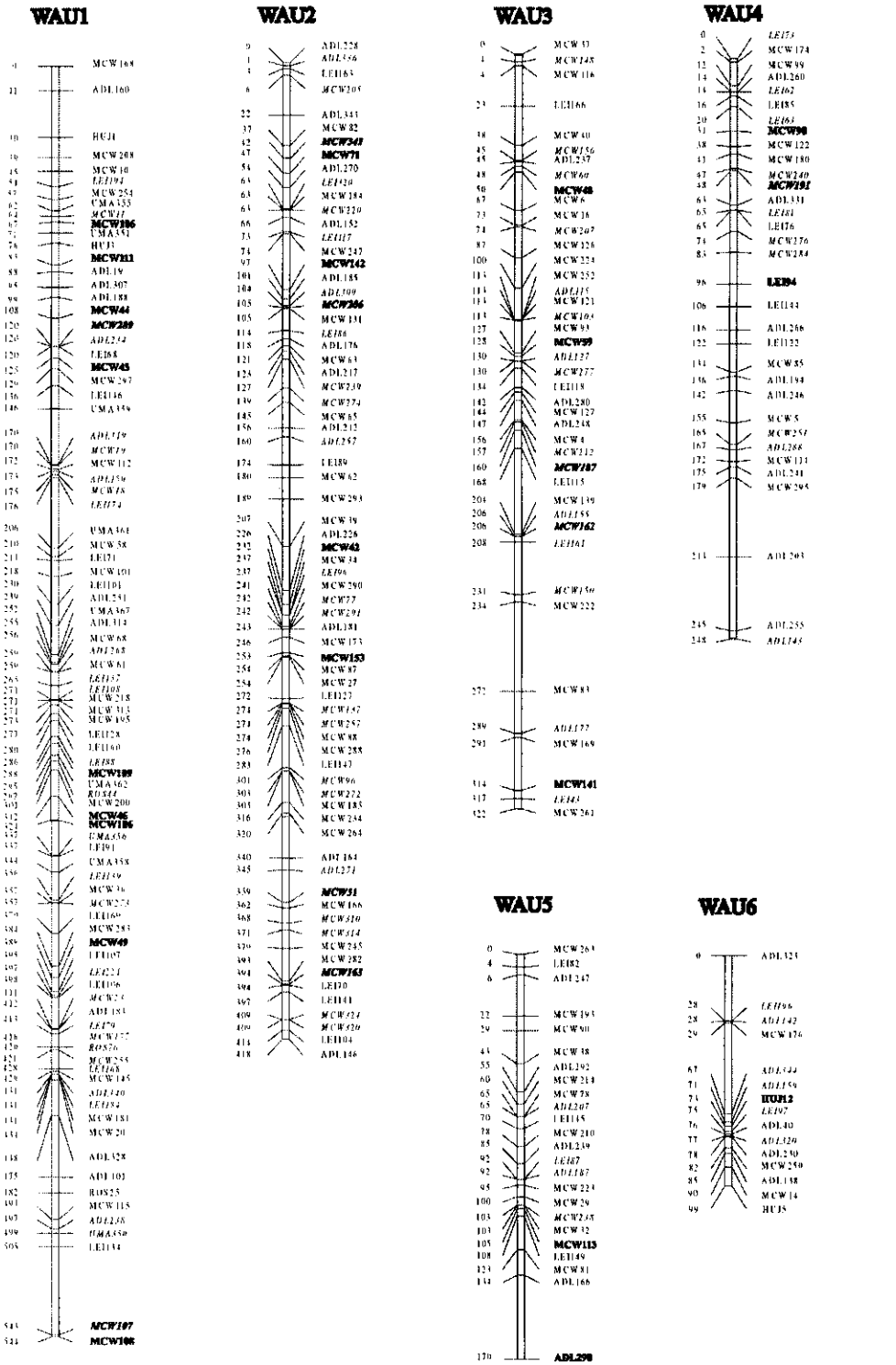
Results

Linkage maps. Twenty-eight linkage groups (Table 1, Figure 1) probably belonging to at least 26 of the autosomes and to the Z chromosome were defined. Six markers (*ADL240, ADL281, MCW188, MCW228, MCW237,* and *MCW248*) appeared to be unlinked to any other marker. Based upon the segregation of the alleles to the male and female offspring, one of the unlinked markers (*MCW237*) could be assigned to the Z chromosome. The total length of the chicken genetic map, excluding the 6 unlinked markers is about 30 Morgans (Figure 1, Table 1). The sizes for the chicken linkage maps, based upon male and female meioses and the percentage by which the male maps differ in size compared to the female maps, are also shown in Table 1. Although there were differences in length for the male and female chromosomes, these differences generally were small. Moreover, for some linkage groups, the male map was larger and for others the female map was larger, resulting in an overall difference between the male and female maps of only 1.15%.

Alignment of WAU linkage map with East Lansing/Compton consensus linkage map and assignment of linkage groups to chromosomes. Of the microsatellites used in this study, 210 were also mapped on the East Lansing/Compton linkage map, making it possible to identify the common linkage groups in both of these linkage maps (Table 1). The corresponding EL/C linkage groups could be identified for all of the WAU linkage groups except for WAU27. This small linkage group consists of only two markers *MCW244* and *MCW340*. In addition, 2 of the 6 unlinked markers were mapped to small EL/C linkage groups (Table 2), and one marker (*MCW237*) is located on the Z chromosome.

Table 1. Summary Chicken Genetic linkage map.

Wageningen Linkage group	Size male	Size female	Size average	% difference male vs female	East Lansing Linkage group
WAU1	555.5	543.0	544.0	+2.3	Chromosome1
WAU2	428.0	405.6	417.9	+5.2	Chromosome2
WAU3	331.8	309.6	321.7	+6.7	Chromosome3
WAU4	241.5	261.4	247.9	-8.2	Chromosome4
WAU5	167.7	173.2	169.8	-3.3	Chromosome5
WAU6	102.1	92.2	98.5	+9.7	E11
WAU7	166.7	144.7	155.7	+13.2	Chromosome7
WAU8	77.7	77.4	77.2	+0.4	E36
WAU9	73.5	82.7	78.9	-11.4	E29
WAU10	80.6	85.8	84.5	-6.5	E30
WAU11	95.4	101.7	97.9	-6.6	Chromosome8
WAU12	48.3	48.7	47.7	-0.8	E21, E31
WAU13	55.4	47.6	51.6	+14.1	E48
WAU14	64.3	84.3	73.6	-31.2	E35
WAU15	44.1	40.6	43.1	+7.8	E18
WAU16	58.4	61.3	59.6	-5.0	E53
WAU17	38.1	50.9	45.9	-33.6	E41
WAU18	23.2	18.1	20.7	+22.0	E46
WAU19	53.5	42.3	47.8	+20.9	E52
WAU20	42.7	52.8	48.5	-23.6	ChromosomeZ
WAU21	10.1	20.1	15.2	-99.0	E49
WAU22	27.9	2.3	28.5	-	E16
WAU23	53.4	47.4	48.8	+12.6	E46
WAU24	18.4	18.6	18.9	-1.1	E11
WAU25	11.2	6.1	10.1	+45.5	E27
WAU26	24.6	24.8	24.8	-0.8	E27
WAU27	0.0	15.6	15.6	-	-
WAUZ	168.0	-	168.0	-	ChromosomeZ
TOTAL	3062.1	3026.8	3062.4	+1.15	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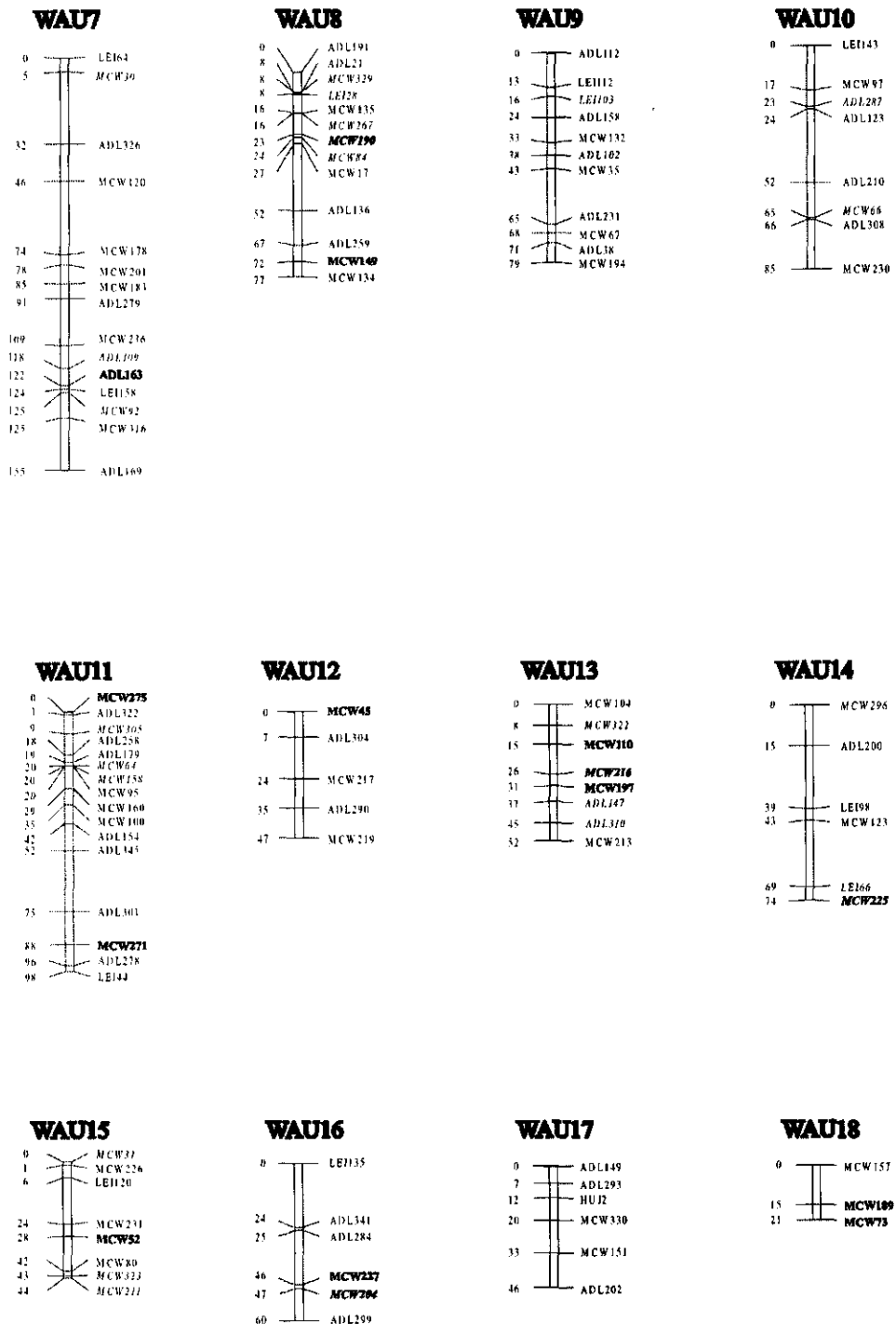
WAU2
WAU3
WAU4
WAU5
WAU6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46 MCW120
74 MCW178
78 MCW201
85 MCW183
91 ADL279
109 MCW276
118 ADL109
122 ADL163
124 LEI158
125 MCW92
125 MCW316
155 ADL169
WAU8
0 ADL191
8 ADL21
8 MCW329
8 LEI28
16 MCW135
16 MCW267
23 MCW190
24 MCW84
27 MCW17
52 ADL136
67 ADL259
72 MCW149
77 MCW134
WAU9
0 ADL112
13 LEI112
16 LEI103
24 ADL158
33 MCW132
38 ADL182
43 MCW35
65 ADL231
68 MCW67
71 ADL18
79 MCW194
WAU10
0 LEI143
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24 ADL123
52 ADL210
65 MCW66
66 ADL308
85 MCW230
WAU11
0 MCW275
1 ADL322
9 MCW305
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19 ADL179
20 MCW64
20 MCW158
20 MCW95
29 MCW160
35 MCW100
42 ADL154
52 ADL145
73 ADL301
88 MCW271
96 ADL278
98 LEI43
WAU12
0 MCW45
7 ADL304
24 MCW217
35 ADL290
47 MCW219
WAU13
0 MCW104
8 MCW322
15 MCW110
26 MCW216
31 MCW197
37 ADL147
45 ADL310
52 MCW213
WAU14
0 MCW296
15 ADL200
39 LEI98
43 MCW123
69 LEI66
74 MCW225
WAU15
0 MCW31
1 MCW226
6 LEI120
24 MCW231
28 MCW52
42 MCW80
43 MCW323
44 MCW211
WAU16
0 LEI135
24 ADL341
25 ADL284
46 MCW237
47 MCW206
60 ADL299
WAU17
0 ADL149
7 ADL293
12 HUJ2
20 MCW330
33 MCW151
46 ADL202
WAU18
0 MCW157
15 MCW189
21 MCW73

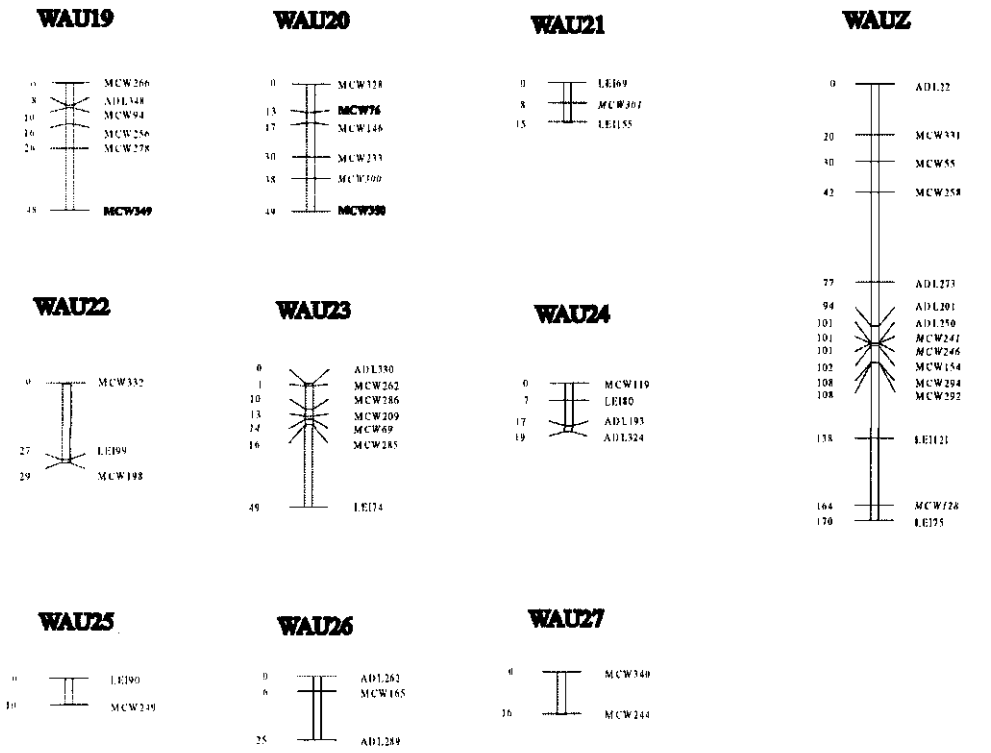

Figure 1. Chicken genetic linkage map. Shown is the sex-averaged genetic linkage map with the estimated Kosambi map distances. Framework loci (loci whose order is supported by odds of at least 1000:1; Keats *et al.*, 1991) are shown in regular script and the remaining loci in italics. Expressed sequences (identified genes and ESTs) are shown in bold.

Because chromosome assignments have been made in the East Lansing linkage map for chromosomes 1 to 8 and 16, the corresponding WAU linkage groups could also be assigned to these chromosomes, except for chromosome 16 (Table 1).

Type I markers. Genetic markers within or adjacent to known genes have been classified as Type I markers (O'Brien, 1991). The inclusion of type I markers will make it possible to access the mapping information that is available in the "map-rich" species such as humans and mice. We have tried to combine the benefits of Type I and Type II (anonymous) markers by developing microsatellites known to reside within known genes and ESTs (Crooijmans *et al.*, 1995; Ruyter-Spira *et al.*, 1996; this paper). Forty-five microsatellites, mapped on our

chicken genetic map fall into this class of markers (Table 3). In addition nine Type II markers showed significant sequence similarity to human sequences in the Genbank/EMBL database (Table 2). Strikingly, the homology often ends at potential splice sites or at the end of the cloned insert, strengthening the assumption that these are significant homologies not occurring merely by chance. The significance of the observed sequence homologies between some of the anonymous microsatellite markers and human sequences is further strengthened by the fact that some of them also show conservation of synteny or linkage between the human and chicken genomes (discussed below).

Table 2. Microsatellites within identified genes.

Marker	Gene	Sequence
HUJ12	Chicken smooth muscle alpha 2 actin gene (*ACTA2*)	genomic
MCW42	Chicken B-cell lymphoma 2 gene (*BCL2*)	genomic
MCW43	Chicken 14 k beta-galactoside-binding lectin gene (*LGAL4*)	genomic
MCW44	Chicken histone H2A, H4 and H3 gene cluster (*HISA*)	genomic
MCW45	Chicken embryonic myosin heavy chain gene (*MYH1*)	genomic
MCW46	Chicken alpha-A-crystallin gene	genomic
MCW48	Chicken N-myc gene (*MYC*)	genomic
MCW49	Chicken lysosomal membrane glycoprotein gene (*LAMP1*)	genomic
MCW51	Chicken vitamin-D-induced calbinding D 28K gene (*CALB1*)	genomic
MCW52	Chicken immunoglobulin gene V26 and V6 gene (*IGVPS*)	genomic
MCW59	Chicken cardiac phospholamban PLB gene (*PLN*)	genomic
MCW71	Chicken engrailed protein gene (*EN2*)	genomic
MCW73	Chicken heat shock factor 3 (*HSPA3*)	cDNA
MCW76	Chicken type I collagen alpha-1 chain (*COL1A1*)	cDNA
MCW225	Chicken Netrin-2	cDNA
MCW341	Chicken activin II B	cDNA
MCW349	Chicken Opsin	cDNA
MCW350	Chicken T Cell receptor alpha chain (*TCRA*)	cDNA

Table 3. Microsatellites within putative genes.

Marker	Gene	Sequence	Human map location
ADL163	85.3 % (163 nt) identity to human intestinal DNA replication protein	genomic	2
ADL240	71.5 % (186 nt) identity to human cosmid LUCA9	genomic	3p21
ADL298	76 % (122 nt) identity to mouse transcription factor C1	genomic	-
LEI94	83 % (115 nt) identity to human EST86460	genomic	-
MCW98	83 % (93 nt) identity to human brain neuron cytoplasmic protein 1 (BNC1)	genomic	4p16
MCW107	76.2 (181 nt) identity to human EST24331	cDNA	-
MCW108	77.6 % (116 nt) identity to human mRNA for GTP binding protein (RAB6)	cDNA	2q14-21
MCW141	72.9 % (1050 nt) identity to human THC (similar to neuroendocrine specific proten C)	cDNA	-
MCW161	78.2 % (110 nt) identity to human mRNA for KIAA0195 gene	genomic	-
MCW186	86.4 % (490 nt) identity to human ZFX (mRNA for putative transcription factor)	cDNA	Xp22.2-21.3
MCW189	66.8 % (292 nt) identity to human adhesion glycoprotein Mac-1	cDNA	-
MCW227	74.5 % (137 nt) identity to human calcium/calmodulin-dependent protein kinase mRNA	genomic	5q21-23
MCW247	66 % (280 nt) identity to human macrophage mannose receptor	genomic	10p13
MCW271	97 % (248 nt) identity to chicken CLFEST63 and 80 % (124 bp) identity to human EST45964	cDNA	-
MCW289	100 % (38 nt) identity to chicken liver adenylosuccinate lyase mRNA	genomic	-

Discussion

Estimated genome coverage of the map. Microsatellite linkage maps are an essential tool in experiments designed to localise loci affecting quantitative traits. Ideally, such experiments require maps with 100% coverage in which the distance between two adjacent markers is 20 cM or less. Based upon the estimated size of 30 morgans for the chicken genome, this would require a minimum of 150-200 evenly spaced markers. To be able to make such a selection in QTL mapping experiments, many more markers need to be placed on the genetic map. The genetic map described in this paper is a step toward this goal. The 430 markers in this paper describe 28 linkage groups that contain 3062 cM (based on the Kosambi mapping function). If one assumes that each of the unlinked markers covers an additional 20 cM and that the markers at the end of the chromosomes cover an additional 10 cM, then the maximum genome coverage of the markers described in this paper is 3750 cM.

New markers that are genotyped generally exhibit linkage to the current map, indicating that most of the genome is covered by the current map. However, it is clear that some of the microchromosomes are poorly represented or not represented at all in the current map. Even if we assume that all the autosomal unlinked markers are situated on separate microchromosomes, it is clear that at least six of the microchromosomes are still missing. The uncertainty of the coverage of the microchromosomes makes it difficult to give an accurate estimate for the genome coverage of the current map. However, based upon a combined comparison between the current map with the previously estimated genome size and the EL/C map, we estimate the genome coverage to be somewhere between 90 and 95%. The poorer coverage of the microchromosomes and the Z chromosome is in good agreement with the results from Primmer *et al.* (1997) who, by *in situ* labeling, showed that these chromosomes particularly have a relatively low concentration of CA microsatellites.

Although the average marker spacing of the map is only 7 cM, there are still several regions on the map where the distance between two adjacent markers is considerably larger than the preferred maximum distance of 20 cM. Particularly the ends of several of the chromosomes clearly are regions in which more markers are needed. At six positions within the linkage map the distance between two adjacent markers is still rather large namely; *LEI134-MCW107* on WAU1, 38 cM; *MCW222-MCW83* on WAU3, 37 cM; *MCW295-ADL203* on WAU4, 34 cM; *ADL203-ADL255* on WAU4, 35 cM; *ADL166-ADL298* on WAU5, 36 cM; *MCW285-LEI74* on WAU23, 33 cM; and *MCW258-ADL273* on the Z chromosome, 35 cM. All but two of these linkages are supported by linkage between more then one pair of markers and LOD scores higher then 4. For example, *ADL298* on WAU5 is linked to both *MCW81* (LOD=3.03) and *ADL166* (LOD=8.12). There are only two exceptions. The first exception is the region between *MCW222* and *MCW83* on WAU3. Here, significant linkage (LOD>3) is only found between *MCW83* and *MCW150* (LOD=6.61). The second exception is the region between *MCW258* and *ADL273* on the Z chromosome, of which the LOD score was only 2.01. Because it is known that both of these markers are located on the Z chromosomes and based on the position of *ADL22*, *ADL201*, *ADL250* and *MCW154* on the EL/C map, it is very likely that this LOD score represents true linkage.

It is our opinion that the mapping of more microsatellites in chicken is still needed, therefore, new marker development will continue in our laboratory and others. Unfortunately, the number of CA microsatellites is about 10 fold smaller than that found in most mammals (Crooijmans *et al.*, 1993; Primmer *et al.*, 1997). Moreover, the observed heterozygosity of the microsatellites is also on average smaller than that observed in mammals. Although, these features, together with the occurrence of the large number of microchromosomes makes the

goal for the ideal microsatellite map more difficult to obtain, the present map provides a good basis towards this goal.

Female versus male recombination: We have observed differences in the recombination rates between the sexes (see Table 1), but the differences are smaller than those observed in other species such as humans (Donis-Keller *et al.*, 1987) and pigs (Archibald *et al.*, 1995). If one looks at the overall difference, the length of the male map is somewhat larger than that of the female map. This would be in agreement with Haldane's prediction (Haldane, 1922) that the linkage map of the homogametic sex (male in chicken) will be larger. However, the observed overall differences between the male and female map is only 1.15%, with several of the female linkage groups actually being larger then their male counterparts. Therefore, although the observed differences in some regions clearly are significant, the overall difference observed (only 1.15%) might be caused by discrepancies in the number of informative meioses between males and females or by typing errors.

Alignment of the WAU and EL/C linkage maps. For most of the WAU linkage groups, the corresponding linkage groups in the EL/C map can be identified (see Table 1). The only exception is the small linkage group WAU22 that contains two markers. Most of the WAU and EL/C linkage groups, particularly the large and intermediate sized linkage groups (WAU1 - WAU11), have many markers in common. Therefore, these linkage groups can be accurately aligned, making it possible to use mapping data from markers mapped only in one of the three linkage maps. The smaller ones, in particular WAU14, WAU18-WAU22 and WAU25 have only one or two markers in common, and therefore make it difficult or impossible to align accurately these with their EL/C counterparts.

In three instances, we were able to connect two previously unlinked EL/C linkage groups. On the EL/C map *ADL228* and *ADL336* have been assigned to the small linkage group E56. Our data clearly show that these markers are located at one of the ends of chromosome 2 (WAU2). Similarly, *ADL298* which was assigned to the small linkage group E34, is linked in our data to *ADL166* and *MCW81*, which places this marker at one of the ends of chromosome 5 (WAU5). Finally, markers assigned to linkage groups E21 and E31 map to linkage group WAU12.

We observed only three discrepancies between the WAU and EL/C linkage maps: (1) *MCW62* is mapped to chromosome 2 (WAU2), whereas it is mapped to linkage group E35 (equivalent with WAU14) on the EL/C map. In our data set *MCW62* is linked to 9 different markers in that region with LOD scores as high as 38 (*MCW62-LEI89*, LOD=38.71;

MCW62-ADL212, LOD=37.83) indicating that it has been placed correctly. (2) *MCW166* is also mapped to chromosome 2 (WAU2) whereas it is mapped to chromosome 4 (equivalent with WAU4) on the EL/C linkage map. Again, *MCW166* is linked to 6 markers in that region with LOD scores as high as 19 (*MCW166-MCW51*, LOD=19.06; *MCW166-MCW245*, LOD=9.88). (3) In our data set *MCW76* has been mapped to the autosomal linkage group WAU20, whereas it has been mapped to the Z-chromosome on the EL/C map. We observed several female F1 animals that were heterozygous for this marker, excluding the possibility that it is located on the Z-chromosome. Although it still might be possible that this marker maps to the pseudo autosomal region of the W/Z-chromosomes, we do not think this to be very likely.

Apart from these three discrepancies, the other markers that are on both maps map to the corresponding linkage groups, and are in the same order. Generally, the observed distances of the linkage groups in our map are somewhat larger than those of the EL map and smaller than those of the C map.

Type I markers. As discussed above, good microsatellite maps are essential tools in localising the QTL that are involved in complex multifactorial traits. The next step, generally, will be to try to identify potential candidate genes in the regions identified with the total genome scan. In animal genomics, comparative mapping plays an essential role toward the identification of potential candidate genes, because this makes it possible to utilise the large amount of data that are available in map-rich species such as human and mouse. Comparable to having a good microsatellite map to perform a total genome scan, for comparative mapping it is essential to have a large number of genes (type I markers) that have been mapped to be able to make a good comparison possible with the maps of other species. We tried to combine both goals by developing microsatellites from cDNA sequences (Ruyter-Spira *et al.*, 1996 and 1998) and genes present in the Genbank/EMBL sequence databases (Crooijmans *et al.*, 1995, this paper). This has resulted in mapping 18 known genes and 27 ESTs on the current map. The map location of several of these genes confirms previously identified regions of conserved synteny between human and chicken (Burt *et al.*, 1995; Klein *et al.*, 1996; Cheng 1997; Heltemes *et al.*, 1997).

For nine microsatellites that have been derived from anonymous genomic sequences, we have identified potential exons that show a high percentage of sequence similarity with identified human genes and/or ESTs (Table 3). This shows that database searches, even with anonymous genomic sequences, will aid in the potential identification of genes and thus help in the identification of common genomic regions in different species. Although care has to be taken in assigning anonymous markers as putative genes only based on sequence homology,

additional information can strengthen these assignments. For example, a stretch of 38 bp of the sequence of the clone of microsatellite *MCW289* shows 100% sequence identity to the chicken liver adenylosuccinate lyase mRNA. This sequence identity at one site continues to the end of the cloned fragment, and at the other end it stops at a putative splice site. Another example is the homology of *MCW247* with the human macrophage mannose receptor gene (*MRC1*). Here, 66 % sequence identity is found for two different regions on the cloned and sequenced fragment of *MCW247*. These two regions, which are separated by 325 bp, show homology to exons 10 and 11 of the human *MRC1* gene. Interestingly, the human *MRC1* gene is mapped to human chromosome 10p13 and is closely linked to the *vimentin* gene. In chicken, the *vimentin* gene is located on chromosome 2, closely linked to *LEI117, ADL152* and *ADL307* (Smith *et al.*, 1997). On the WAU linkage map, these three microsatellites are located on WAU2, and all three are close linked to *MCW247*. This suggests that *MCW247* is indeed located within the chicken homologue of the *MRC1* gene and that the *Vimentin* and *MRC1* gene are syntenic in human and chicken. Other examples are microsatellites *ADL163* and *ADL240*. Microsatellite *ADL240*, which is still unlinked in our data set, has been mapped to the EL linkage group E16 at 15 cM distance from the ADP-ribosylation factor 2 gene (*ARF2*). In human, this gene has been mapped to 3p21.1-3p21.2. Interestingly, *ADL240* shows 71.5% sequence identity with a 186-nt region on a cosmid that has also been mapped to 3p21 in human. Finally, microsatellite *ADL163* which shows 85.3% sequence identity with human p105MCM mRNA (intestinal DNA replication protein) is located on chicken chromosome 7 (WAU7) at a distance 20 to 30 cM from the ribosomal protein-encoding gene *L37A* (Nanda *et al.*, 1996). The *L37A* gene is part of a region on chicken chromosome 7 that is syntenic with human chromosomal region 2q33-34. In human, the gene encoding p105MCM has also been mapped to chromosome 2.

Concluding remarks. The linkage maps of the chicken chromosomes described in this paper, because of the large number of informative meioses involved, will be the basis for a high-resolution map in chicken that will be an effective tool for the QTL-mapping experiments in chicken currently under way. Furthermore, the results of the sequence database searches, clearly indicate that the analysis of random sequences in combination with comparative mapping can be very efficient tools for using information from species with gene dense maps (particularly human) in genome research on species with less well developed maps. Particularly, this kind of analysis will be of great value in the identification of potential candidate genes for the QTL identified in the QTL-mapping experiments currently being analyzed.

Note. The linkage maps described in this paper, the two-point recombination distances, and lod scores are available through our Web site: http://www.zod.wau.nl/vf.
Information regarding the markers used is also available on the same web site. An alignment of the WAU linkage maps with the other linkage maps in chicken (EL, C) and with the chicken physical map will become available through the web site of the Roslin Institute, Edinburgh (http://www.ri.bbsrc.ac.uk/genome_mapping.html).

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Chapter VI

Two-dimensional screening of the Wageningen chicken BAC library

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Abstract

We have constructed a Bacterial Artificial Chromosome (BAC) library that provides 5.5-fold redundant coverage of the chicken genome. The library was made by cloning partial *Hin*dIII digested high-molecular-weight (HMW) DNA of a female White Leghorn chicken into the *Hin*dIII site of the vector pECBAC1. Several modifications of standard protocols were necessary to clone efficiently large partial *Hin*dIII DNA fragments. The library consists of 49,920 clones arranged in 130 384-well plates. An average insert size of 134 kb was estimated from the analysis of 152 randomly selected BAC clones. The average number of *Not*I restriction sites per clone was 0.77. After individual growth, DNA was isolated of the pooled clones of each 384-well plate, and subsequently DNA of each plate was isolated from the individual row and column pools. Screening of the Wageningen chicken BAC library was performed by two-dimensional PCR with 125 microsatellite markers. For 124 markers at least one BAC clone was obtained. FISH experiments of 108 BAC clones revealed chimerism in less than 1%. The number of different BAC clones per marker present in the BAC library was examined for 35 markers which resulted in a total of 167 different BAC clones. Per marker the number of BAC clones varied from 1 to 11, with an average of 4.77. The chicken BAC library constitutes an invaluable tool for positional cloning and for comparative mapping studies.

Introduction

To systematically analyze complex genomes, various large insert libraries are needed. Two host systems have been developed for this purpose: *Saccharomyces cerevisiae* and *Escherichia coli*. Large DNA molecules (100-2000 kbp) can be introduced and propagated in the form of yeast artificial chromosomes (YACs) in *S. cerevisiae* (Burke *et al.*,, 1987). The large insert cloning systems developed in *E. coli* such as bacteriophage P1 derivates (Sternberg 1990), bacterial artificial chromosomes (BAC, Shizuya *et al.*,, 1992), and P1 derived artificial chromosomes (PAC, Ioannou *et al.*,, 1994) can adopt DNA fragments of 80-350 kb. YACs are efficient in covering large physical areas and are, therefore, easier to make contigs. However, the YAC system has a low cloning efficiency, a high incidence of chimeric clones (Neil *et al.*,, 1990; Anderson 1993), instability of insert DNA, and it is difficult to separate donor from host DNA. The BAC/PAC cloning systems do not show these disadvantages. Furthermore, BAC DNA is amenable for direct sequencing of the insert ends, offering a further advantage compared to YACs in building contigs.

Consequently, many BAC and PAC libraries are currently available in human (Shizuya *et al.*, 1992; Kim *et al.*, 1996; Asakawa *et al.*, 1997; Osoegawa *et al.*, 1998) and other model organisms (Osoegawa *et al.*, 1998). Genome mapping efforts to identify genes controlling quantitative traits are currently in progress in all major farm animals including chicken (Groenen *et al.*, 1998; Van Kaam *et al.*, 1998). For these reasons BAC/PAC and YAC libraries have been constructed for cattle (Cai *et al.*, 1995; Libert *et al.*, 1993), pig (Rogel-Gaillard *et al.*, 1997; Al-Bayati *et al.*, 1999), goat (Schibler *et al.*, 1998), sheep (Vaiman *et al.*, 1999) and chicken (Toye *et al.*, 1997; Zoorob *et al.*, 1996; Zimmer and Verrinder-Gibbins 1997). Although chicken PAC/BAC libraries have been described previously (Zoorob *et al.*, 1996; Zimmer and Verrinder-Gibbins 1997), these libraries are not publicly available. Furthermore, the BAC library described by Zimmer and Verrinder-Gibbins (1997) had a limited number of clones that resulted in very low genome coverage. For these reasons, we constructed a chicken BAC library with a high genome coverage that is used in physical mapping and as a key resource in positional cloning. In this paper we present the construction, characterization, and two-dimensional PCR screening of the Wageningen chicken BAC library.

Materials and Methods

Preparation of high-molecular-weight DNA. High-molecular-weight (HMW) DNA was prepared from blood of a female White Leghorn chicken embedded in agarose microbeads according to Zhang *et al.*, (1994). One ml of fresh EDTA blood was mixed with 9 ml HB buffer (10 m*M* Tris-HCl, 10 m*M* EDTA, 80 m*M* KCL, 500 m*M* sucrose, 1 m*M* spermine, 1 m*M* spermidine, 0.15% beta-mercaptoethanol, and 0.5% triton X-100, pH=9.4) and heated to 40 °C, together with 10 ml of 1% LMP agarose and 40 ml light mineral oil (Sigma) both pre-warmed at 40 °C. This mixture was transferred into 200 ml cold HB buffer and stirred on ice for 20 min at maximum speed on a stirrer. Microbeads were centrifuged at 3000 rpm and washed twice with cold HB buffer. Microbeads were incubated in lysis buffer (0.5 *M* EDTA, 1% sarcosyl and 1 mg/ml proteinase K) at 50 °C for 48 h. The beads were washed three times in cold TE and incubated in TE with 40 μg/ml PMSF (phenyl methyl sulphonyl fluoride) for 1h at 50 °C to inactivate the proteinase K and finally washed four times 30 min in TE on ice. For partial digestion, 50 μl of beads were equilibrated on ice twice for 40 min with *Hin*dIII restriction buffer and 30 min with *Hin*dIII restriction buffer and 0.5 U of

restriction enzyme *Hin*dIII (BRL-Life Technologies). Partial digestion was carried out for 5 min at 37 °C and stopped with 1/10 volume of 0.5 *M* EDTA pH 8.0.

Size selection of genomic chicken DNA. Pulsed field gel electrophoresis (PFGE) was carried out on a 0.8% low melting point (LMP) agarose gel in 0.5 x TBE (45 m*M* Tris, 45 m*M* Trisborate, 1 m*M* EDTA). Multiple digestions (30) were pooled and loaded on the gel. Large DNA fragments were selected by three rounds of size selection separated on a 0.8% LMP agarose (FMC, Rockland, Me) in 0.5 TBE. PFGE (CHEF mapper; Bio-Rad) was conducted for 20 h at 11 °C, 4V/cm, 90 s switch time at 120 degree angle. The regions of the gel containing 150-300 and 300-450 kb fragments were excised and stored in storage buffer (10 m*M* Tris, 25 m*M* EDTA, 0.3 m*M* spermine, 0.75 m*M* spermidine and 125 m*M* NaCl) until the next loading. Before the second electrophoresis under the same conditions, the excess of water of the excised agarose plug was carefully removed with tissue to concentrate the DNA and melted at 65 °C. The region of the gel containing 125-400 kb fragments was excised and treated the same way as described after the first size selection. The third sizing PFGE was conducted for 10 h, 10 °C, 160 V/cm, 3s switch time at 120 degree angle on a 0.9% LMP agarose gel in 0.5 TBE. The agarose plug with the congested DNA band of the PFGE gel around 150 kb was excised and concentrated as described above. The agarose plug was melted and incubated with Agarase (1U/100 μl) before ligation.

Vector preparation. The vector pECBAC1 was prepared as described by Frijters *et al.*, (1997). The *Hin*dIII site was used for cloning purposes.

Construction of the BAC library. The ligation was carried out with 90 ng of large *Hin*dIII genomic fragments with 9 ng *Hin*dIII-digested and dephosphorylated pECBAC1 vector in 1x ligase buffer with 2U T4 DNA ligase (USB) overnight at 16 °C. The ligation reaction was transformed into electrocompetent ElectroMAX DH10B cells (Gibco BRL) (1 μl of ligation product with 20 μl cells), with a Cell- Porator system (Gibco BRL) following the manufacturer's protocol (350 V, capacitance: 330μF, Impedance: low ohms, charge rate: fast, voltage booster resistance: 4,000 ohms). Subsequently cells were plated on LB agar containing 12.5 μg/ml chloramphenicol (Cm), 0.5 m*M* X-GAL, and 40 μg/ml IPTG. White recombinant BAC clones were picked directly in 384 well plates (Nunc) containing LB freezing medium [LB, 36 m*M* K_2HPO_4, 13.2 m*M* KH_2PO_4, 1.7 m*M* sodium citrate, 0.4m*M*

$MgSO_4$, 6.8 m*M* $(NH_4)_2SO_4$, 4.4% (vol/vol) glycerol] with 12.5 μg/ml Cm and grown overnight, at 37°C. Three copies were made from every plate with a 384-pin replicator (NUNC), grown overnight and stored at –80 °C. Individual BAC clones were isolated from 4-ml cultures grown overnight in LB with 12.5 μg/ml Cm using an alkaline lysis procedure (Sambrook *et al.*,, 1989). The DNA pellet was dissolved in 40 μl TE, and 10 μl DNA was digested with 5U *Not*I (Gibco BRL) for at least 3 h at 37 °C. Insert sizes were analyzed on a 1% agarose gel in 0.5 TBE (runtime 15 h, 6 V/cm, switch time ramping from 5 to 15 s, and reorientation angle of 120 degree at 11 °C).

Screening of the BAC library. Screening was performed either by hybridization or by PCR. For screening by hybridization, high-density filters were made by double spotting four 384-well plates onto sterile Hybond N+ filters (Amersham), with a BioMEK 2000 workstation (Beckman). Low-density filters were made by manually spotting of 288 BAC clones onto Hybond N+ filters. These filters were hybridized with a radioactive end-labeled probe $(TG)_{13}$ to detect repeats. Positive BAC clones were digested with *SAU3A* and cloned into the *Bam*HI site of pBluescript. From each ligation reaction, between 150 and 300 white transformants were picked in 384-well plates and grown overnight at 37 °C in LB freezing media with Ampicilin 50 μg/ml and stored at –80 °C. Subclones were spotted on Hybond N+ and hybridized with a radioactive $(TG)_{13}$ probe. Positive clones were sequenced with the M13 forward and reverse primers on an ABI automated sequencer 373/377 (Perkin-Elmer), and primers were designed. Markers were tested for polymorphism as described by Crooijmans *et al.*, (1997) and mapped, if possible, in the Wageningen resource population (Groenen *et al.*, 1998).

Screening by PCR was performed by a two-dimensional PCR screening method. Each clone from a 384-well micro titer plate was cultured in 700 μl LB + 12.5 μg/ml Cm overnight at 37 °C in four 96-well deep-well refill blocks (Micronic). In total, 49,920 BAC clones were cultured individually. For each 384-well plate, DNA was isolated of i) the whole plate (400 μl culture of each clone), ii) individual rows A to P (150 μl culture of each clone), and iii) individual columns 1 to 24 (150 μl per clone). BAC DNA was isolated according to Sambrook *et al.*, (1989) and dissolved in 100 μl TE. Identification of particular BAC clones was done in a two-stage PCR process and analyzed on a 1.5% agarose gel. The first step was the identification of the 384-well plate pool containing the microsatellite repeat by PCR, followed by PCR screening of the individual row and column pools of that particularly plate. To confirm the purity of the positive BAC clone, each BAC

clone was plated on LB, 1.5% agar, 12.5 μg/ml Cm, 40 μg/ml X-GAL, and 40 μg/ml IPTG. Two single colonies were picked from the plate with a sterile toothpick and streaked into a well of a 96-well plate. Each clone was dissolved in 6 μl water, and colony PCR was performed in 12 μl as described by Crooijmans *et al.*, (1997) and analyzed on a 1.5% agarose gel in 0.5 TBE. The pure positive BAC clones were finally stored individually at -80 °C. Both random microsatellite markers from each WAU linkage group (Groenen *et al.*, 1998) and microsatellite markers located at the ends of the linkage groups were selected. At least 3 markers were used in case of the large linkage groups. The PCR consisted of 6 μl of DNA (2000 times diluted in TE) in a total of 12 μl as described by Crooijmans *et al.*, (1997).

Fluorescent In Situ Hybridization (FISH). Miniprep DNA of 49 random picked BAC clones and 59 BAC clones with known map location were used in FISH experiments to evaluate the degree of chimerism of the library. BAC DNA was either labelled by random priming with biotin-16-dUTP or with digoxigenin-11-dUTP on chicken metaphases derived from a chicken primary fibroblast cell culture (Ruyter-Spira *et al.*, 1998; Morisson *et al.*, 1998).

Results

Construction and evaluation of the library. The library was constructed from two independent ligations from zones 150-300 kb and 300-450 kb. In total 116 electroporations were performed to make the chicken BAC library. In total, 49,920 BAC clones were individually picked and arrayed in 130 384-well microtiter plates. A total of 152 BAC clones was sized after *Not*I digestion by PFGE, giving an average insert size of 134 kb (Figure 1). The insert size of the BAC clones was examined in the range of 50-255 kb. Non- recombinant BAC clones were not observed. Assuming the chicken genome size of 1200 Mb, the BAC library represents 5.57 genome equivalents. The occurrence of *Not*I sites within the 152 BAC clones ranged from 0 to 4 with an estimated average of 0.77 *Not*I sites per clone. On the basis of the average insert size of the BAC clones of 134 kb, *Not*I sites occur once every 174 kb in the chicken genome. In Situ hybridization of 108 different BAC clones (49 random and 59 with known genetic map location) on chicken metaphase spreads showed specific chromosome location for 107 BAC clones; only one BAC clone identified two different chromosomes (unpublished results; A Vignal, personal communications).

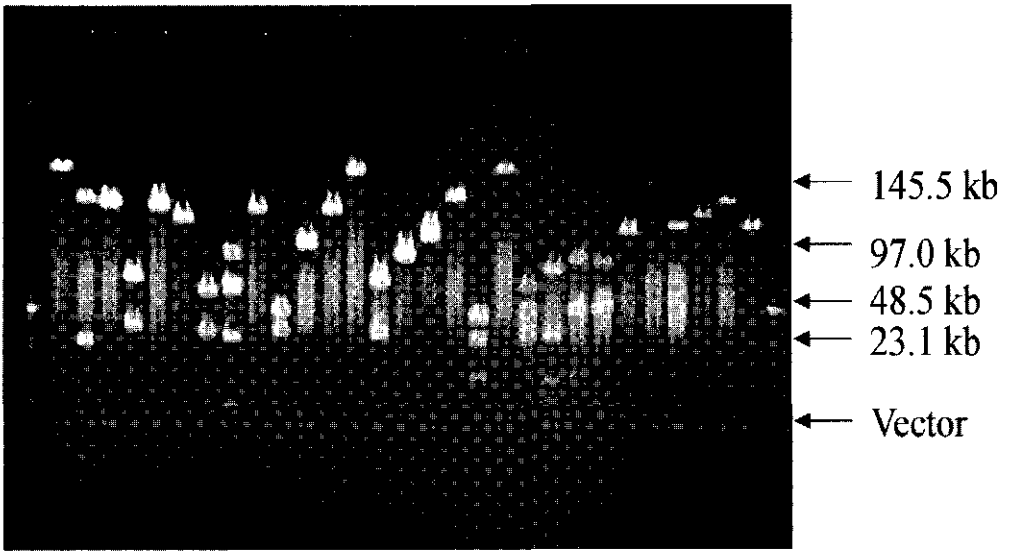

Figure 1. Randomly selected *Not*I-digested BAC clones analyzed after Pulse Field Gel Electrophoresis (PFGE) on a CHEF mapper (Bio-Rad). All BAC clones showed the vector band except the clone in lane 21, which was not digested. Flanking lanes are Low Ranger Marker PFG from New England Biolabs.

Occurrence of microsatellite repeats in BAC clones. The presence of (TG) repeats within the BAC clones was tested by spotting 288 BAC clones on a Hybond N+ membrane filter. Treated filters were hybridized with a ^{32}P-labeled $(TG)_{13}$ probe. A total of 82 BAC clones gave a positive signal (29%), indicating that (TG) repeats in chicken occur once every 470 kb. 24 BAC clones giving a very strong signal were subcloned in pBluescript. From ten BAC clones, the (TG) positive subclones were recovered and sequenced. The number of repeats in these clones varied from 5 to 22. The sequence of two of the clones was already present in the database, and these appeared to be identical to marker UMA1.019 (mapped to chromosome 1) and to marker MCW247 (mapped to chromosome 2). Primers were designed for three loci, and one could be mapped on the chicken linkage map (MCW360; chromosome 2). One primer pair did not amplify any product and one microsatellite was monomorphic.

Two-dimensional PCR screening of the BAC library with microsatellite markers. BAC clones were pooled to facilitate screening by PCR. The two-step PCR screening was performed for 125 markers, mostly microsatellite markers that have been mapped on the

chicken linkage map previously. The screening strategy is described in Figure 2 with an example of the screening results for marker MCW219. At least three markers from every linkage group, including markers from both ends, were used to identify at least one BAC clone per marker. BAC clones could be identified for all markers except for MCW076 (T cell receptor alpha gene), which is equivalent to a success rate of 99.2%. For 35 markers, all BAC clones were isolated from the library, which resulted in 167 different BAC clones. The number of BAC clones per marker varied from 1 to 11, with an average of 4.77.
Specific information regarding these BAC clones and the markers are listed on our chicken genome mapping site Http://www.zod.wau.nl/vf/.

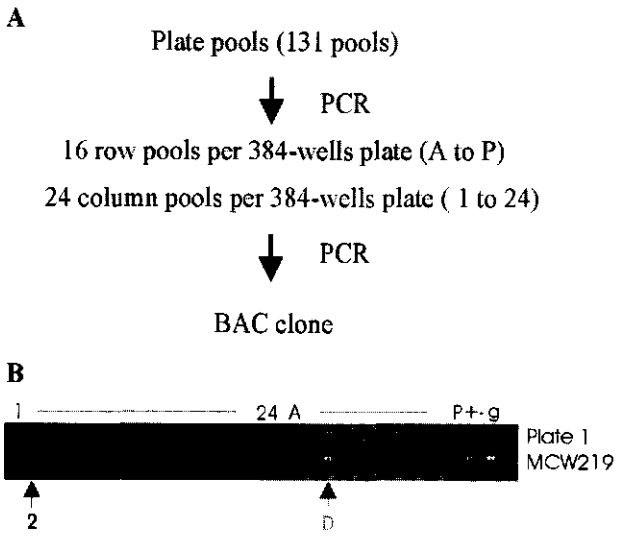

Figure 2. Two-dimensional PCR screening of the chicken BAC library.
(A) Two–dimensional PCR screening strategy. For details, see Materials and Methods. **(B)** PCR screening of marker MCW219 in the row and column pools of plate 1. PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide. The coordinates of the MCW219-positive BAC clone in the 384-well plate number 1 is D2. P+ is the positive plate pool (1), - is the negative control (TE) and g is genomic DNA of a White Leghorn chicken.

Discussion

We have generated a 5.57-genome equivalent BAC library of a female White Leghorn chicken, using partial *Hin*dIII digested DNA. By using a female chicken, we included the two different sex chromosomes Z and W. The average insert size of 134 kb was obtained after modification and adjustment of protocols to obtain large, partially digested *Hin*dIII DNA fragments. One of the major problems in the purification of large DNA fragments is the contamination of small DNA fragments of around 50 kb which are trapped between the larger DNA molecules. These trapped fragments will transform with much greater efficiency, and as a result the average insert size of the library will drop drastically. Our digestion conditions did have optima at much lower concentrations of reagents and shorter incubation times than those reported for the preparation of other BAC libraries. Compared with the protocols described by Cai *et al.*, (1995), Zimmer and Verrinder-Gibbins (1997), Schibler *et al.*, (1998) and Vaiman *et al.*, (1999), the digestion time was only 5 minutes instead of 20 minutes and the amount of enzyme (0.5 U per 50 μl of agarose beads) was less by a factor of 4 to 10. This is probably due to the purity of our HMW chicken DNA. The digestion results of HMW DNA in agarose beads are very good, and the ease of working with agarose beads is preferable to agarose plugs. Microbeads were preferred over plugs because the use of beads increases the surface area surrounding the tissue sample by approximately 1000-fold, thereby allowing for a more efficient and rapid diffusion of chemicals and enzymes into and out of the agarose beads.

The next step of optimization concerns the PFGE conditions and the number of runs. The one-size selection protocol (Schibler *et al.*, 1998; Vaiman *et al.*, 1999) and the two-step size selection protocols used by Cai *et al.*, (1995); Zimmer and Verrinder Gibbins (1997), and Frijters *et al.*, (1997) could not eliminate the small 50-kb fragments from the large fragments sufficiently. The major problem of using a three-size selection protocol, however, is the concentration and the amount of DNA left after the last sizing. The concentration should be high enough (at least 0.8 ng/μl), and a total amount of DNA of at least 40 ng is needed for the construction of a library. This problem was solved by drying the excess of liquid from the excised gelband every time after sizing and before loading on the new PFGE gel. The last PFGE is a very important step to obtain the compressed band containing the DNA fragments. Furthermore, a 2- or 3-s pulse in the last electrophoresis is preferable to a 5-s pulse. Using these modifications, the three-step sizing protocol allowed us to obtain the large sized fragments with a DNA concentration of 1-2 ng/μl. Although the excised fragments were estimated to be between 150 and 250 kb in length, the final

insert size was on average 134 kb, which is probably owing to overloading of the HMW DNA compared with the HMW markers. The overestimation of fragment sizes has consistently been observed during the construction of other BAC libraries (Wang *et al.*,, 1995; Woo *et al.*,, 1994 and Frijters *et al.*,, 1997).

In the 152 chicken BAC clones analyzed, *Not*I sites occur on average at a frequency of 0.77 per BAC within the cloned fragment. This is much higher than the goat BAC library (Schibler *et al.*,, 1998) and sheep BAC library (Vaiman *et al.*,, 1999), where the frequency of *Not*I sites was respectively 0.45 and 0.43. The cattle BAC library (Cai *et al.*,, 1995) did have a frequency is 0.22 *Not*I sites (32 BAC clones tested). These frequencies indicate that the G/C content in chicken is 1.5 to 2 times higher than goat and cattle. The chicken BAC clones described by Zimmer and Verrinder-Gibbins (1997) observed only a limited number of clones with internal *Not*I sites, which is contrary to our observations. A possible explanation for this observed discrepancy is that the BAC clones described by Zimmer and Verrinder-Gibbins (1997) might be due to incomplete digestion. This is further supported by the absence of vector fragments after *Not*I digestion and PFGE. This would also explain the average insert size of 390 kb of their library which might therefore be overestimated.

Screening of the Wageningen chicken BAC library can be performed either by hybridization on high-density filters or by PCR. The two-dimensional PCR screening is much more efficient and faster. In total, 125 markers were used to screen the BAC library, of which 124 did yield at least one positive clone. No BAC clones were obtained after screening the BAC library with microsatellite marker (MCW76), which is equivalent to a failure rate of 0.8%. This value is much lower than the cattle BAC library (Cai *et al.*,, 1995), where 1 out of 33 markers (3%) failed, and the sheep BAC library (Vaiman *et al.*,, 1999), where 4 out of 77 microsatellite markers failed to detect a BAC clone. Similar results were obtained with the goat BAC library (Schibler *et al.*,, 1998) where 1 out of 166 markers (0.6%) failed to detect a BAC clone. The representation of the chicken genome in our BAC library of around 99.2% is in agreement with the calculated representation of 99.6% for a library of a size equivalent to 5.5 times the genome (Clarke and Carbon, 1976). For the 35 markers, the average number of BAC clones per marker is 4.77, which is close to the estimated 5.5 times coverage of the library. Moreover, no empty clones were detected, and one possible chimeric clone out of 108 different BAC clones (0.9%) was found, which makes our library very powerful. The amount of chimerism is much lower than that of the chicken YAC library (Toye *et al.*,, 1997).

As reported by Morrison *et al.*,, (1998), the development of new microsatellite (CA) markers from randomly selected BAC clones is feasible but very time consuming. From the

screening results with a radioactive labeled probe $(TG)_{13}$, resulting in 82 positive clones out of 288, we estimated the occurrence of approximately 2400 (TG) repeats within the chicken genome. From this the frequency of a (TG) repeat is expected approximately every 500 kb in the chicken genome, which is approximately tenfold less than in mammalian species (Primmer *et al.*, 1997).

In conclusion, our chicken BAC library with 5.57 genome equivalents represents a powerful tool for genome analysis, particularly in combination with the two-dimensional PCR screening, and is accessible for other researchers. The chicken BAC library described in this paper will play an important central role in further research: i) Study of chromosomal regions where a QTL is located after a total genome scan by making contigs of the regions. ii) Performing comparative mapping by sequence scanning of mapped BAC clones, followed by sequence comparison (BLAST search) with other species. iii) Alignment of the linkage and physical maps of the chicken, by isolating BAC clones of mapped markers for every linkage group (including markers from both ends) and mapping of these by FISH on the physical map. iv) Obtaining the genomic sequences of specific genes.

The Wageningen chicken BAC library is available for research, and high-density filters can be obtained either at the UK HGMP Resource Centre in Cambridge or at the Department of Crop and Soil of the Texas A&M University USA. For screening of the library by PCR, contact R.P.M.A. Crooijmans (e-mail richard.crooijmans@alg.vf.wag-ur.nl) or M.A.M. Groenen (e-mail martien.groenen@alg.vf.wag-ur.nl) or visit our Webpage at Http://www/zod.wau.nl/vf/.

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Chapter VII

The gene orders on human chromosome 15 and chicken chromosome 10 reveal multiple inter and intra chromosomal rearrangements

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Abstract

Mapping, sequencing and ordering specific chicken BACs improved the comparative map of *Homo sapiens* chromosome 15 (HSA15) to the homologous regions in chicken with more than 100 genes and/or ESTs. A comparison of HSA15 to chicken identifies four conserved chromosomal segments between the two species. In chicken these segments are located on chromosome 1 (GGA1), GGA 5 and GGA10 (two segments). Although also four conserved segments are observed between HSA15 and mouse, only 1 of the underlying rearrangements is located at the same position as in chicken, indicating that this is a rearrangement that occurred after divergence of the rodent an the primate lineages.
A comparison of GGA10 with HSA15 identified 10 conserved segments, indicating the occurrence of at least 8 intrachromosomal rearrangements, which seems to have occurred in the bird lineage during evolution after separation of the birds and mammals. Computer simulations indicate that at least 7 inversions and two translocations have occurred during separation of these chromosomes in chicken and man during evolution.

Introduction

In mammals comparative gene-mapping projects have lead to a comparative physical map for 28 species from eight mammalian orders already in the early nineties (1). Comprehensive maps of mice and human containing a large number of mapped genes proved to be an efficient way to identify relevant genes in livestock such as the muscular hypertrophy gene in cattle (2-4). Much effort is made by sequencing and mapping genes and ESTs in farm animals to improve the comparative maps but so far the resolution is relatively low (5). In most cases the more dramatic evolutionary rearrangements can be identified, but subtle internal rearrangements often remain uncertain or undetected. By comparing the human and mouse maps in more detail many of the syntenically homologous regions appear to be interrupted by insertions, transpositions, deletions, inversions, and other types of rearrangements (6). The chromosomal reconstruction of the ancestor of all primates, suggests that 18-20 human chromosomes have remained unchanged during evolution and the rest have but a single exchange (7). Different exchanges have occurred in the lineages leading to distinct primate families and genera.
Based on 223 genes mapped in chicken the predicted number of autosomal conserved fragments between chicken and human is 96 and for the chicken-mouse comparison this

number is 152 (8). This number appears to be much smaller than would be predicted based on the time of separation between the avian and mammal species some 300 Myr ago.
Although a small improvement of the chicken-human comparative map has been realized by mapping genes in chicken, both by cytogenetics (9) and by linkage analysis within the reference families (10), further progress has been relatively low. To obtain a high-density comparative map, regional mapping of sufficient numbers of coding sequences in the species of interest has to be performed. The recent construction of arrayed genomic libraries of large insert clones such as BACs for many species including chicken (11) are powerful tools to perform comparative and physical mapping. Large insert clones are used as cytogenetic probes and for direct sequencing and therefore useful in screening for orthologous genes. So far, only 8 genes, that are located on human chromosome 15, have been mapped in chicken. Seven of these genes map to chicken chromosome 10, whereas the *RYR3* gene maps to chromosome 5 (10, 12-13).
In this study, we describe the generation of the first detailed comparative map between human chromosome HSA15, its mouse counterparts on chromosomes Mmu7, Mmu2 and Mmu9 and the homologous regions on the three chicken chromosomes GGA10, GGA5 and GGA1, by the identification and mapping of almost 100 genes in chicken. These results indicate the occurrence of multiple inter and intra chromosomal rearrangements during evolution between these chromosomes of the two species.

Materials and methods

Chicken chromosome 10 BAC clones. The BAC library was screened for all microsatellite markers and genes located on chicken chromosome 10 (former linkage group E29C09W09) by two-dimensional PCR (11). All the BAC clones from each of the markers were identified and one BAC clone per marker was selected for both BAC-end sequencing and shotgun sequencing. The BAC-end sequences were used to design specific STS markers for chromosome walking.
BAC-end sequencing. BAC DNA was isolated with REAL Prep 96 plasmid kit (Qiagen), or as described by Crooijmans *et al.* (11), and dissolved in 32 µl 5 mM Tris-HCl pH=8.0. PCR sequencing was performed in 40 µl by using 16 µl of BAC DNA, 8 µl Half Big Dye terminator (Genpak Ltd), 8 µl Big Dye Terminator Rrmix (Perkin-Elmer) and 8µl of M13 forward or M13 reverse sequence primer (10 pmol/µl). The amplification reactions were as follows: 5 min 96°C followed by 45 cycles of 30 s 96°C, 20 s 50°C, 4 min 60°C. The amplification product was precipitated with isopropanol and finally dissolved in 3µl 83%

de-ionized formamide and 17% loading buffer (Perkin-Elmer). The sequences were analyzed on a 4.75 % Long Ranger Gel (FMC) on an automated sequencer ABI377, (Perkin-Elmer). Electrophoresis was performed for 7 h on 36 cm gels and the results were analyzed using sequence software (ABI, Perkin-Elmer).

Sample sequencing of BACs. EcoRI digested BAC DNA was ligated into the EcoR1 site of pTZ18R and transformed to DH5α. Twelve subclones per BAC clone were selected and plasmid DNA was isolated (Qiaprep 96 miniprep kit; Qiagen). The PCR sequence reaction was performed in 10 µl with 200-500 ng plasmid DNA, 2 µl of Half Big Dye terminator (Genpak Ltd), 2 µl Big Dye Terminator Rrmix (Perkin-Elmer) and 1 µl of M13 forward or M13 reverse sequence primer (0.8 pmol/µl). PCR was performed according to the manufacturers specifications, and the excess dye terminator was removed by precipitation with isopropanol. Sequence reactions were analyzed on a 96 well 36 cm 4.75 % denaturing Long Ranger Gel (FMC) according to ABI (Perkin Elmer). All sequences obtained were first analyzed with PREGAP4 of the STADEN software package. Sequences were cleared from vector sequence, Ecoli sequences and bad sequences. The resulting sequences were compared finally with sequences deposited in the public databases using the network BLAST client software of the NCBI (blastcl3)

Fluorescent in situ hybridization (FISH). Two-color *FISH* was performed according to Trask *et al.* (15). NotI digested BAC DNA was labeled by random priming either with biotin-16-dUTP or with digoxigenin-11-dUTP (Boehringer Mannheim) (15). The BAC clones used as FISH markers to identify the specific chromosome are for GGA10: BAC bw016D10 from marker ADL0038 and BAC bw008K20 from marker MCW0228, for GGA5: BAC bw009B13 from marker ADL0298 and BAC bw037H20 from marker MCW0263 and for GGA1: BAC bw038E08 from marker MCW0107 and BAC bw030P07 from marker MCW0248.

Results and discussion

Comparative mapping is a powerful tool to utilize the existing knowledge of a species with detailed mapping information such as man and mouse, in species with less well developed maps. We have used this approach for a detailed characterization of chicken chromosome 10 (GGA10) by using a bi-directional approach, starting to build up the comparative map from GGA10 as well as from the identified homologous region in man.

In chicken, 29 loci have been mapped to GGA10 consisting of 20 microsatellite markers, 8 genes and one EST (10, 12-13). These markers were used in the 2-dimensional PCR

screening of our chicken BAC library (11). The BAC clones isolated were subjected to both end sequencing and sample sequencing. End sequencing enabled the development of STS markers for chromosome walking, whereas the sample sequencing provided information on the gene content of some of the BACs. Of the 8 genes mapped to GGA10 seven have a homologue in human that has been mapped to chromosome 15 (HSA15). The other gene (*GNRHR*) is located on human chromosome 4. Furthermore another gene known to be located on HSA15 (*RYR3*) has been mapped to chicken chromosome 5. Therefore, genes known to be located on HSA15 (16) were selected and used to identify homologous chicken genes whose sequences were present in the public nucleotide databases. Sequences of 36 chicken genes were selected from the database and primers were designed to screen the chicken BAC library.

Table 1. Characteristics of markers developed in chicken genes.

gene	Chicken map location	accession number	PCR size (bp)	forward primer	reverse primer
GABRB3	GGA1	X54243	238	TGAGGTTATGGACAATGTAAC	TTACCAGTGTAACTCTATCAC
UBE3A	GGA1	AJ399379	100	TTTGTCAATCTGTATGCTGAC	AAGGAGATTCATTGGTCACC
ACTC	GGA5	M10607	227	GAACTCCTCCGTCATTGTAC	ACCTAACATGTCCACATCAC
RAD51	GGA5	L09655	173	TGTTCAAATGCTGGCCGAGG	TCAAGCATGTGGTCCTCTGG
THBS1	GGA5	U76994	153	CTGGCAATATGTCTACAATG	TCTGTCTCCTGGTTGTTATC
CAPN3	GGA5	D38028	408	TCTGAACTGTGGAAGTCAGAG	TCAATGTTACAGAGAATGCAG
TYRO3	GGA5	U70045	232	ATTTCCTACTACCCAACGCTG	GCGCTTCCCAGGCAGTTACAG
CHRNA7	GGA10	X68586	128	AAGGAGAGTTCCAAAGGAAG	ATCCATGATCTGCATGAGGC
CKMT1	GGA10	X96403	171	AGCTGGTGATAGACGGTGT	GGCGCATTGATGGCATAAGC
ANX2	GGA10	X53334	191	AATCGAGCCGCCATGCAAAC	AGGCATCCTCAACAAGCACG
FBN1	GGA10	U88872	273	GATGAATGTGTGCTGAACACG	CATGTTCTGCCAACAGGTACC
CRABP1	GGA10	Y12243	230	TTGGTGACCTCAAGCTCTGC	CAGTTCTCCTCTTACCCTGC
AGC1	GGA10	U83593	202	AGTGGCAGCTAATGTGGTCTG	TCTTCTTTGCTATCTCCAGAG
TPM1	GGA10	X57991	211	ATATAGGCATTTTCCACGGTC	CTCACTGATATGGCCTTTCC
NR2F2	GGA10	U00697	167	GTATGTTAGGAGCCAGTATC	CAGACAGTAACATATCCCTG
NEO1	GGA10	U07644	250	CTTAGCCTTGGAACACAAGG	TCTTTCCTTTTGTCTGGATGC
SCK	GGA10	M85039	111	GAGTGCCTTATCCGAGAATC	TCTTCATCACCTCGTAGACG
MEF2A	GGA10	AJ010072	119	TTGTAGCAGCAGAGCAGTAG	CCATGCACCCTCTGCAATAG
CYP11A	GGA10	D49803	125	CCAGTTGGTCCCAGCTTGG	CAGAGCAAATCAGAGGCGAAG
MYO1E	GGA10	X70400	172	CAACGGCAGCGCCAAACTGC	TGTTTGCAAAGCAGCATCGAC
NTRK3	GGA10	S74248	170	AAACTCAAGTGCCTGCTACAC	TGTAACAGCAGCGTCTTTCTG
MYO5A	GGA10	X67251	316	AATCTCGAAGCAGATGATCAG	CAATGACAGCATCACACTCAG

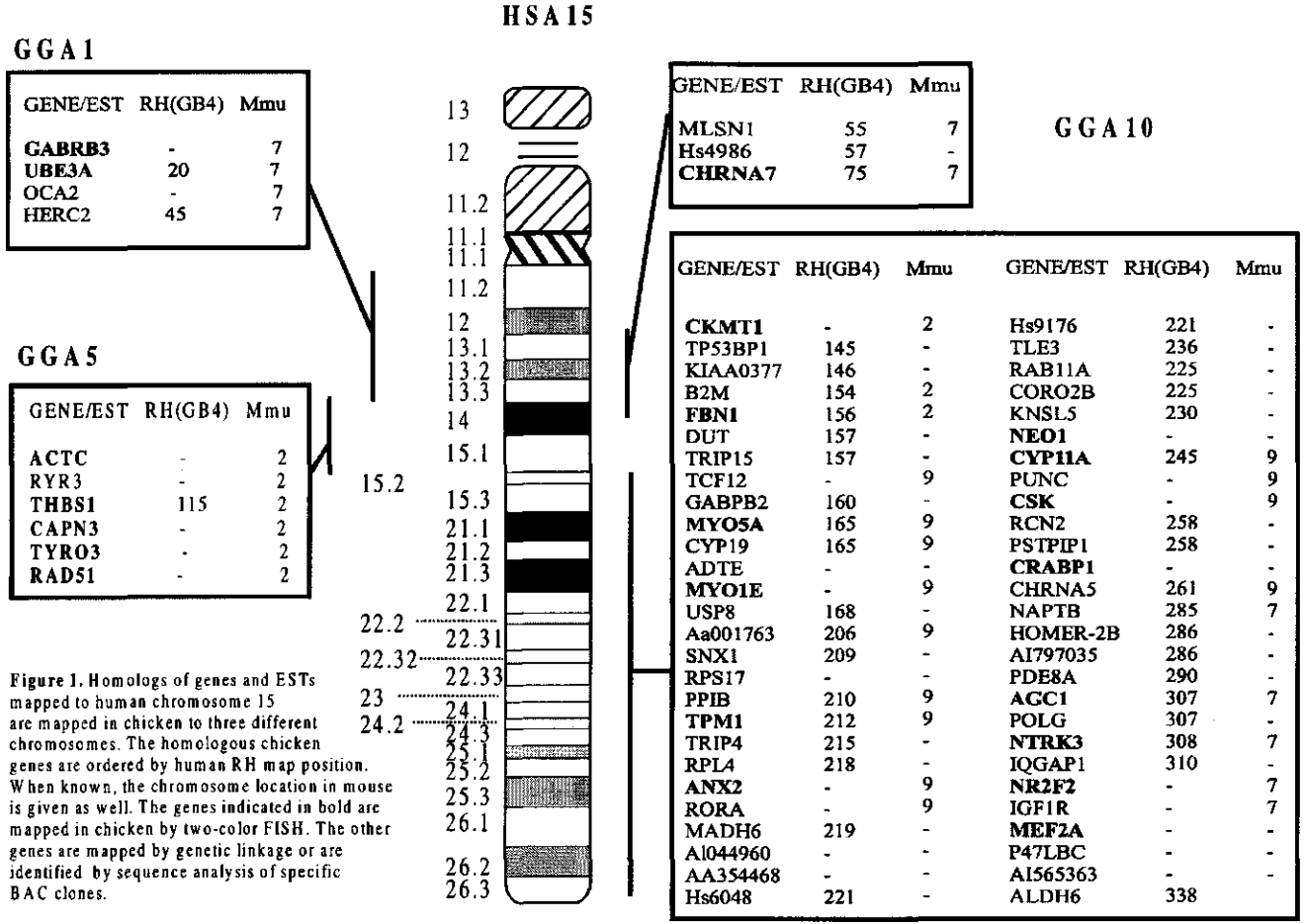


Figure 1. Homologs of genes and ESTs mapped to human chromosome 15 are mapped in chicken to three different chromosomes. The homologous chicken genes are ordered by human RH map position. When known, the chromosome location in mouse is given as well. The genes indicated in bold are mapped in chicken by two-color FISH. The other genes are mapped by genetic linkage or are identified by sequence analysis of specific BAC clones.

For 22 genes we were able to isolate at least one BAC clone which was subsequently used in two-color *FISH* to map the genes in chicken (Table 1; Figure 1, indicated in bold). The precise map location of the genes mapped to chromosome 10 by *FISH* is not known because of the small size of this microchromosome and the absence of a clear banding pattern. However based on the map location in human and mouse and based on the known map location of other genes mapped on GGA10 we can predict the map location for some of these genes on this chicken chromosome. For example, the gene *CKMT1* is mapped by *FISH* to GGA10 and has a HSA15 map location of 39.5-50.8 cM and 145 cR. A sequence derived from a BAC of marker ADL0112 identified the gene *TP53BP1* that is mapped in human to almost the same position. Therefore we expect the *CKMT1* gene is located in chicken close to marker ADL0112.

The BAC clones that were mapped to GGA10 by FISH were also used as starting points for chromosome walking. The approach outlined above has resulted thus far in the development of more than 240 STS markers and the isolation of more than 570 different BAC clones, corresponding with a chromosome coverage of almost 40%. The number of BAC clones per marker varied from 1 to 12 with an average of 4.9 BACs per marker that is in good agreement with the estimated 5.5 times genome coverage of the library of (11).

Seventy different BAC clones derived from GGA10 were selected and used for sample sequencing. All sequences obtained from the BAC-end sequencing and the sample sequencing were compared with sequences in the nucleotide databases (BLAST). In most cases the observed homology to human genes was unambiguous and often several different exons of the same gene were identified. More difficulties occur when homology is detected with several genes belonging to a gene family. This occurred after shotgun sequencing of a BAC clone derived from marker ABR0012 mapped to GGA10. Gene identity was found with transducin-like enhancer protein family *TLE1*, *TLE2* and *TLE3*. *TLE1* is located on HSA9; *TLE2* on HSA19 and *TLE3* is located on HSA15 (15q; 70.1-71.3 cM; 236 cR). According to the gene identity and human chromosome location of the TLE gene family, we assume *TLE3* is located on GGA10.

The sequence results together with the genes mapped by FISH revealed sequence identity to almost 100 human, mouse, rat and chicken genes and ESTs. Several genes and ESTs have not been mapped in human yet, such as the epsilon adaptin gene (*ADTE*). This gene, found after sample sequencing of a BAC clone of marker MCW0357 (*CYP19*), belongs to the adaptin family. Of this family the beta 1, delta and gamma are mapped to human chromosome 22, 19 and 16 respectively. Besides homology found with genes mapped to HSA15, occasionally sequence homology was observed with human genes that

map to other human chromosomes. For example, the gene *HK1* maps to HSA10, *GNRHR* to HSA4 and the gene *TLN* to HSA9. These observed homologies may either indicate the presence of gene families of which one member has not yet been mapped to HSA15 or they might indicate the presence of small regions of homology to other human chromosomes. However, in the latter case one would expect that more genes from those regions would have been identified in the current study. This is further strengthened by the fact that for the three genes that are located on different human chromosomes, on the same BAC clone a gene has been identified that does map to HSA15.

The human chromosome 15-chicken comparative map consists of 67 genes and ESTs and is shown in Figure 1. The genes that are located in man on chromosome 15, are located in chicken in 4 regions of conserved synteny on three different chromosomes; GGA1, GGA5 and GGA10. The majority of these genes however are mapped in chicken to chromosome 10. In mice also four conserved chromosome segments are observed in the order Mmu7, Mmu2, Mmu9 and Mmu7. In an attempt to reconstruct a common ancestor of man, mouse and chicken three time nodules in evolution have been described (8). The first time nodule is 300 Myr ago, when birds diverged from mammals, the second one 100 Myr ago, when the mouse diverged, and finally, 65 Myr ago, where the common ancestor of the primates lived. The reconstructed ancestor of all primates has a chromosome 6 that consisted of a rather unchanged human chromosome 15 and chromosome 14 (7). This reconstructed chromosome is based on the comparison of chromosome paints of primates, where gene order of the conserved segments was not considered. The comparison of HSA15 to chicken and mouse chromosomes indicates the occurrence of three interchromosomal rearrangements (interCR) during evolution (Figure 2). Only one of these rearrangements appears to be at the same location in chicken and mouse, indicating that this translocation occurred in the lineage leading to man after the mouse and human lineages diverged, between 100 and 65 Myr ago. The other two interchromosomal rearrangements in chicken involving the segments on GGA1-GGA10 and GGA5-GGA10 probably have occurred before the man and mouse lineages diverged, either in a predecessor of mammals or in the chicken lineage. In mouse, the other two interCR between the segments on Mmu2-Mmu9 and Mmu9-Mmu7 probably have occurred within the mouse lineage during evolution starting 100 Myr ago.

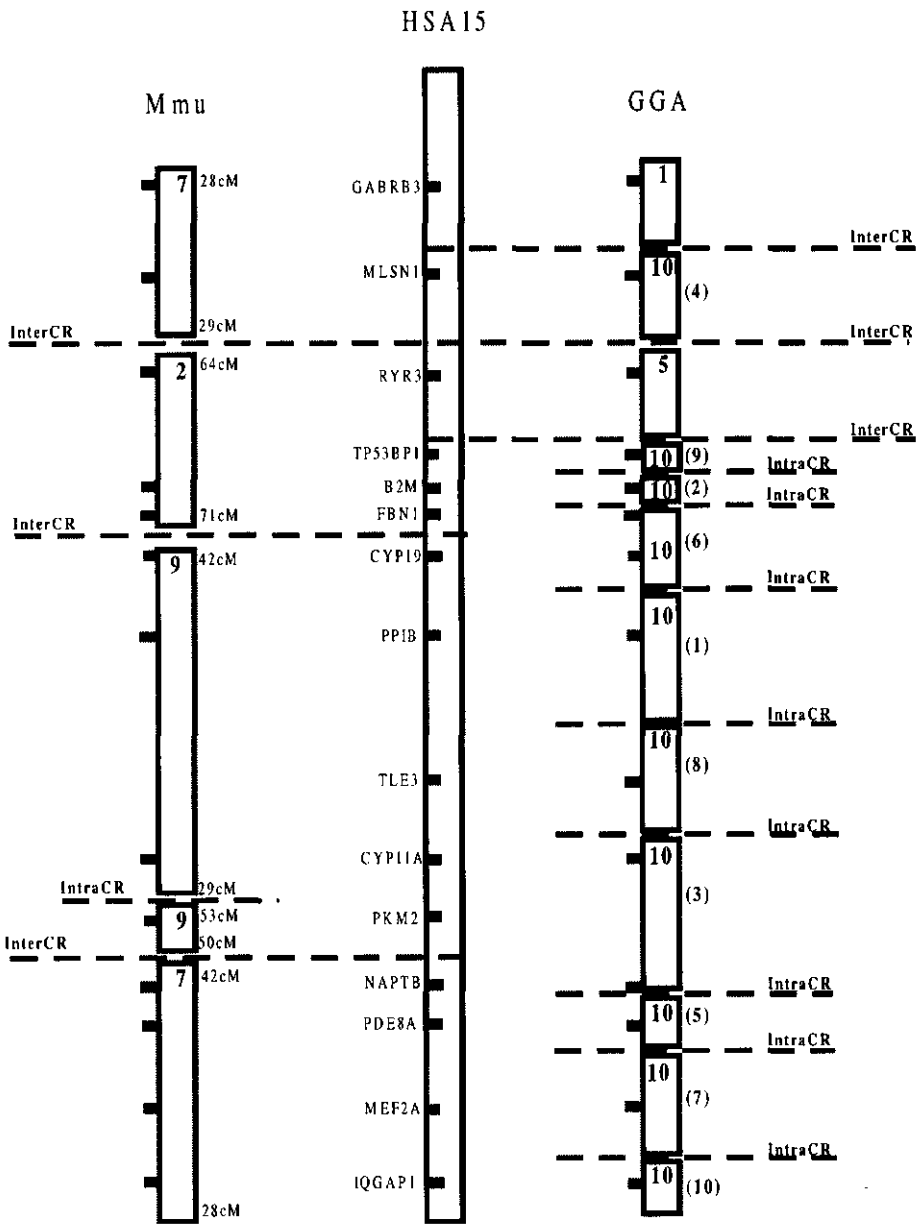
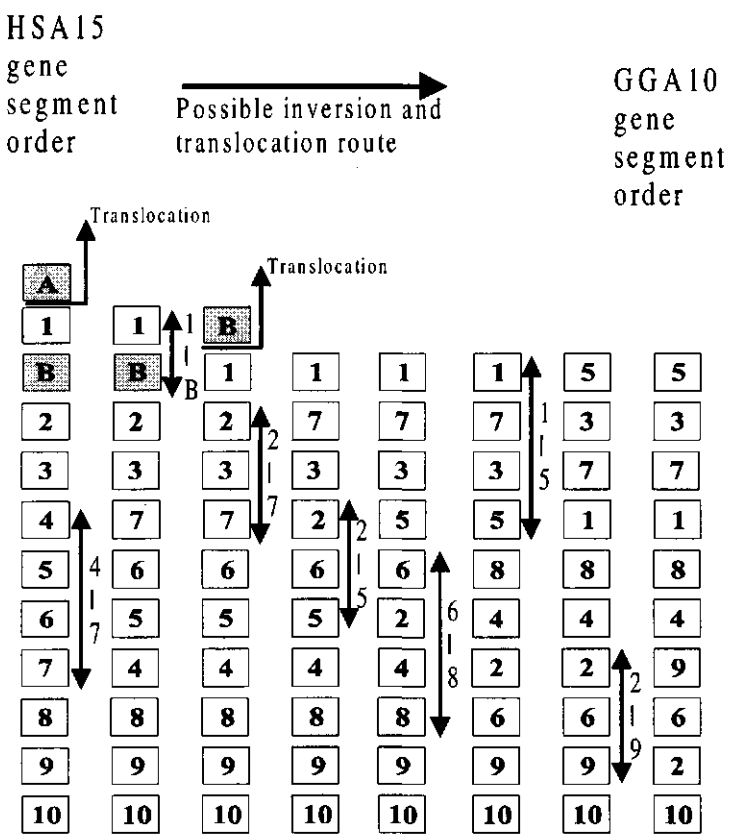


Figure 2. Comparative map of Homo sapiens chromosome 15 (HSA15) to chicken (Gallus gallus; GGA) and mouse (Mus musculus; Mmu). For every chromosome segment at least one gene is indicated. The chromosome segment order for chicken chromosome 10 is given in brackets. Positions of interchromosomal rearrangements (interCR) and intrachromosomal rearrangements (intraCR) are indicated by a dotted line.

A more detailed comparison of the conserved chromosome segments between chicken chromosome 10 and human chromosome 15 reveals a much more scattered picture (Figure 2) than predicted by Burt *et al.* (8). In total 56 homologous genes and ESTs have been mapped to both chromosomes of these two species. The estimated size and order of the conserved gene segments in both species as shown in Figure 2 is based on a combination of genetic mapping, chromosome walking results and radiation hybrid (RH) mapping. The exact gene order will only be known when a complete BAC contig is available for both species. We can identify at least 10 segments, which is the minimum number of conserved segments between GGA10 and HSA15. The number of genes per segment varies from 1 (segment 2 and 10) to 7 (segment 3). This scattered picture clearly unfolded from the sequencing results of a chicken BAC clone identified with marker MCW0228. Gene identity with sequences of this BAC clone was found with the family of the S-cyclophilin like genes of which the cyclophillin B (*PPIB*) located on human chromosome 15 (50.8-58.8 cM; 209 cR) shows the highest homology. In addition to this gene, gene identity with sequences of the same BAC clone was found with four other human genes (*B2M*, *RPS17*, *SNX1*, and *NAPTB*), two mouse genes (*Ckgl* and *Cpeb*) and one rat gene (Casein kinase 1 gamma 1 like gene). The map location of the human genes is scattered over HSA15 (154 cR to 285 cR).

By comparing the chromosome segment order of GGA10 (Figure 2, segment order in chicken between brackets) to human chromosome HSA15 at least 8 intrachromosomal rearrangements can be identified that took place during evolution. Assuming that the most likely intra-chromosomal recombination events probably are inversions, we designed a simple computer program that started with the segment order in man and calculated the minimum number of inversion needed to obtain the chromosomal segment order obtained in chicken. In addition to the two translocations of chromosomal segments to GGA1 and GGA5, the minimum number of inversions is 7 where different routes are possible to reach these observed order (Figure 3). One of these chromosomal segment orders might be the order of genes belonging to the common ancestor of human and birds. However, information from other species is needed to unequivocally address this point. Based upon the rates of chromosomal change observed in mammals, one can calculate that the number of conserved segments between chicken and man would be in the order of 100-600. Based on the comparative mapping data of 223 genes, Burt *et al.* (8) suggested that the number of conserved segments between chicken and man would be in the lower part of this range. However, our data on the detailed comparison between human chromosome 15 and chicken indicates that this probably is an underestimate and that the number of conserved



Other minimum possible routes to obtain the gene order from HSA15 to GGA10 are:

1. 4-7, 1-B, 1-5, 2-9, 8-4, 6-3, 6-1
2. 2-9, 1-B, 1-5, 6-3, 9-7, 4-7, 4-8
3. 6-8, 1-B, 1-5, 3-8, 2-7, 4-3, 2-9
4. 4-7, 1-B, 6-9, 1-8, 2-6, 8-6, 9-1

Figure 3. The minimum number of inversion events between human chromosome 15 and chicken chromosome 10. The two translocations are indicated, where segment A will finally be part of GGA1 and segment B of GGA5. Segment order in both species is derived as shown in Figure 2. Segment numbers 1, 2, and 3 in chicken are derived from one BAC contig where the order is not exactly known. The same is true for segments 9 and 10 that are derived from a single chicken BAC clone.

segments could be as high as 400.

In this paper, we clearly demonstrate the importance of high gene densities in comparative mapping to be able to identify both inter and intra chromosomal rearrangements. Eventually, the development of complete physical maps, either as BAC contig or even as the complete sequence, will further aid in the detailed reconstruction of rearrangements during evolution, which resulted in the chromosomes in the different species as we know them today. A detailed comparative map, as described in this paper, will be of high value in the identification and further characterization of candidate genes in QTL studies in chicken, as well as in the analysis of complex traits in man.

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Chapter VIII

High resolution mapping of QTL in a cross between broiler lines

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Abstract

An advanced intercross line (AIL) has been produced from a cross between broiler lines for the fine mapping of quantitative trait loci for production and health traits. Although, the original description of an AIL is based on a cross between two inbred lines, the basic concept can also be used for fine mapping of QTL in a cross between two different lines. However in that case a sufficiently large number of markers is needed to be able to identify the original different haplotypes. This requirement can be met by the development of marker dense SNP maps. To reach this objective, we started to develop complete BAC contigs for 6 regions of the chicken genome together comprising approximately 10% of the genome. Our strategy is based on a bi-directional approach, using loci from the existing chicken linkage map as well as loci that have been selected based upon the available human-chicken comparative mapping information.

Introduction

The development of a large number of highly informative genetic markers and the development of a highly informative linkage map in chicken (Groenen *et al.* 2000) has initiated a large number of studies aimed at the localization of genes involved in monogenic (Ruyter-Spira *et al.* 1997, 1998; Pitel *et al.* 2000) as well as quantitative traits (Van Kaam *et al.* 1998, 1999a, 1999b; Vallejo *et al.* 1998, Zhu *et al.* 2000). For mapping quantitative trait loci (QTL), like the mouse, the chicken is particularly useful because it has a relatively short generation interval and large number of offspring can be generated from a single pair of parents. Another feature makes the chicken even a better model for the identification of QTL; the relatively small size of its genome (one third that of mammals) and the relatively high rate of recombination (the size of the genetic map is 3800 cM). Although, an increasing number of QTL mapping studies has been described or are in progress, the mapping resolution of the QTLs in such studies is still low, generally being in the order of 20 to 30 cM. For the high resolution mapping of these QTLs several strategies have been described such as the generation of backcross pedigrees, the production of recombinant inbred lines or congenic lines and advanced intercross lines (AIL, Darvasi and Soller, 1995). A detailed description of different fine mapping techniques and their advantages and disadvantages has been described by Darvasi (1998).
In our initial QTL mapping experiment we have used a full-sib/half-sib design for the analysis of a large number of different traits including growth, fatness, amount of breast meat, and resistance to salmonella infections, mal-absorption syndrome and ascites

(Groenen *et al.* 1997; Van Kaam *et al.* 1998 and 1999a,b). Because of the large number of different traits and the complexity of several of the traits, we decided that, for fine mapping, it would be most efficient to produce an AIL. This AIL is based on the families, where the most important QTLs, as detected in the original cross, were segregating. Although, the original description of an AIL is based on a cross between two inbred lines, the basic concept can also be used for fine mapping of QTL in a cross between two outbred lines. However in that case a sufficiently large number of markers is needed to be able to identify the original different haplotypes. This requirement can be met by the development of marker dense SNP maps.

Material and methods

Mapping population. The Wageningen QTL mapping population (Groenen *et al.* 1997) is based on a cross between two broiler dam lines originating from the White Plymouth Rock breed. The breeding and measurements of all the animals from this cross are done by the Dutch breeding company Nutreco. Based on the results of the QTL analysis within the F_1-F_3 generation, F_2 animals were selected from the families in which the QTLs were segregating and used for further breeding. The number of animals produced in the F_0 – F_7 generations that are used for the AIL and the number of animals that were used for the phenotypic measurements are shown in Table 1. The F_7 and F_8 generations are currently being produced.

Table 1. Number of animals used for breeding, genotyping and phenotyping.

Generation	Number of animals: Breeding	Number of animals: QTL analysis
F_0	30	
F_1	20	
F_2	71	456
F_3	105	18,000
F_4	122	
F_5	93	
F_6	108	
F_7	127	3,000
F_8	100	1,500

Markers. A detailed description of all individual loci that have been mapped on the consensus chicken linkage map (Groenen *et al.* 2000) including those used for the QTL analysis of our broiler cross is available at the web site of the Animal Breeding and Genetics Group in Wageningen and East Lansing [1].

Screening of the BAC library. For building the BAC contigs, BAC clones are isolated from the Wageningen BAC library (Crooijmans *et al.* 2000) by a two-dimensional PCR screening method. The first step is the identification by PCR of the 384-well plate pool containing the marker, followed by PCR screening of the individual row and column pools of that plate. To confirm the purity of the positive BAC clone, the clones are plated and two single individual colonies are picked and colony PCR is performed. The pure positive BAC clones are finally stored individually at -80 °C. A more detailed description of the two-dimensional PCR screening can be found in Crooijmans *et al.* (2000)

Fluorescent In Situ Hybridization (FISH). Miniprep DNA of selected BAC clones is used in FISH experiments to evaluate the map location of these clones. BAC DNA is either labelled by random priming with biotin-16-dUTP or with digoxigenin-11-dUTP on chicken metaphases derived from a chicken primary fibroblast cell culture (Ruyter-Spira *et al.* 1998; Morisson *et al.* 1998).

Results and discussion

The use of AIL in an outbred cross. In order to decrease the length of the confidence interval Darvasi and Soller (1995) introduced the Advanced Intercross Line (AIL). An AIL starts of with a cross between two inbred lines. The experiment continues from the F_2 by randomly intercrossing the individuals in each generation to produce an F_3,.....F_t. In this way recombination events, that are required for fine mapping QTL, are accumulated over generations. Individuals in generations between F_2 and F_t are not typed and phenotypic data is only collected in the F_2 and in F_t. As is illustrated in Figure 1 generating an AIL can lead to a considerable reduction of the confidence interval for QTL location. For a QTL with an effect of $0.3\sigma_P$ the 95% confidence interval is expected to reduce from 33 cM in the F_2 to 8 cM in the F_8.

[1] http://www.zod.wau.nl/vf/research/chicken/frame_chicken.html;

http://poultry.mph.msu.edu/

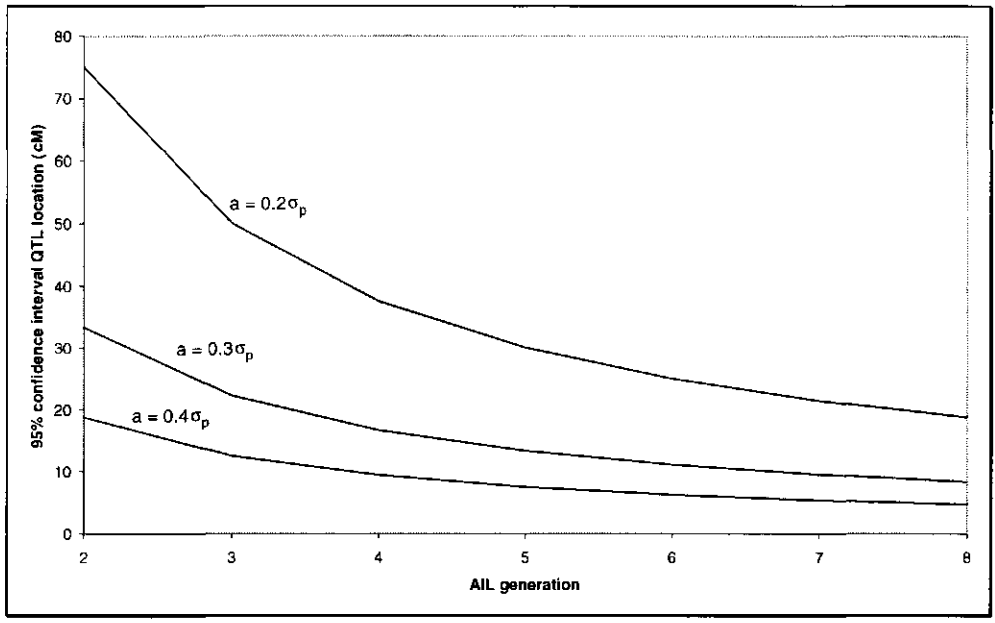

Figure 1. The approximate 95% confidence interval for QTL location as a function of the AIL generation for a sample size of 500 (Darvasi and Soller, 1995).

It is clear that this type of design cannot be applied to all species. A short generation interval is a prerequisite and therefore AIL could be applied to *e.g.* mice or chickens. Another potential problem is the effect of random fluctuations due to small effective population size.

In the original paper by Darvasi and Soller (1995) it is assumed that AIL is used within a cross between two inbred lines and that the two alleles of a polymorphic marker are specific for each of the two lines. However, because we are using AIL within an outbred cross, marker alleles are not fixed within the lines used and the same alleles can occur in the two starting populations.

When, as originally proposed, AIL is used within a cross of two inbred lines there would be two marker alleles, two QTL alleles and complete linkage disequilibrium in the F_1. In such a situation the expected marker contrast (M^1M^1-M^2M^2) can be modelled easily as a function of the generation number and the recombination fraction between the marker and the QTL ($t \geq 2$):

$$\mu_{M^1M^1} - \mu_{M^2M^2} = 2(1 - 2\theta)(1 - \theta)^{(t-2)}\, a$$

where t is the generation number, a is the additive QTL effect and θ is the recombination fraction between the marker and the QTL.

Figure 2 graphically illustrates this relation. Simulation using the actual pedigree of our broiler cross resulted in contrasts that agree closely with predicted contrasts. Based on these calculations we can calculate the number of required markers in order to retrieve a certain fraction of the maximum genotype contrast, *i.e.* the contrast we would get if we would have a marker right on top of the QTL (Table 2).

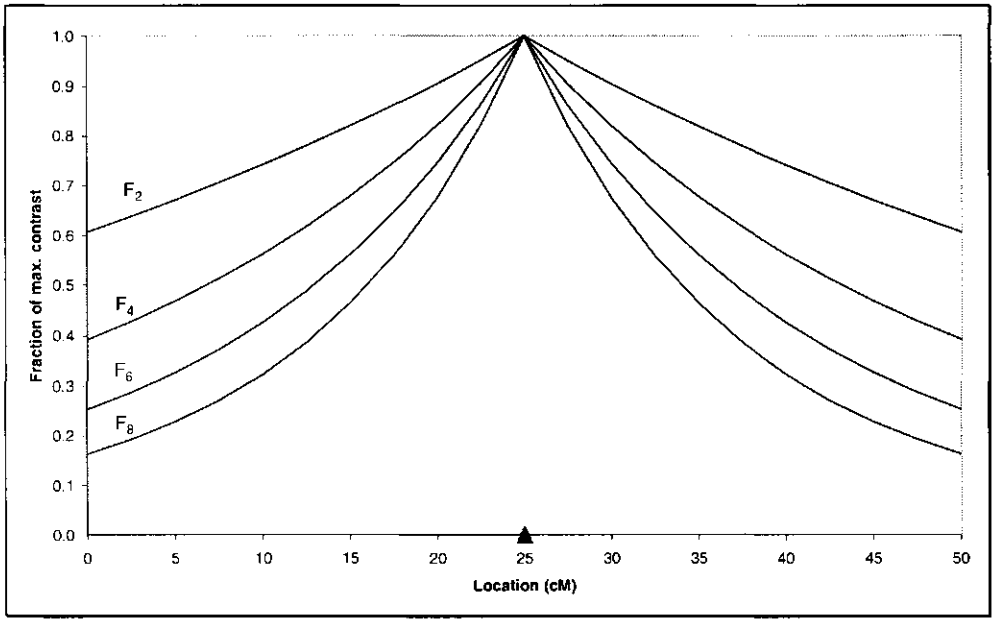

Figure 2. Relation between the expected marker contrast of an AIL depending upon the distance between the marker and the QTL. The position of the QTL is indicated at location 25.

Table 2 shows that in order to pick up 93% of the maximum contrast of a QTL located in a region with a length of 40 cM we would need to have 20 markers in that region. How many markers we actually need to put in this region obviously depends upon the size of the QTL effect and variance of the trait.

Table 2. Maximum QTL genotype contrast versus marker density.

% of maximum contrast	Every x cM a marker required
93	2
81	6
71	10
62	14
51	20

If 71% of the genotype contrast gives us 90% power than we might do with 4 markers in that region. It is good to realise that for actual power calculations not only the expected marker contrast but also the variance of the contrast is important. This variance will depend upon the number of individuals that are typed.

It is obvious that the present experiment is not a cross between inbred lines. Consequently the expected amount of linkage disequilibrium will be lower. For such a situation the contrast of the marker genotypes can be written as (for generation number t≥2):

$$\mu_{M^1M^1} - \mu_{M^2M^2} = \left[\frac{2\,\Delta_M \Delta_Q}{4xy}\right](1 - 2\theta)(1 - \theta)^{(t-2)}\, a$$

where,

Δ_M = difference in marker allele frequency between both lines;

Δ_Q = difference in QTL allele frequency between both lines;

x = marker allele frequency averaged over both lines;

y = QTL allele frequency averaged over both lines.

This formula can be used to calculate contrasts for all sorts of scenario's. What is important to realise is that the contrast reduces rapidly if the situation starts to differ from a cross between inbred lines, *e.g.* if frequencies of marker and QTL alleles are 0.1 in one line and 0.9 in the other (instead of 0 and 1 as for inbred lines) we get only 64% of the contrast for inbred lines.

Similar as for a cross between inbred lines, a strategy to fine map QTLs could be to determine marker contrasts in the F_2 and in the F_7 using an across family analysis. In the F_7 this marker contrast is expected to be reduced by a factor $(1-\theta)^5$. Subsequently, the power can be calculated of significantly detecting this contrast in an across family analysis. However, are there genetic markers in our cross between lines that show in the F_2 a significant effect in an across family analysis? At present, only a limited number of markers have been typed on F_2 individuals for the interesting chromosomal regions. As part of this strategy, the F_2 individuals

could be typed for additional genetic markers: more markers will decrease the expected average recombination fraction between the marker and the QTL and with more markers it becomes more likely that there will be linkage disequilibrium between the marker and the QTL.

Instead of looking at single markers, this approach can also be used if specific haplotypes can be characterised in the F_2 that are associated with the positive QTL alleles. In order to be able to identify these specific and unique haplotypes one might need to perform additional typings on the F_2 population. Once identified, those haplotypes should also give significant effects in an across family analysis of F_2 individuals. For example in the F_2 the haplotype 111-120-140-180 might be significantly associated with an effect. In the F_7 this specific haplotype is not likely to be associated but a smaller haplotype might still be, *e.g.*, 120-140. However, as is illustrated, marker contrast rapidly decrease for situations were the lines used are not completely inbred, which makes it difficult to significantly detect associations in an across family analysis.

Alternatively, we could produce an F_7 consisting of a number of full sib (FS) families. Subsequently, a within family analysis could be performed. As such this does not make much sense because this within family analysis does not help us to fine map the QTL, *i.e.*, the reason for which the F_7 is produced. However, a combination of within and across family analysis could be performed, which would identify haplotypes with positive effects. By lining up the haplotypes with an effect in the F_7 and haplotypes with an effect in the F_2, small IBD regions that cause the effect can be identified.

When performing a within FS family analysis, the variance of the marker contrast (in an across family analysis that is performed later on) will be increased if a limited number of F_6 individuals are selected as parents for the next generations. For example, for 5 FS families only 10 parents are selected. Just by chance certain allelic configurations might be present in this parents that will greatly affect the association between marker and QTL. However, if 40 FS families are selected this chance effect only plays a minor role. To reduce this effect and to eliminate the probability that the QTLs are not segregating in the families produced, we are currently producing 40 FS F_7 families consisting of, on average, 75 offspring each.

Required marker densities in relation to type I errors. An important aspect of the analysis will be the possibility to discriminate F_1 and F_7 haplotypes as being identical by descent (IBD) rather then identical by state (IBS). We therefore need to know what the probability of a type I error is at a given marker density, *i.e.* the probability that based on the IBS of

the haplotypes it is falsely concluded that a chromosomal region is IBD. The type I error in relation to the number of markers and number of alleles per marker for our pedigree is shown in Figure 3. The analysis of the F_7 population most likely will be done by a combination of microsatellite and SNP markers, which means that the average number of alleles per marker probably will be around 3. Given the fact that not all markers will be informative in all animals, we probably will need around 10 markers for the analysis of a particular fragment of a given size.

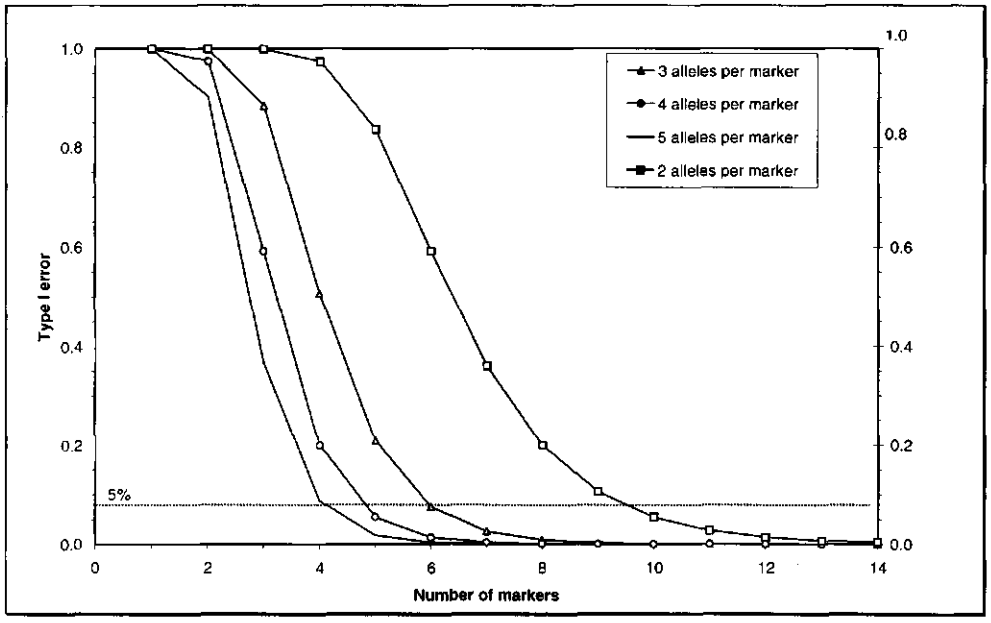

Figure 3. Type I error as a function of the marker density and the number of alleles per marker.

The other important variable we need to take into account is the fraction of the F_7 haplotypes that can be identified as IBD to F_2 haplotypes, i.e. the power. It is clear that a smaller fragment has a higher power because a smaller fragment is not likely to be recombined. For example, in the situation where we have 10 markers in a 10 cM region the power is only 0.688, whereas for 10 markers in a 2.5 cM region the power is 0.905.

As DNA from all animals in the experiment are collected, the possibility is left open to type all individuals in the experiment (F_1 till F_7) for the identified regions. This data can be analysed using a full-pedigree analysis using methods as described by Bink and Van Arendonk (1999). This will provide further power to identify the QTLs.

High resolution physical mapping of target regions. From the calculations described in the previous section, it is clear that a high marker density is needed for the IBD analysis of the haplotypes segregating in the F_7 generation. The number of markers that eventually are needed depends on the number of alleles and thus on the type of marker used (*i.e.* microsatellites or SNPs) and on the required power to identify a certain fragment as being IBD. However, to be able to analyse the F_7 population, a marker density of at least one marker every 1 cM will be desirable. The current average density of the linkage map is only one marker every 2 cM and there are many regions for which this number is much larger. Furthermore, if we consider only the microsatellites, this density even drops to 5 cM. Given the relatively low number of microsatellites in the chicken genome, efforts to increase the microsatellite density of the complete chicken linkage map are clearly not feasible to reach this goal for the regions currently being studied. As an alternative approach we decided to specifically target the marker development to the regions where a QTL had been mapped in the original F_2 population. This strategy is part of a larger effort to develop a high-resolution comparative map between chicken and man for these regions.

To reach these objectives, we decided to develop complete BAC contigs for 6 regions of the chicken genome together comprising approximately 10% of the genome. Our strategy is based on a bi-directional strategy, using information from the existing chicken linkage map and on the available human-chicken comparative mapping information. The first step is, the isolation of all the BAC clones for all available markers on the linkage map for these particular regions by a two-dimensional PCR screening approach (see material and methods). At the same time, all chicken genes with available sequence information in Genbank are identified, whose human homologue has been mapped to the corresponding region on the human map. After developing specific PCR primers for these chicken genes, these are also used to isolate all the corresponding chicken BACs. To check whether these genes are indeed located in the regions being studied, one BAC for each gene is mapped in chicken using FISH. Subsequently, for each marker or gene, one BAC is selected as a starting point for chromosome walking. These BACs are used for direct end sequencing, and the resulting sequences are used for the development of new STS markers, which subsequently are used to screen the BAC library for additional overlapping BAC clones. A summary of the results obtained so far is shown in Table 3.

To increase the chicken-human comparative map, selected BACs are subjected to subcloning and sample sequencing. Approximately 10% of the BAC insert is sequenced (single read) and the sequences are used to identify potential genes by BLAST homology searching. This has resulted already in the mapping of over 130 genes and ESTs in these regions (Table 3).

Table 3. Summary of physical mapping in chicken.

Chromosomal location	Size (cM)	Markers on linkage map	BACs isolated	STSs developed	Genes identified	Estimated coverage (%)
GGA8 (qtel)	30	12	104	44	10	20
E29C09W09	120	41	550	212	75	30
E18W15	71	19	101	34	8	10
E53C34W16	75	20	144	57	21	20
E49C20W21	58	16	49	12	9	5
E48C28W13W27	74	33	53	19	7	5

The approach as outlined above also produces a wealth of information that enables us to increase the marker density in these regions dramatically. First of all; the sample sequencing also identifies many different microsatellites (CA, GA, A, TA). Interestingly, even most of the CA repeats detected so far have not been described before. Secondly, the STSs developed from the end sequences of the BACs for chromosome walking can easily be used for the development of SNP markers. The number of SNPs in the chicken genome appears to be relatively high (1 every 100 bp; Vignal *et al.* 2000). This observation and the fact that the average size of the STS fragments is 200 bp, indicates that sequencing these STS fragments from the original two broiler populations will result in a large number of SNPs. A further source of potential SNP markers is the sequences from the subclones produced for the sample sequencing. Several of these fragments are homologous to chicken EST sequences present in Genbank. Often nucleotide differences are observed between the sequences present in the database and the sequences from our fragments. Although, a number of these differences probably can be attributed to sequencing errors, many will be true SNPs.

It is clear that much work still has to be done before being able to efficiently analyse the F_7 population. However, given the molecular resources currently available in chicken and the amount of data being generated in a short time, we are confident that the approach outlined above will enable us to map the QTL originally found in our F_2 population at a high resolution within the next two years.

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Chapter IX

Future directions of Wageningen chicken research

The isolation and mapping of DNA markers either random or within genes and ESTs in chicken has resulted in a consensus linkage map of the chicken genome (Groenen *et al.*, 2000). Almost 1900 loci are defined and ordered on 50 linkage groups. Of these linkage groups, 10 have been assigned to a specific chromosome (GGA1-8, GGAZ, and GGA16). BAC clones from markers for each of the individual linkage groups are used in two-colour *FISH* to assign the other linkage groups to chromosomes and this has allowed the assignment of another 22 linkage groups to chromosomes 9 to 31 (V. Fillon, RPMA Crooijmans, A. Vignal and MAM Groenen; unpublished results). Identification of all the chromosomes will be possible in the near future with a set of FISH markers.

Moving forward from the chicken genetic map to the physical map, the Wageningen chicken BAC library is an essential tool. This physical mapping can be performed either chromosome-wise or genome-wise.

Physical mapping of a single chromosome can be performed with the bi-directional approach as described in chapter 8. Chromosome walking starts with the BAC clones isolated from previously mapped markers. By BAC-end sequencing, new STS markers are generated which subsequently are used for screening of the BAC library.

Furthermore, specific BAC clones are used for shotgun sequencing to generate sequences that can be used to identify homologous genes in man (BLAST). Where a syntenic region is identified, all known human genes of this region can also be used to identify homologous sequences in chicken. These sequences are used to design specific PCR primers for the identification and isolation of BAC clones that subsequently can be used to map these genes in chicken by *FISH*. This will generate a detailed human-chicken comparative map and new starting points for chromosome walking.

Although this approach has allowed us to obtain BAC contigs for large regions of specific chromosomes, eventually a more general approach directed towards the construction of a BAC contig covering the complete chicken genome, will be more efficient. Fingerprinting of BAC clones is such a technique for genome-wise physical mapping. Briefly, DNA is isolated from all individual BAC clones (50000 in the case of the Wageningen chicken BAC library), which is then digested with a restriction enzyme followed by the analysis of the resulting fragments by electrophoresis. With this fingerprinting technique, contigs can be build by identification of sharing equally sized fragments. The contigs can be assigned to chromosomes with BAC clones isolated from the markers for each of the individual linkage groups. When the complete contig of the chicken genome is available, the next step will subsequently be sequencing of the complete chicken genome by one of the major sequencing centres. The chicken is a world wide model organism for studying genes that

will justify sequencing of the complete genome. Moreover, the human sequencing project will be ready soon and therefore other species will follow.

A total genome scan to identify quantitative trait loci (QTL) for production and health traits already has been performed which has resulted in the identification of many regions containing QTL for a number of different traits. Fine mapping of the QTL regions, is the next essential step for the identification of the genes for these traits. The fine mapping procedure that will be used in our laboratory is described in chapter 8. The advanced intercross line (AIL) technique has originally been designed for inbred lines. Although this method will also be applicable to outbred populations, such as a our broiler x broiler cross, the analysis of this material is far from trivial, and needs further statistical consideration. The F_7-F_8 generation (40 families) will first be typed with microsatellite markers to perform a QTL analysis within families, followed by an identity by decent (IBD) analysis with a high density of SNP markers from the QTL regions of the animals where an effect is shown. In chicken, SNPs occur with a rather high frequency, 1 every 100bp (Vignal *et al.*, 2000). These SNP markers are currently being developed from the STS markers that were obtained after BAC end sequencing. This fine mapping technique in combination with the high-density comparative map obtained with the BAC sequences and the complete chicken BAC contig should make it possible to identify candidate genes.

One of the relatively neglected features in molecular genome analysis is bioinformatics. Generating vast amounts of data (sequences, BACs) makes it essential to order and store this data in a convenient way. Towards this end we have implemented an AceDB database for the chicken genome mapping data (ChickAce) which will be made available through our website (http://www.zod.wau.nl/vf/research/chicken).

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Chapter X

Summary

Molecular unravelling of the chicken genome to identify genes that are involved in production and health traits is on the way. The tools essential towards this goal are described in this thesis. We started with the development of a large number of DNA markers in chicken analogous to similar initiatives in mammals. Two types of markers can be distinguished, the so-called type I loci (with or adjacent to known genes) and the type II loci (random DNA markers). The majority of the markers developed in chicken are type II markers. All markers are mapped, when polymorphic, in at least one of the three reference populations (East Lansing, Compton or Wageningen) which has resulted in a consensus linkage map of the chicken genome with more than 1900 markers. Assignment of the linkage groups to chromosomes is in progress. In chicken this was easily performed for the macrochromosomes but is complicated for the microchromosomes. The development of large insert libraries will enable to solve this problem. The large insert libraries can subsequently be used for physical mapping and as a further tool in comparative mapping.

In **chapter 2** the isolation of highly polymorphic random microsatellite markers is described. These type II loci are also present abundantly in chicken although the frequency is considerably lower than in mammals. Different chicken genomic libraries were made and screened for (CA) repeats. Microsatellite containing clones were sequenced and primers were made flanking the microsatellite repeat, where one of the primers was fluorescently labelled to enable semi-automated genotyping. Special emphasis in the development of the marker was put on the fragment size, fluorescent dye and optimal PCR annealing temperature of the markers. In total, 372 polymorphic microsatellite markers have been developed in our laboratory. Markers developed by other groups (ADL, LEI and HUJ markers) have been tested under our standard conditions. The characteristics of the 644 polymorphic markers are described (372 MCW markers; 174 ADL markers; 89 LEI markers and 9 HUJ markers).

In **chapter 3** markers are described which were developed from microsatellites known to be located within chicken genes or ESTs. These type I loci are very important for comparative mapping. Chicken cDNA libraries were screened for (CA) repeats as described in chapter 2. Moreover, chicken sequences from the nucleotide sequence databases containing a microsatellite repeat (either mono, di and tri nucleotide repeats) were selected. Primer development and testing of these markers yielded 97 markers (51 located within a known gene and 46 within an EST) of which 67 were mapped in at least one of the chicken reference populations. The human map location of 31 genes out of the 67 mapped genes/ESTs is known which resulted in the identification of 10 new conserved

regions whereas the others confirmed previously identified regions that are conserved between chicken and man.

Microsatellite markers are particularly well suited for total genome scans to identify genes involved in production and health traits because of the use of PCR in combination with fluorescent based semi-automated fragment sizing. In **chapter 4** we describe the method to develop sets of microsatellite markers that can be analysed simultaneously in a single lane of an ABI automated sequencer. Combinations of markers with the same fluorescent dye are selected without overlap of alleles. The amount of PCR amplification product of each marker in the sets should not exceed the signal of approximately 1000 on the ABI automated sequencers because of possible read-through. Careful testing of each set will speed up the genotyping. The number of microsatellite markers per set varied from 8 till 21 with an average of 15. In our set-up, a genotyping capacity is possible of almost 3100 genotypes a day.

A comprehensive linkage map of the chicken genome has been developed by segregation analysis of 430 microsatellite markers within a cross between two extreme broiler lines (**chapter 5**). Of the 430 mapped markers, 54 markers are genes or ESTs that identified new regions of conserved synteny or confirmed previously identified conserved region between human and chicken. The average number of informative meioses for the 430 markers is 400. The markers were placed into 27 autosomal linkage groups and a Z-chromosome-specific linkage group. Alignment of the three different linkage maps is possible in most cases due to the 210 markers that are in common. The coverage within the linkage groups is 3062 cM. Including the 6 unlinked markers and the markers at the end of each linkage group, the maximum coverage is 3750 cM. The difference in length of the genetic map between the heterogametic sex (female) and the homogametic sex (male) is small.

In **chapter 6** the construction of the Wageningen chicken Bacterial Artificial Chromosome (BAC) library is described. Partial digested high-molecular-weight DNA of a White Leghorn chicken is cloned into the vector pECBAC1. Almost 50000 clones are individually picked in 130 384-well representing 5.5 genome equivalents. The average insert size of the BAC clones is 134 kb. Screening of the BAC library can be performed either by 2-dimensional PCR as well as by hybridisation on high-density filters. The BAC library provides an essential tool for physical and comparative mapping.

The comparative map of human chromosome 15 with chicken and mouse is described in **chapter 7.** BAC clones have been isolated from markers mapped to GGA10 and from chicken genes homologous with genes mapped to HSA15. To obtain the chromosomal

location of a particular gene in chicken, a BAC clone was used to map that gene by FISH. In total almost 100 genes or ESTs were identified. Human chromosome 15 has conserved synteny with chicken chromosomal segments from 3 different chromosomes in the order GGA1, GGA10, GGA5, GGA10. In mouse conserved synteny was observed with Mmu2, 7, and 9. For chicken chromosome 10 at least 10 syntenic regions were identified which were scattered over human chromosome 15. These results show three inter chromosomal rearrangements and at least 9 intra chromosomal rearrangements have occurred. At least 7 inversions and 2 translocations are necessary to obtain the conserved gene segments in order on human chromosome 15 and chicken chromosome 10.

In **chapter 8** the use of advanced intercross lines to narrow down the QTL regions is described. The Wageningen QTL mapping population has been used to generate an F_7-F_8 population. In these 40 full sib F_7 families a QTL experiment will be performed only for the QTL regions to detect the families where the QTL is still segregating. Fine mapping of the positive families will be performed with SNP markers. These SNP markers are developed from the STS markers obtained by chromosome walking. Identification of specific SNP haplotypes associated with the quantitative trait from the F_7 generation and comparing these with the specific SNP haplotypes in the F_2 will narrow down the QTL region.

Chapter XI

Samenvatting

In dit proefschrift is een start gemaakt met het ontrafelen van het erfelijk materiaal van de kip. Dit erfelijk materiaal is opgeslagen als DNA op chromosomen. In het totaal heeft de kip 39 paar chromosomen waarop alle genen zijn gelegen. Een aantal van deze genen zijn verantwoordelijk voor productie- en gezondheidskenmerken. De kenmerken waarnaar onderzoek wordt gedaan binnen onze leerstoelgroep zijn die kenmerken waar meerdere genen bij betrokken zijn (de "Quantitaive Trait Loci"; QTL). Voor het opsporen van deze genen bij de kip is het noodzakelijk om het genoom in kaart te brengen. Hiervoor zijn in de loop der jaren een aantal moleculaire technieken ontwikkeld welke in dit proefschrift beschreven worden. Begonnen is met het ontwikkelen van genetische merkers voor de kip. Een merker kan in een gen liggen (type I merker) of op een willekeurige plaats op het genoom (type II merker). In hoofdstuk 2 en 3 werden beide type merkers opgespoord in de vorm van microsatelliet merkers. De microsatelliet bestaat in de meeste gevallen uit een repeterende (CA) sequentie die vaak en verspreid over het genoom voorkomt. Deze repeterende sequenties werden opgespoord door genomische en cDNA banken te screenen met een $(TG)_{13}$ probe. Van de positieve klonen werd de basenvolgorde (DNA sequentie) bepaald. Een PCR merker werd ontwikkeld door in de sequentie, flankerend aan de gerepeteerde sequentie (repeat), primers te maken. Aan één van de primers was een fluorescerende kleurstof gekoppeld zodat de PCR merker geanalyseerd kon worden op een automatische sequencer. In het totaal zijn in ons laboratorium 374 polymorfe merkers ontwikkeld. Deze merkers worden, samen met 270 merkers die door ander groepen ontwikkeld zijn, in dit proefschrift nader gekarakteriseerd en beschreven. Het merendeel van deze merkers is getypeerd in tenminste één van de drie zogenaamde referentie populaties die beschreven zijn in hoofdstuk 1. Er zijn 97 merkers ontwikkeld uit genen waarvan er uiteindelijk 67 geplaatst konden worden op de genetische kaart van de kip.

Doordat de fluorescerende merkers gebruikt worden in een PCR reactie en de analyse van de PCR producten tamelijk vergaand geautomatiseerd is, zijn de microsatelliet merkers uitermate geschikt voor het opsporen van genen welke een rol spelen bij productie- en gezondheidskenmerken. In hoofdstuk 4 wordt het gebruik van sets bestaande uit microsatelliet merkers beschreven die gelijktijdig op een ABI sequencer geanalyseerd kunnen worden. Het is van belang dat de microsatelliet sets zorgvuldig samengesteld zijn. Zo mag er geen overlap zijn van allelen van merkers met dezelfde fluorescerende kleurstof en het signaal op de automatische ABI sequencer moet ongeveer 1000 zijn. Binnen ons laboratorium is het momenteel mogelijk om 3100 genotyperingen per dag uit te voeren.

De genotyperingen uitgevoerd op de Wageningen referentie populatie, welke bestaat uit 10 families met in totaal 486 dieren (F_1 en F_2), zijn gebruikt om een genetische kaart van de

kip te maken. Dit wordt beschreven in hoofdstuk 5. In het totaal zijn er 430 merkers gebruikt, waarvan 54 van het type I (genen). Van deze 54 genen is tevens de locatie bij de mens bekend waardoor het mogelijk was om nieuwe geconserveerde chromosoom-segmenten te identificeren en reeds bekende te bevestigen. De 430 merkers zijn op de koppelingskaart van de kip geplaatst in 27 autosomale koppelingsgroepen en een Z-chromosoom specifieke koppelingsgroep. De lengte van de koppelingskaart wordt geschat op 3062 cM.

In hoofdstuk 6 wordt een kippen "Bacterial Artificial Chromosome" (BAC) bank beschreven. De BAC bank is een verzameling van zeer grote stukken DNA van de kip welke gekloneerd zijn in een bacterie. In het totaal zijn er 50000 klonen afzonderlijk opgeslagen wat betekent dat het genoom 5.5 keer gekloneerd is. De gemiddelde lengte van de BAC klonen is 134 kb. Doordat er DNA is geïsoleerd van gepoolde BAC klonen is het mogelijk om deze BAC bank zeer effectief te screenen met behulp van 2-dimensionale PCR. Met behulp van de BAC bank kan een fysische kaart gemaakt worden. Door BAC klonen te sequencen en de verkregen sequenties te vergelijken met die van de mens is het mogelijk om een zeer gedetailleerde vergelijkende kaart te maken. Een voorbeeld van een zeer gedetailleerde vergelijkende kaart is beschreven in hoofdstuk 7. Deze vergelijkende kaart is gemaakt door humaan chromosoom 15 te vergelijken met chromosomen van de kip en de muis. Hierbij werd gebruik gemaakt van een tweerichtingsaanpak waarbij gestart wordt bij zowel de kip als de mens. Startend bij de kip werd de kippen BAC bank gescreend met merkers die afkomstig zijn van chromosoom 10. De geïdentificeerde BAC klonen werden vervolgens gebruikt om een reeks van overlappende klonen (contig) te maken en om sequenties te genereren die vergeleken konden worden met die van de mens en de muis. Uitgaande van de mens, werden sequenties van genen welke op humaan chromosoom 15 liggen, vergeleken met alle sequenties van de kip die in de database aanwezig waren. Wanneer er homologie gevonden werd met een kippensequentie werden voor deze sequentie primers gemaakt. Hierdoor konden 22 homologe genen geïdentificeerd worden. Voor deze genen werd de BAC bank gescreend en de opgespoorde BAC klonen werden met FISH gecontroleerd of deze werkelijk op het te verwachte kippenchromosoom lagen. Door deze tweezijdige benadering zijn bij de kip in het totaal meer dan 100 genen gevonden. Het bleek dat humaan chromosoom 15 homologie heeft met segmenten van chromosoom 1 en 5 en het gehele chromosoom 10 van de kip. Wanneer we de genvolgorde van humaan chromosoom 15 vergelijken met die van chromosoom 10 van de kip blijkt deze behoorlijk verschillend. Om de genvolgorde van

deze twee chromosomen overeen te laten komen zijn minimaal 7 inversies en twee translocaties noodzakelijk.

In de QTL studie (verder niet beschreven in dit proefschrift) zijn een aantal chromosoomsegmenten gevonden die betrokken zijn bij een bepaald kenmerk. Aangezien deze segmenten nog veel te groot zijn zal de in hoofdstuk 8 beschreven techniek van "advanced intercross" lijnen toegepast worden. Hierbij wordt gebruik gemaakt van de QTL populatie die doorgekruist is en waarvan momenteel 40 families gemaakt worden van generatie F_7. In deze families zal eerst een QTL experiment uitgevoerd worden om die families te identificeren waarin het QTL nog segregeert. Hierna zullen deze families met een zeer veel voorkomende merker, de SNP merker (single nucleotide polymorphism), getypeerd worden. Deze SNP merkers kunnen ontwikkeld worden uit de BAC sequenties die beschreven zijn in hoofdstuk 7. De resultaten verkregen met deze SNP merkers zijn te combineren tot zogenaamde "SNP haplotypes". Door de SNP haplotypes geassocieerd met het QTL uit generatie F_7 te vergelijken met de SNP haplotypes uit de F_2 generatie zal het mogelijk moeten zijn om het QTL gebied te verkleinen.

Abbreviation Key

ABR	National Institute of Agrobiological Resources, Kannondai, Japan
ADL	Avian Disease and Oncology Laboratory, Michigan State University, East Lansing, USA
BAC	Bacterial Artificial Chromosome
COM	Compton laboratory, Institute for Animal Health, Compton, UK
EST	Expressed Sequence Tag
FISH	Fluorescent In Situ Hybridization
GGA	*Gallus gallus*
HSA	*Homio sapiens*
HUJ	Hebrew University of Jerusalem
LEI	University of Leicester, Leicester, UK
MCW	Microsatellite Chicken Wageningen
PCR	Polymerase Chain Reaction
QTL	Quantitative Trait Loci
SNP	Single Nucleotide Polymorphism
UMA	University of Massachusetts, Amherst, USA
WAU	Wageningen university, Wageningen, The Netherlands
WS	Wageningen Sequence tag
YAC	Yeast Artificial Chromosome

Appendix 1

Table 1. Characteristics of new polymorphic microsatellite markers (MCW markers).

Marker	Dye[1]	Length[2] (BP)	Forward primer	Reverse primer	PCR[3]	All.[4]	Pop.[5]	Acc. No.[6]
MCW0001	FAM	156-168	TGTCACAGTGGGGTCATGGACA	ACACGTCCTGTGTTCACATGCCTGT	55	3	CE	L40034
MCW0002	FAM	147-149	TCCAGAGACAGTTGCTCCACATTC	GCAAGTTAGTTATTGTAGGGGCTC	55	2	C	L40041
MCW0003	FAM	143-149	CCTAAACATAGCAATGAGGATAAC	ATTCAGTTCCTTAAAGTTCTTGGG	55	4	C	L40036
MCW0004	FAM	149-199	GGATTACAGCACCTGAAGCCACTA	AAACCAGCCATGGGTGCAGATTGG	55	4	EW	L40038
MCW0005	FAM	189-259	ACCTCCTGCTGGCAAATAAATTGC	TCACTTTAGCTCCATCAGGATTCA	55	4	CEW	L40039
MCW0006	FAM	231-241	AGAAACTACTGAGAAAGCCATGCA	CACAAAGTTACAATAATGAACTTG	55	2	CEW	L40037
MCW0007	FAM	313-349	AGCAAAGAAGTGTTCTCTGTTCAT	ACCCTGCAAACTGGAAGGGTCTCA	50	4	CEW	G54469
MCW0009	FAM	162-174	ATGCCTGCAGTTCATGCTGCATCA	TGCACAATATGACTGGTGACTTCC	50	3	EW	G31931
MCW0010	TET	093-109	CTGTAGAATTACAGAAATACA	TAGTACAAGAATCTAGTGTTAAAA	55	5	EW	L40047
MCW0011	FAM	117-119	TAAAATTTATCTTTGAAAATGCCT	GAGAAACATGTATTTCAATTATTC	50	2	W	G31932
MCW0013	FAM	161-169	TTGTTCCATCTACTGGGATTGGTT	GCTGAGCATTAAACGAAATGGATG	50	4	C	
MCW0014	FAM	164-187	AAAATATTGGCTCTAGGAACTGTC	ACCGGAAATGAAGGTAAGACTAGC	55	4	EW	L40040
MCW0016	FAM	164-187	ATGGCGCAGAAGGCAAAGCGATAT	TGGCTTCTGAAGCAGTTGCTATGG	55	4	EW	L40041
MCW0017	FAM	160-178	CAATAGGGTTTCCATGTAACCAGC	CAGCTACTTAGAGGAAGCCAAACC	55	4	CW	L40044
MCW0018	FAM	199-221	GGAATTTGAACACCTGAGATTTCC	CACTATGTTTATGGCAAACTCCTG	55	5	EW	L40067
MCW0019	FAM	114-148	CCCAGATTGATTTCATCATTCAAG	GCATCTCCTCTCTACAGCCATGAA	50	5	W	G31933
MCW0020	FAM	183-189	TCTTCTTTGACATGAATTGGCA	GCAAGGAAGATTTTGTACAAAATC	55	4	EW	L40055
MCW0021	FAM	222-228	GGACCTCTCAGCAGTGCCATAATA	GATGAACTCCTTGGATTTGTCCCA	55	2	CEW	G31900
MCW0022	FAM	129-185	CATGATTCTGTGAATCAGCCCTGA	TTCCAGTCACAAGAGCGATGCTTG	50	5	W	G31934
MCW0023	FAM	156-168	TAAAGCTGAGCCTGGGGAACCTAA	ATCCATTTACTGTCAAGTGAAACAG	55	5	EW	L40065
MCW0024	FAM	146-150	TTTGCAGCTTGCAGAAAGATGCGG	ATTTCTGCTGGTGAGGATCACGTG	45	3	W	G31935
MCW0025	HEX	271-283	ATTGCTGGAGCTCACAGTGCT	ATCGCTCTCAGCTTGCATGTA	55	4	C	L43632
MCW0026	TET	215-228	CAAGAGCTTAAGTAGTTAGCTC	TGCTATTGGGTTCTCTGTAAACCTC	50	4	CE	L43633
MCW0027	HEX	123-155	AAACAGTTGCGGTGAAAGCCTG	AACTCCAAGATAATCTGATAACTG	50	6	W	G31936
MCW0028	FAM	150-180	TCCTCTTCACTTGTAATTACA	TAATGTTTCCTTAAGAACTTAGTT	50	3	W	G31937
MCW0029	TET	149-194	CATGCAATTCAGGACCGTGCA	GTGGACACCCATTTGTACCCTATG	55	6	CEW	L43634
MCW0030	FAM	110-153	AGAGTGTTGTGTCAGTAAGAC	TTTGCTATCATAGCTGGAAGAGCT	55	8	CEW	L40050

MCW0031	TET	080-110	ATACACCATGTAGACCCCTGT	TACAGCCATACAGGACTCATTAAC	55	5	EW	L40066
MCW0032	FAM	273-314	AAGTTCCTTGTACAATTGTTA	TCATTACTAGTACAATCAAGATGG	55	7	CEW	L43672
MCW0033	FAM	192-284	GGTGATAGGCTACAGAAGCTTACA	AAAAACAACAACACCTATATACAG	48	5	CEW	G31938
MCW0034	FAM	227-245	TGCACGCACTTACATACTTAGAGA	TGTCCTTCCAATTACATTCATGGG	55	5	EW	L43674
MCW0035	TET	227-233	CAGAAACATTTGGACTTGGCTT	TTGCTTCATTTCTAGTCTCCAGTT	55	5	EW	L40059
MCW0036	FAM	141-153	CCTCATGTGAAGCATCTTTTCATA	TGTCTTCAGTAGGACTAGTGATAC	55	3	EW	L43675
MCW0037	FAM	155-159	ACCGGTGCCATCAATTACCTATTA	GAAAGCTCACATGACACTGCGAAA	50	3	EW	L43676
MCW0038	FAM	138-163	GCAGTAGAGACTGAACATTGGGAC	ACTTATAAGAACTAACCACTGACA	50	3	CEW	L43677
MCW0039	FAM	111-123	CATTGGACTGAGATGTCACTGCAG	ACATTTGTCTAATGGTACTGTTAC	55	5	CW	L43678
MCW0040	TET	135-150	ACCGAAATTGAGCAGAAGTTA	ACTCAAAAATGTGGTAGAATATAG	55	3	CEW	G31939
MCW0041	FAM	144-160	CCCAATGTGCTTGAATAACTTGGG	CCAGATTCTCAATAACAATGGCAG	55	3	C	V00439
MCW0042	FAM	202-220	CCGAAGCATCAAAGCGTCGCGTTC	GGCATCGCACCGTTAAGTTACACC	50	7	CEW	D11381
MCW0043	FAM	134-154	TGACTACTTTGATACGCATGGAGA	CACCAAGTAGACGAAAACACATTT	50	5	EW	D00311
MCW0044	FAM	135-148	AGTCCGAGCTCTTGCTCGCCTCATA	ACAGTGGCTCAGTGGGAAGTGACC	50	6	W	X02218
MCW0045	HEX	145-151	CCAAAGGAAACAAATACTATACGA	GAAAGAAAAACTGACACTGTGACT	55	3	EW	M20006
MCW0046	TET	150-158	ATGAGACCACTTGTTGCCTCAAGG	GATCGTAGTGGTCTTTTCCAACCT	55	3	W	M17627
MCW0047	HEX	100-107	GGATTACGGCCGTTTGTGCACAAA	AATGGAACGCCGAACTCGCGTGCA	55	4	CE	X63083
MCW0048	HEX	165-201	CGTATAGGAGGGTTTCTGCAGGGA	AAGGAGGAACGCACCGCACCTTCT	55	5	CW	D90071
MCW0049	HEX	116-127	AGCGGCGTTGAGTGAGAGGAGCGA	TCCCCAACCCGCGGAGAGCGCTAT	55	5	CEW	M59361
MCW0050	HEX	260-272	GGTGTCCGCACCCCGGAGCTTCTT	GCAGCATCGCGCAGCACCGCGGAT	50	4	E	S43620
MCW0051	TET	067-090	GGAACAAGCTCTTTCTTCTTCCCG	TCATGGAGGTGCTGGTACAAAGAC	55	5	CEW	M33143
MCW0052	HEX	229-258	ATGGCTAATAGCAGATGACACCTG	GAGGTTAGTGCATCAGTTGTACCT	55	3	EW	D13439
MCW0055	TET	186-195	TTTGTAGTTACCTGGTACTGA	GTTTGCATTGTCTACAGCTCCTTG	55	3	W	G31940
MCW0056	TET	175-207	TGGTAACCTCTAACCTTGACG	AGTGAAGGAGACTCCACAGCCTCT	50	4	EW	L43673
MCW0057	TET	152-157	AGACCAGTCCATGACCTCTCA	CATCAACCCAGTATGCCTTCTGGA	50	2	W	G31941
MCW0058	TET	185-286	GGGCACACAGAGTGGACACCA	TAGCATTTTCTCCAATGACTCCGG	55	4	CEW	L40057
MCW0059	TET	158-172	AAGTGCCTTTGCTATCCTGATTGG	AACTCCTATTGTGCAGCAGCTTAT	55	6	CEW	M59037
MCW0060	FAM	206-224	CCAGCAGGTAAAAGCCGACTCTGC	AGGCCTGACTGGCTCAGAAGGCAA	55	2	EW	G31942
MCW0061	HEX	108-128	ACCTGTATAGTGCACTGCTGATGG	TTTCATGCTAAGCCGATCTAGGTC	55	4	EW	L43679
MCW0062	TET	125-169	ATACTATCAGTAGCATATTATACC	GCACTTCAATCTTTCCATGTACAT	55	9	EW	L43680
MCW0063	HEX	134-153	GAAAACCAGTAAAGCTTCTTAC	GGCTCCAAAAGCTTGTTCTTAGCT	55	6	W	G31943
MCW0064	HEX	144-150	CTTCAAGAGCCATAGGTGGTCT	TCTCAGCACTACAAAATACACAGG	50	3	W	G31944
MCW0065	TET	100-110	TCAGCAACAGAAGTGAAGGGCAAT	CAGGCATTACTTCAATAACGAGGC	55	5	CW	L43681

MCW0066	HEX	110-126	CTGGAATCACTGTTGTGGACTT	GGCCTTGAGATTTCATTCAGAGAC	50	5	CEW	L43682
MCW0067	TET	175-184	GCACTACTGTGTGCTGCAGTTT	GAGATGTAGTGCCACATTCCGAC	55	3	CEW	G31945
MCW0068	HEX	171-193	CCTCACTGTGTAGTGTGGTAGTCA	GAGAAGCTTGAACCTACCAGTCTT	55	5	CEW	L43683
MCW0069	HEX	159-168	GCACTCGAGAAAACTTCCTGCG	ATTGCTTCAGCAAGCATGGGAGGA	55	4	CEW	L43684
MCW0071	FAM	104-108	TGGCGTTATTTCAAAACGACCGTA	GCGGTCGTTCGGTCCTTATTTTAA	55	3	W	L12696
MCW0072	TET	191-199	TAAACTGACTTCACTACTCAGCAT	AAAGGACATCTAACTTCAAAACAG	50	2	W	X54093
MCW0073	HEX	156-166	TATTTCACCCACGGGGACGAATAC	AGGGTGCTGAGAGCTGCCAATGTC	55	6	CEW	L06098
MCW0074	FAM	122-124	GCCAATTGCTCGTTGGAGTATCAG	TGGCCAAGGGTACGCGCTTACAGC	55	2		D10287
MCW0075	FAM	170-180	CGTCAAGCCAGATGCTGATGAGTG	ATTCCAACCAGAAGTTTGACTCGC	55	2	C	X13026
MCW0076	TET	121-134	GCTCCGGACCATGAATTTGGCATT	ATTATTACTCTCTCCTGTCACGCG	55	5	EW	M17607
MCW0077	HEX	157-162	CACAGAATTTAAATTGATTACT	ATCTAAGAGGCAGTGATTACCACC	50	4	CW	L43685
MCW0078	HEX	131-137	CCACACGGAGAGGAGAAGGTCT	TAGCATATGAGTGTACTGAGCTTC	50	3	CEW	L43686
MCW0079	HEX	260-284	TGCTACGGGGGCTGTGATGCATTC	AAGCTGTCCTTGATGTGGTCCCGC	55	5	CEW	L12469
MCW0081	HEX	112-134	GTTGCTGAGAGCCTGGTGCAG	CCTGTATGTGGAATTACTTCTC	55	4	EW	L40045
MCW0082	HEX	100-118	GATCTTTAAGGGGAAAGATAT	CTTTTGATGCCTCTCCATTTC	55	3	EW	L43636
MCW0083	HEX	066-093	GCCTTTCACCCATCTTACTGT	TACATTTCAGAAGGAATGTTGC	50	2	W	G31946
MCW0084	HEX	087-118	TTTGAAGGGATGCTGCATGCA	CTGATTTGCAGCTTGGCTGAG	50	4	W	G31947
MCW0085	FAM	272-282	GTGCAGTTATATGAAGTCTCTC	GGTATCAGGGCTTCTGAAACA	50	5	CW	G54426
MCW0086	FAM	270-290	AGCAAAACATGCCTTCAGAGC	AGCAGGAGAGAAGCTGCAGTA	50	3	W	G31948
MCW0087	HEX	272-287	ATTTCTGCAGCCAACTTGGAG	CTCAGGCAGTTCTCAAGAACA	55	7	CEW	G31949
MCW0088	FAM	280-306	TTGCAAATGAACGTATCATGC	TCCAATCCTAGAAGTGTTATG	55	9	EW	L40062
MCW0089	FAM	281-311	CAAAACATGCCTTCAGAGCAAC	AAGCAGGAGAGAAGCTGCAGT	55	6	W	G31950
MCW0090	FAM	107-122	GATCCTTCTTCCTCTCTCCTG	CCTTCAACTTAAAACATTATAGAG	55	5	CW	L43639
MCW0091	FAM	270-272	CTTTGTGTTTCCAAAGCACTC	TGTTCCACCTACTCATTACTG	55	3		G31951
MCW0092	FAM	071-075	GATCCTCCATGAATACAGGTT	GATCGCAAAACGCCTTTGTG	50	3	W	G31952
MCW0093	FAM	254-265	TCTTTTTGTTGTTGTTGGCTT	TCATGTGTTCCTGTACGTATG	55	3	W	G31953
MCW0094	FAM	077-095	GGAGCTGGTATTTGTCCTAAG	GCACAGCCTTTTGACATGTAC	55	7	EW	L40063
MCW0095	FAM	072-091	GATCAAAACATGAGAGACGAAG	TTCATAGCTTGAATTGCATAGC	55	7	EW	L40060
MCW0096	FAM	182-313	ATCTAATAGTTTTGCTACCATC	AGAACATTAGGTACTACAGTTC	55	7	CW	L40046
MCW0097	FAM	263-309	GGAGAGCATCTGCCTTCCTAG	TGGTCTTCCAGTCTATGGTAG	55	5	EW	L40053
MCW0098	TET	260-262	GGCTGCTTTGTGCTCTTCTCG	CGATGGTCGTAATTCTCACGT	55	3	CEW	L40074
MCW0099	TET	279-310	CTGAATGGGTTAATATGTTTATG	GATCTGGGGACACTGTCTGCA	55	6	EW	G31954
MCW0100	TET	080-096	GATCTAAACAAAAACAGACACA	TGTAGGCGATTAAACATACTTC	55	3	CW	G31955

MCW0101	TET	273-279	GTTTGTTTGCATCTGTAGTCTG	CCATATTCTGTTAGAAAGTAGAG	55	5	CW	L40049
MCW0102	TET	227-239	AACACAGAACTGTTGGAATGG	TGTTAAAACCAAAATCTATCAGG	55	4	EW	L40073
MCW0103	TET	269-274	AACTGCGTTGAGAGTGAATGC	TTTCCTAACTGGATGCTTCTG	55	4	W	G31956
MCW0104	TET	191-208	TAGCACAACTCAAGCTGTGAG	AGACTTGCACAGCTGTGACC	55	5	EW	L43640
MCW0105	TET	255-261	CTTGTTCTATTCTTCAGTGGC	TCTCAGTAAGGCTTGGCACTC	55	2	EW	G32111
MCW0106	HEX	125-129	GGCAACTAAGTTGTGGACTG	GCAGCATTCAGTGGGATAAT	55	6	CEW	L48902
MCW0107	HEX	113-121	GAACAGAACTCTGTTTACTG	TCTGCTTACCTCAACTGACA	55	5	EW	L48906
MCW0108	TET	091-109	GTCTCGTGGAGATGATCTAT	ATAATGAAGACACCGACATT	55	6	W	L48903
MCW0109	TET	131-159	TGATTCACTTGATGGTCGAG	CTATCAATTTATTCTGCCTT	55	8	EW	L48904
MCW0110	TET	100-118	CATCTGTGTTACTGTCACAG	TCAGAGCAGTACGCCGTGGT	55	8	EW	L48908
MCW0111	HEX	102-110	GCTCCATGTGAAGTGGTTTA	ATGTCCACTTGTCAATGATG	55	4	EW	L48909
MCW0112	HEX	263-277	ATCTCTCTGCCCATGTTTCAG	GATCACTAAGGTCCCTTTCAA	55	7	CEW	L40080
MCW0113	HEX	100-102	AGTGTATCCAGCCCACACTT	GAAGTGGTGTCATCAAGGAC	55	2	W	L48905
MCW0114	TET	261-293	AGCAAACTGCTCAGTGCTGTG	GCGTTGAAAGTAGTGCTTCCG	55	7	CEW	L43641
MCW0115	HEX	252-260	ATACCAACATCTGCCTCTGAC	GCAGTGTGTCTGACTAGCTCT	55	5	CEW	L40076
MCW0116	TET	287-289	GTATTTGAGTCTCAACAGCTC	AGGCCAAAGATGAGACACCTG	55	2	EW	L43687
MCW0117	HEX	213-236	GTCTCGGACCTCTCTTACCAG	AGGGAGATGTCAGGGTGTTCG	55	5	CEW	G31957
MCW0118	TET	166-168	ATGATGAAGCATTTAGTCTAAG	CAATTTACTCAGAGATGCAGTG	55	2	E	L43642
MCW0119	TET	118-180	TGTGCTGCTCCACAGGCCAG	GATCTGTGTCTGGCATTGTGT	55	5	CEW	L43643
MCW0120	TET	270-284	CTATGTAAAGCTTGAATCTTCA	ATTCCTGGGTGCTAATTTACC	55	8	CEW	L43644
MCW0121	HEX	207-212	ATGGATAGGGGTAACTGTTGC	CTACGTGTGTTTGACAGCTGG	55	2	W	G31901
MCW0122	HEX	267-277	TCCTTTGGAGCACGGAGGAAC	AGATGCACAGGCAGAGCTCCA	55	6	CW	G31958
MCW0123	TET	084-094	CCACTAGAAAAGAACATCCTC	GGCTGATGTAAGAAGGGATGA	55	4	EW	L43645
MCW0124	HEX	259-266	GACTAGATGCTATGTCTTCAC	CCTTGCAACATTACCTTCAGC	55	2	W	G31959
MCW0125	TET	068-080	GATCATCCTTCATTTTTTCTTTA	AAGAGCGAGATAAAGTTGTAG	55	3	W	G31960
MCW0126	HEX	129-164	ACAGAGGAAGCCTGAATGAGT	TGGTGTACAGCACAGGCAACA	55	3	W	G31961
MCW0127	HEX	229-245	TGCAATAAGAGAAGGTAAGGTC	GAGTTCAGCAGGAATGGGATG	55	6	CW	L43646
MCW0128	HEX	170-176	CACATCCTTCTTAGCAGTCAC	CTGATGAAAAATTGTCCAAGTAG	55	4	W	G31902
MCW0129	HEX	108-118	ATTTGGTGAACACAAACCTGC	CCACTTGAATGAAGCACCTAC	55	4	W	L40077
MCW0130	HEX	256-258	AAAAGCAGTTTTAGACTTGCTC	AGGTGAGCAGGGAGTATCTTC	55	2	EW	G31962
MCW0131	HEX	195-217	GTTGCTGATTCTAAGGCAGGC	TTGCAGTTGTAAAGGTGTAGC	55	7	CW	L43647
MCW0132	FAM	116-130	TTTACTACACAGTCCTGCAGC	ATCTTCTCCGCGCTTTTGCAG	55	3	CW	L43648
MCW0133	FAM	128-148	GATCTTTCTGTACAATGAATAC	TTAGGAGCAACTCAGTTGGAG	55	5	EW	L43649

MCW0134	TET	260-284	GGAGACTTCATTGTGTAGCAC	ACCAAAAGACTGGAGGTCAAC	55	9	EW	L43650
MCW0135	TET	124-150	ATATGCTGCAGAGGGCAGTAG	CATGTTCTGCATTATTGCTCC	55	7	CEW	L43652
MCW0136	TET	140-167	CACAGTCTTTGCTTGGAGTG	GCTTTAGTTCATGTGAGACTA	55	2	E	L43653
MCW0137	TET	244-258	GATCACTTTCCCTTAGGAAGG	TTCATTCTGCTGACTCTCCTG	55	6	CEW	L43654
MCW0139	TET	172-168	TCTGCCACACTTCATTTATA	AAGTAGTTGCTACTGTACTTG	55	5	CW	L43655
MCW0140	TET	260-262	GACCAAGAATTTCACAGAAAT	TCTGCAGTTTGAAGGTCTGAT	55	2	W	G31963
MCW0141	TET	255-259	GTATGAGTATAGCTGTATTG	CTGAAAGATCTAAGCTTTGT	55	3	EW	L48883
MCW0142	TET	068-091	GTCTAAAGAAATACACATAC	CTGAAAGATCTAAGCTTTGT	55	3	W	L48882
MCW0143	TET	100-105	ATCTGTTGCACACTCATTGC	TACTGAGCATTGTGCATGCG	55	2	C	L48880
MCW0144	TET	089-091	TGGCGTAACCTCCTGCTGCT	GCAGCCGAACTGTTTTGGCA	55	2		L48877
MCW0145	TET	164-212	ACTTTATTCTCCAAATTTGGCT	AAACACAATGGCAACGGAAA	55	8	CEW	L43656
MCW0146	TET	164-169	CCGTGTGGTGAACAACGATGA	CAAATCTGCCCTGACGTCAGC	55	3	W	G31903
MCW0147	FAM	116-178	GATCCATTTATAAAGACCCCA	CCTGGTTTGCCAATACACTTG	55	8	E	L43657
MCW0148	FAM	098-108	TTGCCAGGTCAGGACTACAGT	TCACTCTGTAGCTTTTTTTGC	55	3	W	G31964
MCW0149	FAM	074-106	ACTCCTACAACAGCATACAT	TGCAATTAAAGGAGTAACCT	55	7	EW	L48895
MCW0150	HEX	227-235	TCCTGACTGAAATGGTACAGC	CATGAAAACCTTTGCCCTCAG	55	3	CW	L43658
MCW0151	HEX	255-269	CATGCTGTGATACTACAATTCC	AACATCCTGGAGTTTGGGAAG	55	5	EW	G31965
MCW0152	HEX	224-229	GAGGTATTTCTCAGAACTTCC	CAAACTATTAGTTCTTCAGCTG	55	3	W	G31904
MCW0153	FAM	073-075	ACTGCCTGATGTAAACAAGT	CATATGGAAATGGCGCAGCT	55	2	W	L48885
MCW0154	FAM	171-193	GATCTGTTTTATCACACACAC	CCATTTCCTTTGTTATCAGGC	55	6	EW	L43659
MCW0155	TET	136-138	GGTTAGTAATGTTCCTCATC	AGACATCAATGAGTCAGTCA	55	2	W	L48886
MCW0156	HEX	242-300	TCTGTAACATTTTTCCTTTTGTG	TTAATGTGGCAGACTCAAAGG	50	5	W	G31966
MCW0157	HEX	291-301	GTGTGATGTAGGCCAGATGTC	GTGCTGCATTCTGCCAATAGG	55	3	CEW	L43660
MCW0158	FAM	164-224	GATCCATTTATAAAGACCCCAC	TTCAATACTCCTTTGTAAAGCA	55	8	EW	L43661
MCW0159	FAM	076-081	AGGAGACTATCCAGGACTAGC	CTATAAAGGATGCCAAATGAA	55	2		G31967
MCW0160	FAM	208-226	GATCTTCTTGGTTTGGAAACC	AACAGCATCCATCACTGCATG	55	4	EW	L43662
MCW0161	FAM	221-227	TACAAATTCCTGAGACAGATG	GTAGACATTGAGGAATTGCTG	55	2	W	G31968
MCW0162	FAM	076-085	TCATCTCCAGACCTGGCCTG	GCATTTACATTGTAACAACT	55	2	CEW	L48891
MCW0163	FAM	100-102	GAAGTGTGTTCTACAGTCTG	ATAGGCATTGTTAATGTACC	55	2	C	L48890
MCW0164	FAM	241-248	TTGGTTCTGACTGGAAGTACA	ATCAGAATATACTGTAGAACAG	55	3	W	G31905
MCW0165	FAM	118-120	CAGACATGCATGCCCAGATGA	GATCCAGTCCTGCAGGCTGC	55	2	E	L43663
MCW0166	HEX	194-210	GATCAGAAAGAACTGGAACTG	AGGAGTTAGTTGAACCAGAAC	55	7	EW	L43664
MCW0167	HEX	095-120	GATCCCAAAACAAATGCACAC	CTTACATGAGTGCTATCTGCT	55	4	EW	L43665

MCW0168	FAM	115-121	GATCAGATTTATTTCCCCTCA	CTGATTTCTAGAGCTGACTG	55	2	EW	L43666
MCW0169	HEX	092-096	GATCCCACTTGTTAAGAAGTG	CCTGACCTTACTGAGCTTGGA	55	3	EW	L43667
MCW0170	HEX	263-287	TTGTGAAACTCACAGCAGCTG	TTATAGCAGGCTGGCCTGAAG	55	7	EW	L43688
MCW0171	HEX	217-230	CACATCATAAATCAGAGTTGCC	TAATATTCAGCCATCAGGACC	55	3	CEW	G31969
MCW0172	HEX	299-305	GGAGGTTTAGCTTAGACTGAG	CAGCCTGAGATAGCACGTATC	55	2		G31970
MCW0173	HEX	250-273	TTCAAGCTGCTTAGAGGTTCC	TCTTGTTAGCCAGGCTGAAAG	55	5	W	G31906
MCW0174	HEX	261-278	TGGACTTAACACTGCTATTGC	CTCTCTACCTTGGAGGGCTGA	55	7	W	G31907
MCW0175	HEX	262-264	GATCATAATAAAGTATAATGCTG	GTGTTTCTATTCACATAAAGCC	55	2	C	G31908
MCW0176	HEX	257-270	AAAGAGAAGTATAAAACATGCC	TCCATTCTTGGCAGTGCATAG	55	4	W	G31971
MCW0177	TET	293-316	TTAAAACTGTGTCCATGTAAGC	GTAGAAACATGAACACTCTACC	55	3	W	G31972
MCW0178	TET	076-096	ACTGGAATTTTAGGGCAACAG	AACTGTTAGCTAATATGACCTG	55	5	EW	L43668
MCW0179	TET	239-246	ATCTGGCTTCCAGGGCAGCT	TTAGACACTAAATCAGCATGAG	55	4	EW	G31973
MCW0180	TET	071-088	GATCACATCACGTTAATTTT	GGTGGAGAAAAGTGAAAGAC	55	5	CEW	L43669
MCW0181	TET	249-255	GATCTCCAGAGGTCCTTTCC	GTGATGTTGAACACTGCAGTC	55	5	EW	L43670
MCW0182	TET	291-301	AATGTGCTTTGACCTCCTACC	CATGCCTTATGATTGCAGATG	50	3	CW	L43671
MCW0183	TET	290-311	ATCCCAGTGTCGAGTATCCGA	TGAGATTTACTGGAGCCTGCC	55	3	W	G31974
MCW0184	TET	240-293	CATTTGATACTTACTGAAGCAC	CAAGTCCGGAACTAGTGATGT	55	6	W	G31975
MCW0185	TET	200-226	GATCTACTGTCATTTTAGTTT	TGAATAGATTTCAGTGAGTGC	55	3	CEW	G31976
MCW0187	HEX	117-132	ATCTAGTTTGGACAAGTTAC	CTAACTTATTTCAGTCAAAT	55	5	EW	L48899
MCW0188	HEX	177-183	GTGACAGCGGCAGAGATGGA	CGCACAGCCCCACTCGCACA	50	2	EW	L48897
MCW0189	HEX	110-124	CACGACCTTTGCACGTCTAT	CCGAGCGCGTGTGGTGCATG	55	3	CW	L48893
MCW0190	HEX	060-105	GTGATCATTTCTACATGCAG	ACAACAGAACTAAACAAATA	55	4	CEW	L48881
MCW0191	TET	202-232	TGTAATCAGCATTTAATAGA	AGGCAAACAGTTGTGAAACT	55	4	EW	L48878
MCW0193	TET	302-317	TATTCAATAGAGTTACGCTGTC	ATTACGTCTGCACCAGTACAG	55	6	CEW	G31977
MCW0194	HEX	162-170	GAACAGAAATAATCATTGGAGA	TCAGTAGGGACTGCTCTGAAC	55	4	EW	G31909
MCW0195	HEX	185-214	TCTGAAGTAGAGTAAAGGCAG	GAATAGCACAGTGTCTGCTGG	55	2	W	G31978
MCW0196	TET	192-200	ATACTTGAAGAAAACTAAACTTC	GATCAGTGTTGCAGATTTTGG	55	6	C	G31979
MCW0197	TET	093-112	GTGCTGCTGGGTTTAACCTA	CTCACACGCGCACATACTTA	55	6	CEW	L48901
MCW0198	HEX	264-267	GATCTTTGCTACCATCCACTG	ACCCATCTGGTTGGACTATGC	55	2	EW	G31980
MCW0199	TET	282-286	ACCATGTGGAATTCAGAGAGG	CCAAAGATAAGTAGCCATAGG	55	3	W	G31981
MCW0200	TET	241-263	GAGACATTGCAAATACTCAGC	TAGTCAGGGAGTTCAGGAAGG	55	3	W	G31982
MCW0201	TET	302-312	ATGTGAAGGCCTTCCAAATCC	AACCTTCACAGAAAGCTCATG	50	4	CEW	G31983
MCW0202	TET	305-307	TGTGAACGTGGAGGTACCATC	AACATATGGCTCCACAGCCTC	55	2	EW	G31984

MCW0203	TET	183-187	CTGTTGTAATGATAGAAGAGC	CTGTTCACGTCTAGTCCCCG	55	2		AF030581
MCW0204	TET	096-108	AGATTCGTGCAAGCGCTGCTG	TCAACTAATGGCACAACCGTG	55	4	EW	AF030578
MCW0205	HEX	282-290	GATCCTGACCCAGAGGACC	TCTTTGAGGAAGACCAGGTGG	55	4	CEW	G31985
MCW0206	HEX	226-235	CTTGACAGTGATGCATTAAAT	ACATCTAGAATTGACTGTTC	55	4	EW	AF030579
MCW0207	FAM	278-284	GATCCTTACAGCCTGCAATGC	ATACTGTTGGAAGATGTATGCG	55	4	EW	G31910
MCW0208	HEX	228-239	TGACACCCAGATGCAGCAATC	TACATGACTGAGAGGCTGGTG	55	3	CE	G31911
MCW0209	HEX	190-229	CAAGGATAAAGAGCAGCTGCC	ACCAGAGGTGGTGGCCATAG	55	4	CEW	G31986
MCW0210	TET	153-186	TGCCACTTTCTGCCAGCTAAG	CAATGATATCCACTGCATGAG	55	4	EW	G31987
MCW0211	FAM	224-256	GATCCAGGCTGAAAAAACAC	AACAGAGGCATGGGAGCCAC	60	6	W	G31988
MCW0212	HEX	188-210	AAAACCTTGAGAGCCATCTAG	GTGATTTCCCTTTTCAGTGAC	55	5	CEW	G31989
MCW0213	FAM	293-311	CTGTTCACTTTAAGGACATGG	GACAAGTCAACAACTTGCCAG	55	5	CEW	G31990
MCW0214	FAM	274-291	CAACAGTAACCATACATCTGC	TACCTGGATTCTTTCATCAGG	55	5	W	G31991
MCW0215	FAM	288-296	CCTAAGGCTACAATTAGACTG	GAGTGGCTCTTTCTACCAAGG	55	2	W	G31992
MCW0217	FAM	153-174	GATCTTTCTGGAACAGATTTC	CTGCACTTGGTTCAGGTTCTG	50	4	W	G31912
MCW0218	FAM	258-281	TCCCTAGGCAAACCTGCTTAC	AAGACCCCACAACTTGACTTG	55	5	EW	G31993
MCW0219	FAM	227-241	CCACAGCTATAAATGCTATAGC	GACATGACTTACTGAAAACTAG	55	4	EW	G31994
MCW0220	FAM	251-261	TTGAATGCCTCCACAGCAGCA	GAGGACTGCTGTAACAATTACC	55	4	CW	G31995
MCW0221	HEX	172-180	CTATAGGGACGCACAGCATG	TCGCCCACTGTAGCCCTGAG	55	3	W	G54427
MCW0222	FAM	221-225	GCAGTTACATTGAAATGATTCC	TTCTCAAAACACCTAGAAGAC	55	3	W	G31996
MCW0223	FAM	178-188	TCCAGAGATAGTCTGTAGTGC	AGCACGTACAGCAGTGTTGCT	55	4	CEW	G31997
MCW0224	TET	291-301	ATTACCTTTCTTCATTAACGCC	TTCATAGACTTGAGCGAGGAC	55	4	CEW	G31913
MCW0225	FAM	176-186	AACGGACTCTTCTGTCTATAG	TGCTTTGCTCCTCATTAAAGG	55	5	W	L34550
MCW0226	FAM	297-310	ACTTATCTGGCTTGCTCTCAG	GTCTCTCTAACCATCCTAAGC	55	3	W	G31998
MCW0227	FAM	242-260	CAAGTTTGCCCACATGGACAG	CGTGGTGGCATTAGTGGG	55	5	W	G31914
MCW0228	TET	222-251	GATCTCTGCATTACAAGCATG	TTGCTGACCTGCTCATGCAAG	55	6	CEW	G31999
MCW0229	TET	086-092	AGCGATGATTGTAATCAATGG	TCAGCTCGGCCGTCGCTC	55	3	CW	G32000
MCW0230	FAM	281-298	TGCACAGAGCCAAGCTGCTTC	GATCCTCTGATGGCTGCCG	55	2	W	G32001
MCW0231	FAM	266-272	CTTTCTGAAATTCACAAATGTAG	ATTCCTTTCTGTGCTTCTCAC	55	4	CE	G32002
MCW0233	TET	208-218	TCCAGCAGTAAGTATAGCTGC	TGTTAGCTGCAGGGTATTAGC	55	3	W	G31915
MCW0234	TET	262-269	GAAGCTTTGAAACTAGCCAGC	AGAAGCTAGTTGCTGAAAGGC	55	3	CW	G32003
MCW0235	HEX	190-196	CTGTGACAATTAACTCTCCAG	AATTCTCAGGTAAACTGGAGC	55	3	CW	G32004
MCW0236	HEX	310-330	GATCCTACAGCCTCTGAGTC	TTTCGAAAAGAAGCTGCCTGG	50	5	CEW	G32005
MCW0237	TET	196-234	ACTGCTGGAGTTTGCCTGTC	TGAGAGTTGCCTCTGTCACC	55	5	W	G32006

MCW0238	FAM	187-217	GATCAAGGATACACACAC	CTATGCTTAGTGTCACCAGC	55	5	EW	G32007
MCW0239	FAM	158-170	GGTAATAACAGGAAGTGTTCG	CATCAAATGGTGTGCATCTGG	55	5	CEW	G32008
MCW0240	FAM	172-197	CAAAACCGGTGTCACCTACTG	GGTTATTTCTTCAGTGACTTCC	55	8	CEW	G32009
MCW0241	FAM	272-278	AACCAGTTTGTTAACATCAGC	ATTGGAGTTGGTACCATACTC	55	3	W	G32010
MCW0242	FAM	154-158	GAAGCTGATATTACCCAAGAC	AGACATTCAAATGGTCACTGG	55	3	CEW	G31916
MCW0243	FAM	193-232	GGCTATCTGCTTGGCAATAGG	ATAGTTGAATAAATTACAGATGG	55	4	CEW	G32011
MCW0244	FAM	166-174	CAGCTCCATGGAGTCCTGATG	TCACATGGGAGCATCCCGATG	55	3	W	G32012
MCW0245	FAM	284-290	ATCTATGGCCACCTCAAACTG	GATCTGTGCTGAACACAGCAG	55	2	W	G32013
MCW0246	TET	230-238	TCATAAGGCAGAGAATTCATC	TTTCCATTCAGACAACAAGGC	55	4	CW	G32014
MCW0247	TET	200-212	CTTCACATGCTCCACTTGATG	AGTGACTATACTTCTTCACGG	55	3	CEW	G32015
MCW0248	TET	216-225	GTTGTTCAAAAGAAGATGCATG	TTGCATTAACTGGGCACTTTC	55	4	EW	G32016
MCW0249	TET	245-260	ATCCGAATCCTGTAACTGATG	TGCATGGAAATCCATCAGCAC	55	2	W	G32017
MCW0250	TET	228-238	CAGAATTTAGAGACTGTCTAC	ATACGGTAGCTCTGTTGCAAG	55	4	CEW	G32018
MCW0251	HEX	175-177	GCCACTTTGAGTCATACATCG	TTCCCACATCACTTTAAGCAC	50	2	EW	G32019
MCW0252	TET	254-296	CTGCTCAAGCCCATCAAATGG	CGATAACATCTGACACTGCC	55	3	CW	G32020
MCW0253	HEX	202-232	ATCAACAGGAACCAGTTTCTG	CTATAGCCATAAGGACTCAAG	55	2	C	G32021
MCW0254	TET	115-121	GAACCAATGAAAGCGAGATGC	GTGTTCAAAATGCTGAGAAGTG	55	3	CEW	G32022
MCW0255	TET	167-173	CATTTTTACAGTGTCTCGATTC	GAACAGCAAATTCCATTAACAG	55	3	EW	G32023
MCW0256	TET	169-181	GATGGGGCACTGTGGGTCC	TGGTTTCCATCAAGCAGTTCC	55	4	CEW	G32024
MCW0257	TET	288-301	AGTCCATCATCAGATGCTTGC	TCTTGAGTGATTCTGTAGAGG	55	4	CEW	G32025
MCW0258	FAM	141-162	TTCTTAGTCCTTGCCAGAGGC	CTGCAGGAGGATGTGTCCTAG	55	3	EW	G32026
MCW0259	FAM	299-313	GATCTTGAACTATTTCAAACTTG	TAATTTAAGTTCAGTTCAGTACC	55	2	CW	G32027
MCW0260	FAM	173-194	ATCTGCCTTCTGCCAGACAG	TTGTCATTGCTCCTCTGATGG	55	3	CW	G32028
MCW0261	FAM	245-257	GTAGTAGCAGCTACACCAGAG	GAGCAGTTCATATGAAGTGCAG	55	4	EW	G32029
MCW0262	HEX	065-076	GATCCAGGCTTTAAGAAGAGG	GATCTTGTACATGCCAGCAC	55	4	EW	G32030
MCW0263	HEX	240-254	TGTTCAAACATAAGGGCGTTC	ACTTCTCCTTGGAGAACACTG	55	4	CW	G32031
MCW0264	HEX	227-241	AGACTGAGTCACACTCGTAAG	CTTACTTTTCACGACAGAAGC	55	5	CEW	G32032
MCW0265	HEX	130-137	GATCCTTCTTGCATGCATT	ATCACTGACATCACTGTGTAG	55	2	W	G32033
MCW0266	HEX	165-181	GATCCCCATGCGCACAC	TTGCTACACTTCCACCTTTGG	55	4	W	G32034
MCW0267	FAM	263-284	CTTTGCTCCTTTCACTCCTGG	TGAGCGAGTTTTGCAATGGAG	55	5	CEW	G32035
MCW0268	HEX	180-182	TTCAGGTTCTGGTCTGTTTGG	AGTGAACGCTATTCCTTAGG	55	2	CE	G32036
MCW0269	HEX	196-207	GATCCAAAACACAACCTTTT	GATCCTTCATTTGAACTAAATG	55	5	EW	G31917
MCW0270	HEX	198-217	TCCACAGAGATTTCAGGCTAC	ATAGCCCCATCAGACTCTAGC	55	4	CEW	G32037

MCW0271	TET	196-212	GTTGCTTAAAAGCATTCTGT	ACACTAACAACTCGTTTATG	55	4	CEW	AF030577
MCW0272	TET	135-145	CTCAGGTTCCAGCTGATGC	ACTCAGAGCCACTTAGCTGC	55	3	CW	AF030580
MCW0273	TET	176-181	TGTTATGCCATTGCTTTTAG	CAGCACCCACATCAAGATCC	55	2	EW	AF030582
MCW0274	TET	161-165	TCATGTAATGTAACCTCTGC	GATGTACCAGTGCCTCAGCG	50	3	W	AF030587
MCW0275	TET	147-151	TTTTTTTCGAGTTTCTGCAG	AAACCGACTTCGATACC	55	2	CEW	AF030584
MCW0276	TET	218-239	ACTCTGAGTGGAATTACCTG	ATTTCTGTTAGAAGCAGCTGC	55	4	CEW	AF030585
MCW0277	TET	257-269	ACAAGTAGAACTTGGTGAGTG	GAACTGATAACTAGCAGGTAG	50	5	EW	G32038
MCW0278	TET	234-253	CTACCCTTCTTTCCTGCATTG	TAATGGGATCGTCATAAGTGC	50	2	EW	G32039
MCW0279	TET	94-96	GCTCTTTGGAAGCATAAATGC	GCTTCCAGGTGAGTCTTATTC	55	2	EW	G31918
MCW0281	TET	268-296	CGCCTCTATCGTTATGGTAAG	AGCCCATAACCAAGGCATCTG	55	6	EW	G32040
MCW0282	HEX	287-308	GATCCTAAGGTTCTACTACAG	AGTATTTCACTAGTGAACTACC	45	3	CEW	G32041
MCW0283	HEX	112-155	GATCCTAAATATTTTAATTAACAC	TTTCTGTGAATGCTGACTGAG	50	7	EW	G32042
MCW0284	TET	238-246	CAGAGCTGGATTGGTGTCAAG	GCCTTAGGAAAAACTCCTAAGG	50	2	W	G32043
MCW0285	TET	176-194	AGTTGGAGGTTATATTACGGG	TATGACATAATCCACGCTGAG	55	4	EW	G31919
MCW0286	FAM	198-204	GATCCCTGCTGTTGTTTCATC	AATGGCTATTGCAGCCCAGAC	55	2	EW	G31920
MCW0287	TET	237-258	GCCGTGTGACATCAGTGCTC	TTGCACCAGCGCTGCAAACTG	55	3	CW	G32044
MCW0288	TET	118-122	GATCTGCTTCTCTGCCCCATG	GGTACTGTCACCAGAATGAGC	55	3	CW	G32045
MCW0289	TET	217-234	TAGTGCAGAAAGACAAGGCAG	GATCCTTCCTCCTTCACTGG	55	5	CEW	G32046
MCW0290	TET	190-202	ATTCAACATTTGCTTTCGGTAG	GATCTTCTGATTTTTTGTTATCTG	55	2	W	G32047
MCW0291	TET	183-191	ATCTAAGAGGCAGTGATTACC	CTGTTCCTGCTAATGAGAAAC	55	4	CW	G32048
MCW0292	HEX	94-130	GGAGGCTCTGGGTGTTCC	CAGCAGATGGGGCTGAGAG	55	5	EW	G32049
MCW0293	TET	224-232	GATCATCCATGCAGATATCGT	GGAGAAGACTGAGGAGAAATC	55	3	CEW	G32050
MCW0294	HEX	306-317	ACTGAACAGAAACAGTCTTCC	CTTCTCTAGATGTCCACTACC	55	3	EW	G32051
MCW0295	HEX	94-107	ATCACTACAGAACACCCTCTC	TATGTATGCACGCAGATATCC	55	5	EW	G32052
MCW0296	HEX	245-249	GTAATCCTGCACAATCCCTGC	GCACTACAATGCTGTCACTGC	55	2	CW	G32053
MCW0297	HEX	288-303	TGCCAAACATGACCTCCAGTC	ACTTCACTGCAGGGTGGTGAG	55	4	EW	G32054
MCW0298	TET	133-153	AACACTGACACGAATAAGGCC	GATCCAGCCTGTCCAAATCC	55	3		G32055
MCW0299	HEX	096-102	TGTTTGGAATTGTAAACTGC	TGAAGGAAAGGTGAGTGAGAC	55	2	C	AF030583
MCW0300	FAM	123-130	CAGAGAAACGTGCATGTGGAC	TGTGCACATTTCTCTGCTGAC	55	3	CEW	G32056
MCW0301	FAM	266-284	GGAGAGGAGACAACTGTATTC	AGGGTGAGAGGTAACAAGTGC	55	4	CEW	G32057
MCW0302	FAM	134-138	CGCTGGCGTCAGGCAGTC	GATCCCTTGTCGGGCCGG	55	3	W	G32058
MCW0303	FAM	290-296	AGCCGATAACATCTGACACTG	CTGCTCAAGCCCATCAAATGG	55	3	W	G32059
MCW0304	FAM	284-296	TCAGTATGAGAGCTTCTCAAG	TTGTTACAAGGTCTTCTGGAG	55	4	CEW	G32060

MCW0305	TET	258-268	TCAGAAACAAAGCAGGAGCTG	TGACATCTTTCAAACGAGACC	55	9	CEW	G32061
MCW0306	HEX	133-167	AATGTACTTTCTGGATGCAGC	TGTTAAAGTATTTCTCTATCAACC	55	7	CEW	G32062
MCW0307	FAM	172-178	AATGCTGTATTTCAATAAGTGG	GAATACCACAATCCATGAAGC	55	3	EW	G32063
MCW0308	TET	201-207	AACTCTCAACCATGAGTCTAC	ACAGCCATTCTCACTTTCTGC	55	2	EW	G32064
MCW0309	HEX	258-280	TTTCCATCCTGGAAATAATGG	TGCCATTCTTCATCCTGAATG	55	2	CW	G32065
MCW0310	FAM	318-320	CCAACTAGGTGGAGGAACTTC	GATCCCAGGTAGGGCTGGAAG	55	2	EW	G32066
MCW0311	FAM	250-266	TTAGCATGCTTCTCCTGGCAG	CACTGTGTACTGAAGTGGCTC	55	3	W	G32067
MCW0312	TET	217-221	TTTGTTCGGGATTAAGCTTGG	CCTAAATCAGGATGTTTGGAC	55	2	EW	G32068
MCW0313	HEX	226-252	GATCTGCTGGCAAACTTT	TCTCTCAGCCTCATAAACTAG	55	6	CEW	G32069
MCW0314	TET	278-284	GCCAGGCTACACCTCTTCTAG	GTTGGTATGATGGTATGATGC	55	2	EW	G32070
MCW0315	FAM	250-266	GATCCAAGCCTGGAAGTATG	TGATGCTGGAGGCAAACATC	55	4	W	G32071
MCW0316	FAM	168-182	TCTTACTTTCTGTGCACAGTG	AAAGGTCTTAGGAGATGAACC	55	2	EW	G32072
MCW0317	HEX	235-257	ACTTGTTGGCTGCTTGAGATG	ATGCATGCATTCACAGAAAGC	55	5	CEW	G32073
MCW0318	FAM	204-224	TGAGCTGCCCTATTTTGCTG	TTCCCTTTTCTCACCAGTGC	55	2	E	G32074
MCW0319	TET	178-180	ATCTGTCATGAAGACCTACAG	ATGATGTCTCCTAACAGTCAC	50	2		G32075
MCW0320	FAM	169-177	CACAGGCAAAGGGCCTAAAG	GATCATCCTAGAGTTTAGCCC	50	4	CW	G32076
MCW0321	FAM	115-117	GCTCACATTCTACCGTCCC	CTCAAGAGACCAGCCACCAC	55	2	E	G32077
MCW0322	FAM	256-258	GATCTCCCTAGCTACAAACC	CTTCCGCCTTCTTGAGAGTC	55	2	W	G32078
MCW0323	HEX	110-114	TGAATTCTCTCGGCTTCCATC	GAAATGGTACAGTGCAGTTGG	55	3	W	G32079
MCW0324	TET	279-285	ACCAGAAATCTAACACCAACG	AGGCTTTGCTCTGGTGTCAG	55	3	CEW	G32080
MCW0325	FAM	154-160	CTCCTGGACTGGAAAACCAG	GAGCTGCTGTGCACATATGG	55	3	W	G32081
MCW0326	TET	171-173	GATCTTGAGAGAAGGCGCAC	ACTTCATAGAAGGCAGACATG	55	2	CE	G31921
MCW0327	FAM	193-203	GTCCTTGCCATGTATTGACTG	CAGCACTAAGTGGCTGACATC	55	4	EW	G32082
MCW0328	TET	255-267	ATGGAAACAGATGGAGCTGGC	CTCCAATCCCAGGCTCCAAC	55	7	CW	G32083
MCW0329	FAM	268-312	CATTGAAGGCAGTCTGCTGC	CAAAAGAGTGCAGATTGAAGG	55	4	CEW	G32084
MCW0330	FAM	260-290	TGGACCTCATCAGTCTGACAG	AATGTTCTCATAGAGTTCCTGC	55	4	EW	G32085
MCW0331	FAM	216-222	CAGAGCAGCTGGAGATGTAAAG	AGAGGGTAAGAAATCCTGCTG	55	3	EW	G32086
MCW0332	FAM	197-201	TGGGTTTGCAACGGGACATAG	GAACAATGGTGAGAGCACTGC	55	2	CW	G32087
MCW0333	TET	201-207	TAACAAGGTGGAGACCAGAAG	TCTCACCTTTTGTGTGAATGC	55	4	CEW	G54427
MCW0334	FAM	162-192	ACAGTACGCAACAAATTCCAG	ATCCCTCCATGAAGACCACAG	55	4	CEW	G54462
MCW0335	TET	133-135	GATCTCAGGTGCAGCTGCC	ATCTTACCTCTTCAGAGCTAG	55	2	E	G54429
MCW0336	FAM	242-266	CATGCAGACAGCAGCTCCC	GATGAAGTAAATGGGAATGCG	55	5	CEW	G54430
MCW0337	HEX	104-106	CCTGCCAGTATACTCTGTCAG	ACCAGCTGCAAATCTCTTAGG	50	2	W	G54431

MCW0338	FAM	215-227	GTTACTACAGCCAATTCCATC	TCGGTCTAAATGTATACCAAC	55	6	CEW	U51641
MCW0339	TET	116-129	GTGAAACCCAACCTGAACCAG	AATGCTGCAGTAGCACAGTAG	55	2		G54432
MCW0340	HEX	179-185	ATTATCTGATGCATCAGCTGG	CACCGATTGTAGCGGAACATC	55	3	CEW	G54433
MCW0341	TET	216-226	ACAGAGCACGGCCAAAGTCAG	TTTTCTCGCGAAGGAGCAGCG	55	4	EW	U31223
MCW0342	FAM	144-175	AAGAGAATCATGGAGTGGGAG	CTCATTACTAAACCCATAAACC	55	4	CEW	G54434
MCW0343	HEX	203-211	AGACCATGCCCAGAGACTCTG	CATTAAAGGTTTGTCATTACCG	55	4	W	
MCW0344	HEX	198-200	CTGCGCTGCGAATCACAGCT	GCTTTGCACGGGAGTTTTCTC	55	2	C	D28600
MCW0345	HEX	230-240	GTCCTACGATAATCCACTAGG	CCTTACTGGAGCTGTTAGGTG	55	5	W	G54463
MCW0346	FAM	137-156	TGCTCCTTTCAGAAAGAATGC	TGAAAGTGCTTGATCGGATCC	60	4	EW	S78786
MCW0347	TET	270-290	GCTTCCAGATGAGCTCCATGG	CACAGCGCTGCAGCAACTGC	60	3	W	X89248
MCW0348	FAM	199-201	CAGTTTGTGGACTCTTGGACAC	TGACTTCCTGCGCTCATGTCC	55	2	W	Z71594
MCW0349	TET	188-198	GATTAAATGAGGCAGAAGGC	AAGCCTCCTCTTTATAAAGCC	55	3	W	U87449
MCW0350	TET	168-174	CAGCATTCTGCTCATTTAGC	AGTGTTGCTCAATTCAGACAG	55	3	EW	U04611
MCW0351	FAM	150-167	GTAAAGGCTCTTTACAAACGG	GAGTAGGGCTTAGGAAGTAAG	55	5	CEW	D26339
MCW0352	FAM	150-178	GTAGGACAGTGCTGGAGCAG	CCAGGTCCTCCAGCGTGATG	55	5	EW	G54464
MCW0353	TET	222-228	AGGACACATACTGTAAGTCAG	ACTGTGTTAGCATCCCCTCG	55	2	E	D16184
MCW0354	HEX	120-122	GCTGCCGTGCGTAGTCCTG	CGGTCGTTCCGTCACTTTGG	55	3	CW	U71183
MCW0355	TET	261-271	TTGACCTGCACCGTCAATGG	CACATACTGGAGATCCTGAGC	55	3	CW	Y14347
MCW0356	TET	211-250	ATATAGGCATTTTCCACGGTC	CTCACTGATATGGCCTTTCC	55	2	CW	X57991
MCW0357	TET	210-218	GATAGTCCATGGTCACGTTTG	GCATGCCTCTAGTGTTAGTAC	55	3	EW	D50335
MCW0358	TET	138-146	CGGATAGAGTAGAGCTCAGAG	AGCGGTGAGAGCGGGAAGC	55	4	CEW	G54465
MCW0359	TET	234-249	TGATGATGGCTTAGGACAACG	CTTGAATACCGTCACCTGAAG	55	4	CEW	D28598
MCW0361	TET	121-131	ACCACAACGCTGAATTCGTGC	CTCGCAGAGCCGCGTTGAG	55	7	CEW	AF022151
MCW0362	HEX	153-157	CATACGAAAATTTCTTGGTCC	ACTTCAGACATTCCTAACACC	55	3	CEW	X57339
MCW0363	FAM	161-195	ACTTCCTCAAGGAAGCATCAG	CTCAGATGTTTGTACGGTGAC	55	7	EW	
MCW0364	FAM	170-200	GCGGTGAAGGGAGATGTCAG	ATCACCCCTATCACATCATCG	55	6	CEW	
MCW0365	HEX	132-166	GATCGCTTCCCCGGAGCG	GATCCCTCTGCACACGGG	55	6	CEW	
MCW0366	FAM	181-212	ATTCATGGGGCACACAGTTG	AAAGTGCCGTACGGTTGAAC	50	6	EW	S40818
MCW0367	TET	282-295	CAAAGTACGTCTACACAGTA	TCTACTAAAGTAGCTGCAAGG	50	6	CEW	
MCW0368	HEX	213-241	TACAAACAATCCTGTCAGAAG	CCTTAGTTACCAACACATCAG	50	4	CEW	
MCW0369	FAM	183-221	GAGGATCATGCTTTCCAGAAC	ATGTTGGTGACTTGGTGTAAG	50	9	CEW	
MCW0370	TET	182-193	GAAAGAGGAGAGTAGTTCACG	TCCCACCTCATCATGCATTCC	50	2	W	AL023516
MCW0371	HEX	202-211	TTTCATGGCATCCTAAGATGG	CTGCTCCGAGCTGTAATCCTG	50	2		AL023516

MCW0372	FAM	196-202	ACTTGAATGTGAAGGCACTC	GCTGCAGAAACTAGCCATTG	50	4	EW	
MCW0373	FAM	160-172	AAGCTTAGCTGGGGGAAAAA	ACTTTTCAGCCTTAGCTAGG	50	3	EW	
MCW0374	TET	180-192	AAGCTTCTAGATGTAAATAC	GTAAGAGCCATGTGCAGTAC	50	5	EW	
MCW0375	HEX	184-190	GCCATCAAAGTATGATTTGGC	TGGGATACAAGAATCTCAACATG	55	2	W	
MCW0376	FAM	275-287	GCTTGATGGCTCTTTGCACA	CTTTTATCATCTCTCTAAGTG	55	4	EW	
MCW0377	TET	261-268	GATCCCTCAAGATTTGTTAGC	ATAAGAGCCCACTTCATTTGG	55	3	CEW	
MCW0378	FAM	87-91	GATGATGTGTTTCAAATACACC	AGGTTTTGCAGGCTTTGTTTGG	55	4	CEW	
MCW0379	TET	127-133	AATCAATTGTGCATCAGTTAGG	TAGTTTGCAACCCACTGCAAC	55	5	C	
MCW0380	TET	277-301	ATAAGAGCCCACTTCATTTGG	GTAGTCATTTTGGCTGTTAGG	55	5	CW	
MCW0381	TET	123-125	AGGACAATTCTACAAAGATGAAC	ACCTTCTGCTGCAGAATTTGG	55	2	EW	
MCW0382	FAM	101-130	TAGAATACTGACGTTTCATTGG	AGGGCAACTTAAACTACTCAG	50	4	CW	AJ397960
MCW0383	FAM	149-160	GTAATTCTCACGTACTGAGAC	GAAAAACGCGCAGCAGAGTGG	50	2	CEW	AJ394144
MCW0384	HEX	168-177	CTTGGACAGGTGCAAGAGGAC	AAAGTGTATGTTGCCCTGCAC	50	2	W	AJ394484
MCW0385	HEX	163-165	TACTGCCAGTGTTTTTGAAGG	TGTATCTAAAAAATGTTAATGCTG	50	2	W	AJ393912
MCW0386	TET	180-194	TAGGCTTGGCTCATTTGTTGC	TGGAAATCTCTGAGCATCTGC	50	5	CEW	AJ397995

Table 2. Characteristics of chicken microsatellite markers derived from sequences from Genbank (LEI markers).

Marker	Dye[1]	Length[2] (bp)	Forward primer	Reverse primer	PCR[3]	All.[4]	Pop.[5]	Acc. No.[6]
LEI0028	FAM	224-252	CATCAGAGTCTGCCTGCAGTG	AAACTCTTGGAAGACAGTGAC	55	7	CEW	X83979
LEI0029	TET	220-240	TGAGAAGGATGTCCTACTATC	CCCTCAAGTATGATCATCTGG	55	3	W	X83982
LEI0043	TET	106-120	GGCCATTAGGACCCGGTTGG	CCTCCAAACCTCTGAGAAAGC	55	4	EW	X78623
LEI0044	HEX	176-207	CTGGACGTATGAATCCCTGAG	GAAGGTGGCTGCTGGAGGAAC	55	7	EW	X78624
LEI0062	FAM	81-113	TTCATTTCCACGTGGAGTGAC	GGAGCTGTCATCCACTCTTGG	55	7	CEW	X82792
LEI0063	HEX	206-290	ATCACTGTGTACCTGAGGCC	AGGTCTTCAAGTGCACACCTG	55	7	EW	X82797
LEI0064	HEX	295-304	TGGTTGTCTCAATACAACGTC	CTGTAAAGATTTCTCAGAAACAG	55	6	CEW	X82808
LEI0066	FAM	299-313	GATCAGATGCATCCAAAGTTC	GAAGCAGGAAAATAGAAAAGGC	55	6	CW	X82813
LEI0067	FAM	250-264	GATCAAACGTGCGTGGTGGTG	TGTTGCTTCTCGCAGTGAAGC	55	2	CW	X82862
LEI0068	FAM	173-199	GTGCAGAAAGACAAGGCAGTC	AGCAGGTAAAGAGGCTACAGG	55	5	CEW	X82867
LEI0069	HEX	249-271	ACTGGGGCTGAGCACTGCTC	GATCTGCAGCTTCTGGAGCG	55	5	EW	X82868
LEI0070	HEX	185-223	TGCGGAGAGCAATTAGTCTGC	GGAAAACAATCACTGCCTCG	55	6	CEW	X82869
LEI0071	HEX	281-330	TCAGGTTAGTCTGACCATTGC	TGAGTGTAAGATTGCTAATGGA	55	7	CEW	X82814
LEI0072	FAM	84-100	TAAGCTGACATTCACCACCAG	GACTCTTTCAGTACATACTGG	50	5	CW	X82815
LEI0073	HEX	163-221	CCATATCATTTGTCAAGCACC	AATTCCTGACCTCCATGATAC	55	8	CEW	X82871
LEI0074	FAM	303-315	AAACGTCTGCCTTCATGCGAG	CATCAATTAGAGCGAAGCCTC	55	3	CEW	X82861
LEI0075	FAM	226-259	CTATGCTATCATTGAAACACAGC	ATCCAGTGCGTGTCTGGTCAG	55	5	EW	X82794
LEI0076	HEX	254-280	GATCTGAATTTCTCGTTCATCC	CACAGCAGACCTTCACCTGAC	55	5	EW	X82801
LEI0077	HEX	161-190	CTTGAGGGGAGTGAGTCTGAG	AAACAAGGAGGGATTACCTCC	55	4	CEW	X82788
LEI0079	FAM	200-234	AGGCTCCTGAATGAATGCATC	TCATTATCCTTGTGTGAAACTG	55	5	CEW	X83242
LEI0080	HEX	191-211	GTTAGAGCCATACAGAAACTTC	ATCACACAAGCTTTCTTCCTG	55	8	CEW	X82863
LEI0081	TET	218-260	ACTTACCTTTTCTTAGCTACTG	GATCCTTTCAATGCTCATGCT	55	5	EW	X83249
LE00I82	HEX	253-280	TATCCATACAGTACCCTCCTG	CCTTAGCTGGCTCAGTGGATG	55	5	EW	X83251
LEI0083	FAM	193-238	CAAAACCCTCACACCCATTGC	TGTATTGCTTCAGCTCTACTG	55	4	CEW	X83248
LEI0084	HEX	219-231	TCTGAAGGTCACGCCTGACTG	CGTTGTCGCATGTTCTCGATG	55	4	CEW	X85514
LEI0085	HEX	259-273	GATCCAAAGGATGAAGTGCC	CCACTTCTCTGTCAGCTGTC	55	5	EW	X82800
LEI0086	HEX	233-285	GATCAGATATGAGGTGCACAG	GCCCTAGAAGCTGTACTTGTG	55	5	CW	X82795

LEI0087	HEX	265-304	AAGTACTAATGCAACACCCAG	AAAACTTCTACCTATGTAGCC	55	6	CW	X83245
LEI0088	HEX	253-277	TGATTCACTTGATGGTCGAGG	GATCTAATGTGGAATGCCTGA	55	7	CEW	X82816
LEI0089	HEX	180-198	GATCCAGGTGGCTCTAACACG	TTAGCTCCTGCTTGTCACTGC	55	5	CEW	X83239
LEI0090	TET	210-218	TAGTGCAGCCCTATGGAGCG	GGTGAGTGTGCGTTACACGC	55	3	EW	X83250
LEI0091	TET	238-264	TCTTGGATACCAGTGAGAACC	ATCACCTTCATTAGACACTGC	55	6	W	X83244
LEI0092	TET	164-212	GATCTACATTTGTGCAGTGTC	TCCTTGGTCTGACTCTCCATG	55	7	CEW	X82810
LEI0093	TET	245-256	TCCTTGAAGTATTCCAAAGCTC	GTGCCCAGTCTTATAGCTAGC	55	4	CEW	X83260
LEI0094	TET	253-285	GATCTCACCAGTATGAGCTGC	TCTCACACTGTAACACAGTGC	55	5	CW	X83246
LEI0096	FAM	228-246	GATCAGATTGGTTCCCTGTG	TGGGTGAAGTTTCCTCGTAG	55	7	W	X83257
LEI0097	TET	292-304	TCCTCAGAGCATGAAGAAGCC	AATAGTGACAGCTCTTTCTGC	55	7	CEW	X83259
LEI0098	FAM	156-170	CAGTTAGCAGAGATTTTCCTAC	TGCCACTGATGCTGTCACTG	55	6	CEW	X82860
LEI0099	FAM	115-131	GATCTGGCAGAACAGAAACAG	ATATTTCACACCTGACCTGCG	55	4	CW	X83237
LEI0100	FAM	242-274	ATGTAATCAGCAAGTGCCTGG	TTCAGAGGCACTGACTGAATG	55	2		X82859
LEI0101	FAM	268-292	CCAGCTTCAGTGGCTGTGAAG	AGTCCTATCTTAGCCTGCTCC	55	7	CEW	X82805
LEI0103	FAM	225-251	GGCAGTTGCAGTCTATGTCTG	TGCCAATTTCAGCTTAGCTATG	55	4	CEW	X82796
LEI0104	TET	223-234	GATCTAGTGGTGCATGTCCC	CTCCACGTGCAAAGCAGCCA	55	4	CEW	X83240
LEI0106	FAM	289-300	AAACCTTCAAATGGTTAAAATGC	GTCAGCATGACAGCAGCTGAG	55	3	EW	X82854
LEI0107	HEX	208-230	ATCATTGCTACACCATGGTTC	GCTGCTCAGAAGCATCTGTGC	55	4	CEW	X83253
LEI0108	FAM	223-231	AGTTCAAGGTTGCAAGATGTG	TACATGCCATACTAGTCCTTC	55	4	CEW	X85517
LEI0112	FAM	271-305	GGGAACATACAGGGTGCTGCT	TATCATACCAGCGCAGCTCTG	55	6	CW	X82789
LEI0115	HEX	273-292	CCAAAATGACTACTTCCACCG	TAATACTGCCCTAGATTATGTC	55	4	CW	X85529
LEI0117	FAM	192-217	CGCTGCTCAGCGTGAATAGG	ACAGATGCCCACGACGAAAG	55	4	CEW	X85539
LEI0118	FAM	67-91	AAGACCAATGGGAGCAAATCC	GGCTAAACAGCCATTTGTACC	55	3	C	X85540
LEI0120	FAM	278-316	CGTAACACATGCAACTCAATG	TTAGAATGAAAAGGCTGTTCC	55	6	CEW	X85511
LEI0121	FAM	263-280	TTGACGTCCTGGATAGATTAC	ATTATCCAGAACTAACATCAAC	55	3	EW	X84436
LEI0122	HEX	289-300	AATCCCTATAGAACTTTGTGC	GATCTTACTGGATTACCATTC	55	6	EW	X85533
LEI0123	FAM	253-283	TCTTCCACCAAGTTGGGCATG	TTTTCCTAGAGGCTTGCTGAC	55	4	W	X82790
LEI0124	FAM	74-79	ACACATGCATGTACCCTCGCC	CCTGATGACTCAGTTAGAGCC	55	2	EW	X82793
LEI0125	HEX	265-275	GTTAGGCATCCTGTTTCTTCC	CTGTTGTCTTCCTGCAGTGCT	55	2	EW	X82798
LEI0126	HEX	189-231	GTCAGAGAAGGAAGATACATC	CTAACTACAATGCTGGAATGC	60	8	CEW	X82799
LEI0127	HEX	217-249	CATAGAAATCAGGAAATTGATGC	GTCAACTTTAGTAAGAACTGC	55	6	EW	X82804
LEI0128	HEX	165-272	TCTCCAGTAATACACATGGTG	CAAACTCAGAGCTAAGTCTAG	55	5	CW	X82806
LEI0130	HEX	248-273	CTTAGGTCCTTACTTGTCTCC	TTCTCAGCAGACATGATTGGC	55	6	CE	X82809

LEI0131	FAM	183-191	GATCCAGTACAGACGGGCAG	TGTGCATGAAATGAGTGACTG	55	2		X82855
LEI0132	FAM	241-243	CCTCTCAAATGAAGCGCTCAG	ACACTTTCAGGACGTGGTCAC	60	2	W	X82856
LEI0133	HEX	269	GTGAGTAATTATGCACACGCG	CAAAGAGGAGAGCTCAGCAG	55	1		X82857
LEI0134	HEX	294-298	ACATTCAAGCCCTGACTCAGC	AATTTCCTGCACGTCCAGCTG	55	3	CW	X82858
LEI0135	FAM	134-145	CACAATGAAGGATGAATAGTGC	AATTCACAGTTACACCTGAGG	55	2	W	X82864
LEI0136	FAM	160-280	CATTTGTAACAAGTGCACGTG	TCAGCTCTCCTAGACCTAGTG	55	10	CEW	X82865
LEI0137	FAM	193-197	AATTCAGCAGCTGTATAGAGG	ATCCAGCCTGTAAACCGTTAG	55	2	EW	X82866
LEI0138	HEX	191-196	CTCCTAGTTAAGTGCTAGTGG	TTGTTTGGTTGGTTTGCTCCG	55	3	E	X82870
LEI0139	HEX	265-273	ACATTTGAGATGAAGCTTGCC	GGTATCTAGTGCATATGATGC	55	3	CW	X82872
LEI0141	TET	226-250	CGCATTTGATGCATAACACATG	AAGGCAAACTCAGCTGGAACG	55	5	W	X83235
LEI0143	FAM	263-275	GATCAATGAGTGCCGGGAGAG	CGGAGGTGATACGGATGGAG	55	3	W	X83238
LEI0144	TET	261-275	GATCAGTTATCAATGGCTGGC	CACTGAGGTCTTCTAGATGAG	55	4	CEW	X83243
LEI0145	HEX	296-326	CTGTTCATCTTCCTCTCAGTC	GATCTTGAATATAGACCTTGG	55	6	EW	X83252
LEI0146	TET	258-276	TCAAGCCACCAAAGTGCTTGG	GATCACTCTGCTCATAGCAGT	55	5	CEW	X83254
LEI0147	HEX	263-273	TCAGGCCTCTTGAACTCAGG	GCTATTAAGATACCTCAGCTC	55	4	CEW	X83256
LEI0148	TET	299-307	GATCAATCTGCATGCAGACTG	AGACCTATGTGGCTGGGAAAG	55	3	W	X83258
LEI0149	FAM	160-240	ACTGTCTATATAGCCTCTTAG	TTCTATTGGAAGTCTACATGG	55	5	CEW	X83261
LEI0155	FAM	101-113	GTACGTGTAGCTCGGCTCACC	GTCCGTGCATGGCTCCGCTC	55	5	EW	X85516
LEI0158	FAM	90-104	ATTGTTATCTCCAGAGAGGAC	GTCCTTGATGAATTGGTTAGC	55	3	W	X85520
LEI0160	FAM	248-254	CAAAGTAATCAGCTTGTGCTAC	CATTTCCACCGCATTGAGCAG	55	4	CEW	X85523
LEI0161	HEX	90-100	CAGCCTTTTCAAGCTTGCTGC	GTTCACTTTAGACATGAATCGG	55	4	CEW	X85524
LEI0162	FAM	193-223	TAATGCTTTCATTTCCTCAGC	CAAAATACCTTCTTCTGCATC	55	3	EW	X85525
LEI0163	FAM	189-207	ACTTGGGCATACTCTTGTTGC	CTGCAGGTACCGTGAGATGTG	55	3	EW	X85527
LEI0164	HEX	205-211	TTCGCTTTCACTTGCTGCCAG	TATCGGAATGCAAGATGACAC	55	2	W	X85528
LEI0166	HEX	254-267	AAGCAAGTGCTGGCTGTGCTC	TCCTGCCCTTAGCTACGCAC	55	5	EW	X85531
LEI0168	TET	209-212	ATCTAGATGACCCTTGAGGTC	GATCTGTGTTGTAGATATCTAAAG	55	2	W	X85534
LEI0169	FAM	236-253	TTGCTTGTTTGCTGCCTTTTAG	ACAGTGTAGCATGGACAACAG	55	3	CEW	X85535
LEI0171	HEX	350-365	GAGTGTAGACAGTAGTGTATC	CTCAGGGCACCATTTTCACTG	55	3	E	X85538
LEI0173	HEX	278	TGGAGTATCTATGCTATGGTC	TGTTAAGGATATGAGCCACAG	55	1		X85542
LEI0174	TET	230-263	ATCATACATGTTCTAGGGCTG	AAAGGGCATTCCCGCATGAG	55	4	EW	X85543
LEI0320	FAM	270-280	CTGCATGGATTGCTGCTCTC	GCTATCATGCCATACTGAATC	55	4		X83984

Table 3. Characteristics of HUJ markers.

Marker	Dye[1]	Length[2] (bp)	Forward primer[7]	Reverse primer[7]	PCR[3]	All.[4]	Pop.[5]	Acc. No.[6]
HUJ0001	TET	151-180	CCATCCGCTTATACAGAGCACA	ccctttgttaacacctactgca	55	8	CEW	L05542
HUJ0002	FAM	124-142	CATCTCACAGAGCAGCAGTG	gaatcctggatgtcaaagcc	55	9	CEW	L10228
HUJ0003	FAM	147-182	GACAGCAAGGATTAACCTGAG	GTCTTGGAGACTGTTAGTTGG	55	8	EW	L05590
HUJ0005	TET	114-119	tcccttccaaccttacagt	aaaagcaacacgaataacagat	55	3	CW	L10231
HUJ0006	FAM	107-117	ggaacatgtagacaaaagca	agcagtccatttcacagcca	55	3	EW	L10294
HUJ0007	TET	152-156	cataaactaaagtctcaacac	ttcttccacacatcttgcta	55	5	EW	H93646
HUJ0010	HEX	249-255	cttgctgacggtgcactcag	GCAGTCACAAGCATAGCAGG	55	4	CW	J02714
HUJ0011	TET	123-130	gttggcgctccggaccagga	ctctctcctgtcacgcgttgg	55	4	EW	J00836
HUJ0012	TET	120-140	gtctcatgctatgagagtgg	cctctggttgaatcagtctg	55	7	CEW	M13756

Table 4. Characteristics of monomorphic chicken microsatellite markers.

Marker	Dye[1]	Length [2](bp)	Forward primer	Reverse primer	PCR[3]	Acc. No.[6]
WS0001	FAM	304	TAGTAAATGCGTCAAGAGGA	CAGCCTCATATCTGCCTCCA	55	D13225
WS0002	HEX	110	TTCTCGAGGCTCAGAGATAG	TGCCACCCAGCTCCAGAATT	55	G32088
WS0003	HEX	112	GTGCAATGGGCGTGTTAG	TCCCAACACACCCTACGT	55	G32089
WS0004	HEX	175	TCCACATCCTTCCAGAAACA	TCGTGTATCAGGGACGAAATG	55	G32090
WS0005	TET	123	CTTAAGCTTGGAAGTTATTAGG	GCATTTTGGTGCCTGCCACG	55	G32091
WS0006	TET	125	TTTGCACGATCTTGTGCAAG	GATTCCTTGGTAACAAGCTG	55	G31922
WS0007	HEX	125	AGTGTTCTCCAATTTGTTCT	CTTACTCCCAGGATCTACTC	55	G32092
WS0008	FAM	113	GATTGGCTTCGTTCAGACAAG	CATGCATTTACTAGATTCTAGC	55	Z49898
WS0009	HEX	94	GAGTTCACTTGGTCAATGGGT	TCATTAAGCCTTAGCCCTTATC	50	G32093
WS0010	TET	238	GATCAATGCTGATTGCACTTC	TGTTGCACTGCATACTGTGAC	55	G32094
WS0011	HEX	274	GATCTAAACTCAGCCAGAGTG	CTCCAGGTGATGTCTCACCAG	55	G32095

WS0012	FAM	274	TGGCTGACGTGGGCTGTTGG	GAGCAGTACTCCAAACGCTTC	55	G32096
WS0013	HEX	286	GATCTCCTTTATGTCAGATT	ATTAGATTCCTGGTAGTTCTG	55	G32097
WS0014	TET	165	GATCTTAATGGAAAAGGAAAAAC	GATCAGGCGTCAAACTTAGCT	55	G32098
WS0015	HEX	289	TGCTGCCCCTCTACTGAAAC	CCAATTTTCCATCCCTCGATG	55	G31923
WS0016	HEX	231	TCCCCTAGACTAACCTAATGG	CCACAGGACTGAACAAGACTC	55	G32099
WS0017	HEX	289	AGACTTTGACTTGCGAAACCG	ATTCTGTTTTGATTCCTGATGC	55	G32100
WS0018	HEX	240	CATCAAATCCTCCATTACTGG	GATGGTCATCATAGACACCAG	55	G31924
WS0019	HEX	116	GATCTTAGCTTTCAAAAGGC	CAAGAGTAATTTCCTAGCTCAC	55	G32101
WS0020	HEX	200	GATCATCCTTCTTGCCACCAC	CGTACTGCTTAGCATCATACC	55	G32102
WS0021	FAM	113	TTCAGTCTCAAGAGTGATTGC	TAAATCACATTTTCAACAGCAAC	55	G31925
WS0022	FAM	236	CCTAATTAGACTTTGACCTCC	TAGCAGAAAGCAAGGCAGCC	55	G32103
WS0023	HEX	115	CTCATACAGACCTCCTGGAAC	GTAATATCCACTAACCGTTGG	55	G31926
WS0024	FAM	184	ACAGATATGTTTCATCCAGAG	CCAATCAGTGCTATCACTTTG	55	G32104
WS0025	HEX	170	CCAGAGAACATTTTGTCTAGG	ATCAGCCTAACCACACCACTC	55	G32105
WS0026	TET	261	TTCTCCTGTGCAGTATTTCCG	CGCTTCGTCCTTTCATTGGTAC	55	G32106
WS0027	FAM	266	ATGGGCTTGGTGGCAAGAATG	AAACTGTGCTCCCAAAGTCTG	55	G31927
WS0028	FAM	252	CTAATGGAGGCTCTACATCTG	TTACCCAAGAGCTATGAGCTG	55	G32107
WS0029	HEX	152	AAAATGATGTGCCAGCATCAG	AGGTAGCGTAGAGATGTAGC	50	G32108
WS0030	FAM	218	AGTGCCACAAATTCAACTAGC	AATCTTTAGCTGGAGAACTCC	55	
WS0031	HEX	271	AAGGCTATGAATGTGAACATG	GATCTTGACTCACCTAAAAC	50	G31928
WS0032	FAM	149	GATCAGCTCACATATGAGTG	GCTAATAAATCAGGAAGCGGG	55	G31929
WS0033	HEX	281	TTAACGTTCTGGGTGTGTACC	TCAATAACAGCTCCTGCCTCC	55	G31930
WS0034	TET	240	CACGGGCTTCTTTTCTGAAG	AATAATGCTGACAGTAGCCTC	55	G32109
WS0035	TET	281	GATCCAGAGGTGAAATTTACAC	CCATGAACCAGGTAAGTACAG	55	G32110
WS0036	TET	202	AGTGGCAGCTAATGTGGTC	TCTTCTTTGCTATCTCCAGAG	55	U83593
WS0037	HEX	123	TGATCAAAAACGAGCTCATGG	TTCACGAAACAGGAAAACGAG	55	X17612
WS0039	TET	170	TACGCGCTCTAAGCTTGAATC	GCTTCATGTTGAATCTGCAAC	55	M36662
WS0040	HEX	300	GACTGTTTTTATTCTGTG AGTG	CCTCTTGGTTGCAGATGGATC	55	X54944
WS0041	FAM	097	GGGCCTGAAAGAAAAAGGAAG	GGTCATCGGTGGGTTTAATGG	55	M59937
WS0042	TET	235	TCGCCGCTGCCAAATTTTGG	CCCTCCTTCCATTTCCTGATC	55	X52669
WS0043	TET	261	AGGCGTAGGGCAGTACTAAT	GTCCCAGGAACCATCTGATTC	55	L34006
WS0044	TET	184	ATGACTCAAACATTCTGTGC	TTCATCACACTGCATGGCTCC	55	X80792

Table 5. Characteristics of microsatellite ADL markers.

Marker	dye[1]	length[2] (bp)	Forward primer	Reverse primer	PCR[3]	All.[4]	Pop[5]	Acc. No[6]
ADL0018			TTGCAGGTCCCTCCCATACA	GCACACCTTCCTCATCCCCC				L23886
ADL0019			TGCTGCCTAGACCAGTTCAA	TCTGCTGGGATTATGTGTCA				L23887
ADL0020	TET	88	GCACTCAAAAGAAAACAAAT	TAGATAAAAATCCTTCCCTT	55	1		L23888
ADL0021	HEX	165-180	GCTGGTCGCTTTGCTCTGAA	GCTTAGCCTCATCTCTTGTA	55	2	CE	L23894
ADL0022	HEX	144-165	GCATCAGAGGAAGAAGGAAA	GGTCAAGGAAATCATAGAAA	55	4	EW	L23899
ADL0023	FAM	167-171	CTTCTATCCTGGGCTTCTGA	CCTGGCTGTGTATGTGTTGC	55	2	CEW	L23905
ADL0024	FAM	145-151	TGAAGCAAAAACCCAGCAAG	GTTCCATTACAGAGTGAGGT	55	3	CEW	L23906
ADL0032	HEX	112-116	ACTCATNGGTGCTGTGGGCT	CTCGCTGTTCTTGCTGCTCC	50	2	E	L23907
ADL0034	HEX	117-143	AACCTAAAAACTCCTGCTGC	GGGAACCTGTGGGCTGAAAG	55	6	CEW	L23908
ADL0036	HEX	294-304	GTTTGGCTTACATTTATTAT	TTTTTAGGAGTTATTTGACA	55	5	E	L23911
ADL0037	TET	180-182	ATGCCCCAAATCTCAACTCT	TCTCTAAAATCCAGCCCTAA	50	2	C	L23912
ADL0038	TET	139-150	TCGCCTGTGAACTCTTACCC	AGACATTCTCTAATCATTCC	55	6	CEW	L23916
ADL0039	TET	152-156	GCTACAACGCTTCAAACCTG	ACAAACAAACCAAAAAACCT	50	2	EW	L23917
ADL0040	TET	207-215	TTTCCCCAGATTTACAACTT	GCCAGTGATACTCCAGCAGC	60	3	W	L23919
ADL0102	FAM	091-107	TTCCACCTTTCTTTTTTATT	GCTCCACTCCCTTCTAACCC	48	3	CEW	G01547
ADL0105	HEX	235-237	CATTCCGTGCTCAACTTCAA	CCCAAACGCTGCTCTTGCTG	50	2	E	G01553
ADL0106	TET	160-162	CATTCTCTGATTCTGCCTTT	AACTCCTGGTGTGCTACAAA	45	2		G01550
ADL0111	HEX	125-135	CCTTCCTGACCTTCCACTTC	CCACAAAAATACCCACCATC	55	4	CEW	G01724
ADL0114	FAM	165-185	GGCTCATAACTACCTTTTTT	GCTCTACATTCCTTCAGTCA	50	5	EW	G01726
ADL0117	FAM	174-190	TCTTGTTTTCCTTTTGTTGT	GCATACGGCTCCTTCAGTTG	55	3	E?	G01728
ADL0118	FAM	161-165	GATCACTCTTAGATGCCACA	AGAGAGGGGTTACAAGGCTG	50	3	EW	G01729
ADL0120	FAM	155	GCATTCCAACTCCCCTTTGG	ACCAGATATAACAGTCCTCT	55	1		G01731
ADL0121	FAM	121-140	CTGGAACAAGAGGGCTTTGC	GGATGTGAAAAATCTCCTGG	55	5	CEW	G01732
ADL0122	FAM	230-236	TGAAATACCAAGGCATCTGT	TGGCTAAGAAAGTGGAACTG	55	2	E	G01733
ADL0123	HEX	105-128	GCTGTGTCAAGATTAGAATCAC	AACAATGAAAAACACTACCTGA	48	4	W	G01557

ADL0124	FAM	254-278	ATGCGTTACAAGGTNGGAGG	ATATGATGACTGGAGGTTTT	50	3	CEW	G01734
ADL0125	FAM	130-144	TAAACGGGGAATGTTAGGCA	TGGAAAATAAATAGAAGGCA	55	4	?	G01558
ADL0127	HEX	147-151	GAACCAGCAATTATATTAAATA	TTAACACAAAAGAACCAGGCAG	50	3	CW	G01736
ADL0132	HEX	184-190	GCTGTTTCTAAGCCATCTTC	CCACTTGGAAGTAATCTCCT	50	2	E	G01740
ADL0134	FAM	112-124	TTCCATAAGCCATCAATCAG	TTTTCCTCTCCCTCCATTTT	55	3	CE	G01754
ADL0136	HEX	148-154	TGTCAAGCCCATCGTATCAC	CCACCTCCTTCTCCTGTTCA	50	2	CW	G01561
ADL0138	FAM	114-126	GCTTCAGGAACTATTTACAT	CTTTAGAGATTATGCCAGTA	50	6	EW	G01563
ADL0142	HEX	233-237	CAGCCAATAGGGATAAAAGC	CTGTAGATGCCAAGGAGTGC	55	3	EW	G01567
ADL0143	HEX	156-172	CCTGTCTCTGGTCTTTATCC	AGTTTACTTCCTTTTCTTGC	55	4	CEW	G01568
ADL0144	TET	163-210	TCAGAAAAAGGAAAACAAAA	TTATCACCAAGTCAGCCATC	55	5	CEW	G01569
ADL0145	HEX	118-148	CGTGGTGTTGTGTATCATTT	CTCTTTTGCAGTCCTCCTAC	50	5	CEW	G01570
ADL0146	FAM	150-166	GACCTGCATTGTCAGTGACC	TGCTTCCTACCCATTCTCCT	55	5	CEW	G01571
ADL0147	TET	214-220	CTGGTGAATGAGAAGCGATG	GCTGCGGCAATAAACTCCCT	55	3	EW	G01572
ADL0148	FAM	136-162	AAAGCTCAGAAAAGCACACC	CCCATGNATGTCAAAAGACC	55	3	CEW	G01573
ADL0149	TET	218-222	ATAGCATACACCCAGCCACC	GAATAAGAATGTTNCCCTGC	50	5	CEW	G01574
ADL0150	TET	148-162	ATGCCAAGCATTACAGAAGC	CCTGCAGCACCTTTATCTCT	55	5	CEW	G01575
ADL0152	TET	175-185	AGATTAGTGCAGATCATCCA	TGTTTTGCCATTTCAGAAGC	55	3	EW	G01577
ADL0154	TET	130-166	GCTGCCACCTTCAAAACCTG	CTCACCATCTCATTCTTCAT	55	6	CEW	G01579
ADL0155	TET	107-109	GGTCCGACTGAAAGCATTAT	TTAAGACTGAAGCCAACCAG	55	2	C	G01742
ADL0157	TET	138-161	CTCTGTCAGGAAGGGGTGTA	GTGCCTGTCCTCTGTTTCAT	50	5	CEW	G01581
ADL0158	HEX	189-216	TGGCATGGTTGAGGAATACA	TAGGTGCTGCACTGGAAATC	55	4	CEW	G01582
ADL0159	TET	109-121	GCCATTATTTTTCCCTGTGT	CTCCCCANAGTCATTAGCAG	55	3	CEW	G01583
ADL0160	TET	125-129	TGGCAGAAATAAGGCAGTGC	ATTCATCGCTGGCATCTTGC	55	3	CEW	G01584
ADL0161	TET	130-140	TGGAACTTTTCCCTATGTTA	AAGGAGTCAATTGTAGCACA	50	3	CEW	G01585
ADL0163	HEX	168-178	TGTGTAGCCTACAGGATTGC	AGCCAAAATGGAGGTTCTGG	50	4	W	G01587
ADL0164	TET	197-210	GGTAGCATGAACAAAGCATC	TCCTCAGGCCTTTCAACATA	55	3	CW	G01743
ADL0166	TET	136-156	TGCCAGCCCGTAATCATAGG	AAGCACCACGACCCAATCTA	55	4	CEW	G01588
ADL0167	TET	169-177	AACTTCCTCTTGGTTGATAA	CTGTTTAGCCTCCCTCATAA	55	3	CEW	G01589
ADL0168	TET	107-117	TAAGAAATAAAAATCAAAGC	ACGAAGTGGGTGTCAGTGTT	50	2	E	G01589
ADL0169	HEX	097-119	CCACACCAAACTGCTTCATA	ATTCCGCCTCCCCATTAGTG	50	5	CEW	G01591
ADL0170	HEX	127-131	CAATGAGGATAACAAAACAA	CAGTTCCTTAAAGTTCTTGG	50	2	CW	G01592
ADL0171	TET	092-107	ACAGGATTCTTGAGATTTTT	GGTCTTAGCAGTGTTTGTTT	55	2	E	G01593
ADL0172	HEX	134-160	CCCTACAACAAAGAGCAGTG	CTATGGAATAAAATGGAAAT	50	4	EW	G01594

ADL0174	TET	174-178	CCTCTTTTTCCTGGTGCCCT	GCCTAAGTGGACAACGCAGC	50	3	?	G01596
ADL0176	FAM	186-196	TTGTGGATTCTGGTGGTAGC	TTCTCCCGTAACACTCGTCA	55	4	CEW	G01598
ADL0177	TET	152-158	TGGGTGCGAGTGTGCGTGAG	TGGGAAAGCGGAGAAAAATC	55	2	CEW	G01599
ADL0178	FAM	127	AACAAAGACACACTCGGTAT	GGAATCATCTTGCCTGGATA	55	1		G01600
ADL0179	HEX	132-136	CCCAGAGGAGGGCAGGATGT	AGACGTGGGCACAAGCACAC	55	3	EW	G01601
ADL0180	TET	132-146	ACCAGAGCATCTACTGAAGA	AAACCTGGAAATGAAAGCAT	55	7	CEW	G01602
ADL0181	HEX	180-184	CCAGTGAAATTCATCCTTTT	CAATCTTTTGTGGGGTATGG	50	3	EW	G01603
ADL0183	FAM	103-111	TTGTGAAGTGGATAAGATGA	ACAGAAATGGAAAGCGAGAC	55	4	CEW	G01605
ADL0184	FAM	131-139	GCCTCCTCACCCACAAAACC	TCAGTAACACCACGAATGCC	50	5	EW+bg	G01606
ADL0185	TET	128-150	CATGGCAGCTGACTCCAGAT	AGCGTTACCTGTTCGTTTGC	55	7	CEW	G01607
ADL0186	HEX	147	GAGTGCTGCCTTCAAGTATC	ACCCAAGAATTGCTCTGATG	55	1		G01745
ADL0187	TET	088-105	AATTGTTTGTTTACGCTTCT	GCTGGTGGCACAGATGAGAG	50	5	CEW	G01608
ADL0188	TET	152-156	CACTTCCAGTATTAACGTGA	GTGGACACAATGAGTTCCTC	55	3	W	G01609
ADL0190	HEX	208-222	GGCTCTGCCACGTCCCACTC	TGGTACCGTGTGTTTCAACT	45	3	EW	G01611
ADL0191	HEX	138-146	TCAGCTCTTCAGGCAAAAAG	AACTTGGACCACAATCTTAT	50	4	EW	G01612
ADL0192	HEX	137-150	AAAGGAAAGCCTATGTGAAT	AAAGCACCAAGCGAGATACA	50	3	EW	G01613
ADL0193	TET	122-150	TGCTAGGAGAGGAGTTTTGC	TGTGCACATGATTCAGAAGG	50	5	CEW	G01614
ADL0194	TET	200-222	ATTTTGTTGTGTGGGATTAT	GCCTTGATTGCTGTTATTAC	55	4	W	G01615
ADL0195	FAM	118-130	TTGAGACCAGAACAGGAAAT	CAAACGCAGGCAGGAAGCAG	55	3	W	G01616
ADL0196	HEX	111	TGTGTAGCAAAAGAGCAAGC	TGATGAAAGATGATGACAGC	50	/		G01617
ADL0197	TET	102	TTTTCTGGTTTTCTTTGGAA	TCTAACAGGGGAAGGTATGC	50	/		G01618
ADL0198	HEX	117-124	TGCTTTTGGTCCTTCCTTCG	GCTAATATTGCTGCTTCTGC	55	4	CEW	G01619
ADL0199	FAM	158-174	GACGAAAGCAAGAGCAAAGC	ACAAAGCCAGAGGAAACATC	55	3	EW	G01620
ADL0200	FAM	127-137	TGATGGACTGGAATAGTGTA	ACAGGAAATTGTGCTGGATT	55	4	EW	G01621
ADL0201	HEX	138-144	GCTGAGGATTCAGATAAGAC	AATGGCTGACGTTTCACAGC	55	2	CEW	G01622
ADL0202	HEX	243-261	CTGCTTGTTCTTCCCCTTCA	CTCTGCTCTCTGTGCCTCAA	55	5	CEW	G01746
ADL0203	TET	176-196	ACCCCTCCCCATCTCACTGC	GCTCCACCACTGCTCGTGTG	55	7	CEW	G01623
ADL0204	TET	211	TTCGGTACCGTGTGTTTCAA	TCTCCAGCCGTTTCCCTCAT	50	1		G01628
ADL0205	TET	124	AAGTGCAAAGAACATTGACT	ATCTTCAGCGTCCTCTGTGT	55	1		G01625
ADL0206	TET	104-136	TTTCTATCCTTCATCTCCAG	AGACATCCTGCTTTCTCGTG	55	6	EW	G01626
ADL0207	TET	183-197	ATTTTGGGCTGCCTCTGAGA	TATGCAATGGCGAAGCAAGC	55	3	W	G01627
ADL0209	TET	136-170	GGTTAGCTCCCTCCTTCCAG	TCACTCCAGCTTGAGACAGG	55	4	CEW	G01629
ADL0210	FAM	124-147	ACAGGAGGATAGTCACACAT	GCCAAAAAGATGAATGAGTA	55	5	EW	G01630

ADL0211	TET	105-123	TTTGGTGGTAGAAAAGACAC	TGTAGCTTAGCCTCATCTCT	55	5	CEW	G01631
ADL0212	FAM	101-114	TTCCTCGCTAAACTATGCTG	TTTCAAAAGTGCCCTCACAC	55	4	EW	G01632
ADL0213	FAM	104-118	AATTGGAGTTGAGTTTTGAT	CCCCCATCTTCATTCACCTC	55	5	CEW	G01633
ADL0214	HEX	110-116	CTGTTAACCCACCCGCATCG	AGCAGNCGGCCAGCATTTGG	50	3	EW	G01634
ADL0215	FAM	170	AGATCCATTTTCTTCATAGA	GGAATCATTTTCTTTCATAC	55	1		G01635
ADL0216	FAM	240	TTTATTCTTTCCCACTTTTGG	TTGGACAGTCTAGCAGCTGGTC	50	1		G01636
ADL0217	HEX	148-162	TCTACTTCGTTGGAGTGTCA	GGAAAACAGAGGAGAAATGG	55	5	EW	G01637
ADL0218	FAM	168-171	TGGAAGAGAAAGGCTAAAAC	GGATTCGGTGACTATGTTGC	50	2	W	G01638
ADL0219	HEX	102-112	AATATGTTACACTGCCATTT	GGACCAAGAATCTGTTCCAG	50	4	CEW	G01639
ADL0221	FAM	105-109	GTGTGCCCGTAATCCTGTAT	GTTCCAATGCCCCCTAATGC	55	3		G01641
ADL0223	HEX	190-214	CCTCGAGTCTGAGAACACTT	AGATTGGCTGTAGGCTCTGT	50	3	CW	G01643
ADL0226	FAM	186-198	ACAGGCTGGGGAGAAAATCC	AGCTGCGGAACACAATGACG	55	3	W	G01646
ADL0227	TET	172-174	AATAGCGCTTGCTCATTTTT	GCAGGGATAAACTGGCTGAT	50	2	E+bg	G01647
ADL0228	TET	086-113	CCGTTTTTCTTTTCCTGATG	AGCCCTTGGGCACTTAGCAG	55	4	EW	G01648
ADL0229	FAM	72	AAAGATGCAAAGTTTTCAAA	AAATGCCTTACAGAGTGTGG	55	1		G01649
ADL0230	FAM	105-120	GCCAAATAGTAATCCACTGC	TCGCTCTTGCCATTGTAAGT	55	7	CEW	G01650
ADL0231	FAM	102-140	ACTATTAGCCTGGGGAGAGC	AAGGAAACAAAGAGAAATCC	55	6	EW	G01651
ADL0232	HEX	134-141	AATCGACGCGCTGAAAATGA	GCAGGGGACTCCATAGTGTC	55	3	CE	G01652
ADL0233	FAM	103-118	GCCCTTTAAACCCAAGACTC	GGGGGAAAAGGATGCTTAGC	55	6	CEW	G01653
ADL0234	HEX	161-165	CCCTGGGGCTCCCTCAGCAC	CTGGACGCGTGAAAAAGTTC	50	2	EW	G01654
ADL0235	FAM	136-156	TCATTCTTCTAAACACCACT	AAACCAGAAAGCAAAATACA	50	3	EW	G01655
ADL0236	FAM	126-134	CTGGTTGTCAGTTGAAGGAC	ATAAGGTGGTGAGCAGCACT	55	4	EW	G01656
ADL0237	TET	136-154	GCTTGTGCCTAAGAATGAAC	TGTATGGAGTCTCAGCAAAT	55	4	CEW	G01657
ADL0238	FAM	161-163	AAACCCAAACAAAAGCAGAC	GCTCCTCATAAGCAAAATGC	55	2	W	G01658
ADL0239	TET	175	GAAAAAGCAGAGCAGTGTCT	GTGATGGGAAAATCTTCAGG	55	1		G01659
ADL0240	TET	120-130	ACCTGGGAGATTGGATTCAA	CGTCCCGTCCTGANTGTTTG	50	3	EW	G01660
ADL0241	FAM	205-221	AAAATAGCATGGCAAATCAT	CAGATGCATCAGCACAGAAA	55	6	CW	G01661
ADL0242	FAM	141-145	GACCTGCCCACTTTTGGAAA	TATCTCCCCCTCCCCTCTCC	55	2	W	G01662
ADL0243	HEX	121-153	TCTGTGCCTATGTATTTTTA	GCATCTTTTTCTCCTGTTTA	50	5	CEW	G01663
ADL0244	FAM	142-157	AGGGTCTGAAGAGAGGTGTT	GCAAGATGCAAAGAGATTTC	55	3	EW	G01664
ADL0245	HEX	110-140	ACAAGGGTGGTGCTTAGTCC	AAGTGAAACCAGGAAACGAT	50	3	W	G01665
ADL0246	TET	149-171	GCAGGCTGATAGAAAAATGC	CTGCAAGCTGCTCTGGTATT	55	5	CEW	G01666
ADL0247	HEX	165-171	CTCTTGTTGTCTGTCTTGTG	TGCATGTTGTCAGTTTTCAG	55	4	EW	G01667

ADL0248	HEX	125-156	AGCCAGTAAAGGTAAAATGC	TTTCCAGCCTCTGTATCCAA	55	5	EW	G01668
ADL0249	FAM	135-149	TGTTCCTGTGACATCTCTGT	TACTTGCTTGTGGCTGTTGT	55	2	W?	G01669
ADL0250	HEX	164-168	AAGCCGTACTGAGAAGCACT	CAGGCACAGTAGAAAAGAAC	55	2		G01670
ADL0251	FAM	123-127	TTTGGCTTAGGGTGATGCTG	CGTGCTCCACACAGGAATGT	55	2	W	G01671
ADL0252	TET	182-193	AGCTCAGCCTCGGATACCTG	GTGAAGGGGTCTCTCCTCTG	50	3	CEW+bg	G01672
ADL0253	HEX	124-148	GCTGTTTTGTCGGCAATAGC	CCCGTATACAGTAACTCTGA	55	4	CEW	G01673
ADL0254	FAM	131-153	CAGCGTGGAAGCAATAAATG	CACTCATCCCACAACCACTG	55	7	CW	G01674
ADL0255	FAM	106-116	GGGTATTGGTCTTCAAAATG	GTAAAGGCCTTCCTCTTCTT	55	3	EW	G01675
ADL0256	TET	124	CCTAAAATGCAGACAGAAGC	ACAAACGCAGTCATCAAAGG	55	1		G01676
ADL0257	HEX	167-197	ATCTTGAAACCTCACAAAGC	TCTTCCAACCTATTTTTAGT	55	6	EW	G01677
ADL0258	FAM	168-178	TCATTTCAGCTCACATTTTA	TTTTCAGGTTGTCTGGTTGC	55	3	CEW	G01678
ADL0259	TET	112-146	GTAATGGAGGATGCTCAGGT	CTCATTGCAGAGGAAGTTCT	55	5	W	G01679
ADL0260	HEX	118-144	ATCAGCCCATCCCAGTATCG	GGAGCTGTCATCCACTCTTG	55	6	CEW	G01680
ADL0261	FAM	158	TGCTCGCAGCGTGATAAAGC	CCCCGTGCACAGAACCAAGC	55	1		G01681
ADL0262	FAM	106-110	GTGCAGACACAGAGGGAAAG	TCACATGCACACAGAGATGC	55	3	CEW	G01682
ADL0263	TET	136-150	AGAGTCAGAAAGTGGGAAGG	NCTGTTCGGTTGGTTGTTGG	55	5	CW+bg	G01683
ADL0265	FAM	168-192	GCCTTCTGAAGGTAAGTAGC	TGTCAGCAGAGCAGCAATAC	55	6	EW+bg	G01685
ADL0266	HEX	109-136	GTGGCATTCAGGCAGAGCAG	AATGCATTGCAGGATGTATG	55	5	CEW	G01686
ADL0267	FAM	105-119	AAACCTCGATCAGGAAGCAT	GTTATTCAAAGCCCCACCAC	55	3	E	G01687
ADL0268	TET	105-117	CTCCACCCCTCTCAGAACTA	CAACTTCCCATCTACCTACT	55	5	EW	G01688
ADL0269	HEX	137	CGTATTCGTGACATCCTTTT	CCACACTCGCCCTACAGAGG	55	1		G01689
ADL0270	HEX	091-125	TGGGGTTGGGTTGGTTTTTA	CCCTGGCAGTTGGTTATTCT	55	6	CEW	G01690
ADL0271	FAM	137-144	ATGAATGAAACCCATCTAAC	TCATCAGAAGCCCAAGCACA	55	2	W	G01691
ADL0272	TET	168-176	TATGGTAAGGTGAGCAAACC	GGGAAAGCTATGAAAGATTT	55	2	E	G01692
ADL0273	TET	139-146	GCCATACATGACAATAGAGG	TGGTAGATGCTGAGAGGTGT	55	3	CW	G01693
ADL0274	HEX	153-174	GCAGCCCTTCTTGTAAATGG	GGAAAAACAGCCAAATCAGG	55	4	CE	G01694
ADL0275	FAM	292-287	CCAAACCAAAGAAGCAATAC	AGGGAGAAGACAGGGAAGTT	50	3	EW	G01695
ADL0276	TET	185	CAGTTTCTCCTACTTATCCA	TGTGCTATCATTGTCTACCC	55	1		G01696
ADL0277	FAM	123	CCGCCAAGGTGATGCTGTTC	GGGAGTGGGGTTGTCAGACC	55	1		G01697
ADL0278	FAM	112-122	CCAGCAGTCTACCTTCCTAT	TGTCATCCAAGAACAGTGTG	55	4	EW	G01698
ADL0279	TET	097-117	CATGGCTGTTGCTTTACATA	GTGAACCCCAATGCTCTCTG	55	5	EW	G01699
ADL0280	FAM	170-180	CCCCTATAGCACAGCAGTCC	GGAACCTCAGCCTTGACATT	55	4	CW+bg	G01700
ADL0281	HEX	164-184	GAAGAGGTGGAATGGACTGC	ATGCCCTCAGCAAAGTGTTG	55	4	EW+bg	G01701

ADL0282	TET	104-124	TGTTCGCGAAAGTCCTCCTC	CGGCGATACAGCCTGGTTCC	55	3	CEW+bg	G01702
ADL0283	HEX	125	GCAGATTTCCTTTATTTACC	ATTTCTATTTCACAGAATTG	50	1		G01747
ADL0284	HEX	128-149	CAGAGTTCATCCGCCACTGC	CCTCCCCCACTAACATTGGAA	55	5	CEW	G01703
ADL0285	TET	154-160	CTTCCAATCCTGACTGTAAG	TGATCTTGAAAAATCCACTT	55	2	CEW	G01704
ADL0286	TET	114-124	GAAGTGAAGAGTTGGAGACG	GCTAGATGCTGGCTGAATAA	55	3	CW	G01705
ADL0287	TET	123-138	TGGTGATAGGCTACAGAAGC	CCCCACCACCCTACAAACAC	55	5	CEW+bg	G01706
ADL0288	FAM	150-160	AAAACTTGCCTCCCAACATT	ATCCCCCACGTAATACTTAT	55	4	CEW	G01707
ADL0289	TET	168-178	ATGCTATCTTAGAGGTGCTC	AATTGAAATCACTTTGCTCA	55	3	EW	G01708
ADL0290	FAM	168-195	AAAGGCCAGGATGGATTTTC	CCCACCGGTCAGCACGAAGG	55	4	CEW+bg	G01748
ADL0292	FAM	120-126	CCAAATCAGGCAAAACTTCT	AAATGGCCTAAGGATGAGGA	55	4	EW	G01710
ADL0293	TET	112-121	GTAATCTAGAAACCCCATCT	ACATACCGCAGTCTTTGTTC	55	4	EW	G01711
ADL0294	FAM	138	TGCACGCAAGCAGGAGAAAA	CAGCAGAGTGGGGTCACCTC	55	1		G01712
ADL0295	HEX	145	GAGAAATGCCCCTCCCTTCA	TCTCCCGGCTCTGGCGTTGC	55	1		G01749
ADL0297	FAM	106	ACCCATCTAGGCACCTTGTG	TTCCCTGCTCACCACCAGTC	55	1		G01750
ADL0298	FAM	100-120	CAAGGCTGGGATTGATGAAA	TGGCGTGTGGGTTTACAAAA	55	3	CEW	G01714
ADL0299	FAM	159-161	GTCTAGGCCCCTTGCCAAAC	CCACCCCCATGTTCAGGTCA	55	2	EW	G01751
ADL0300	FAM	160-180	TTTCCATGCAAGGNTAGGTG	CTGAAGGCATTCTCAAGGAG	55	5	CE	G01715
ADL0301	FAM	128-136	TCCTCCCTGAAGTCCTTACA	GGATGCGTTTGTGAAGTTTG	55	3	CW	G01716
ADL0302	TET	105-109	GGGGAGGCAGGTTGGAGCAG	CCCAGGGTCAGCAAAGAGCA	50	2	E	G01717
ADL0304	FAM	141-163	GGGGAGGAACTCTGGAAATG	CCTCATGCTTCGTGCTTTTT	55	5	CEW	G01719
ADL0306	FAM	118-137	GTTACTGTATCTTGGCTCAT	TCAGTTTGACTTTCCTTCAT	55	3	EW	G01721
ADL0307	FAM	209-224	GCTGCTTAACTAAATGTTTG	CAAGCGNCACTGACCCTGTC	55	4	EW	G01752
ADL0308	FAM	160-165	CCTCTGAATGTCTGAATGAC	GGATGACTCCTTGGCAACAC	55	2	CEW	G01722
ADL0309	HEX	106-125	CCCCAGTACTGCTTCCTCAG	TGTGGCTGCACTTCCCAATA	55	4	CEW	G16078
ADL0310	HEX	137-149	GTCTCTGGATCTCCTCTTCG	TGCACACTTCCCACTACAGG	50	4	EW	G16079
ADL0311	TET	188-207	GAGGAAATTGAAGCGTAAT	TAGACCAGGTGGACCCAGAG	55	5	EW	G16080
ADL0312	HEX	152-157	AAGCTGGAACTTGAAGAAGA	TCAGGAGGGTTGGAGGTGTG	50	3	CEW	G16081
ADL0313	HEX	132-156	AGCCACACAGNAGCGTCTCC	ACTGGCAGCCATTGAAGGAC	55	4	CE	G16082
ADL0314	HEX	182-185	CCCCATAATTCTTTCAGTGC	CATCCAATGCAGACAGGACA	55	2	EW	G16083
ADL0315	TET	250-252	TCCTTGGGCAGTAGTTTCAA	CTCCCATGTTGCTTCTTTAG	55	2	EW	G16117
ADL0317	TET	188-206	AGTTGGTTTCAGCCATCCAT	CCCAGAGCACACTGTCACTG	55	5	EW	G16085
ADL0319	TET	112-116	AACGCTGTCAACCACTGTGT	TTGGCTTTCAAGGTTATGTG	55	2	EW	G16118
ADL0320	TET	116-138	AGGGGTTATTGCTGCTCTGC	GTCCTCAGTGTGCCAAATGC	55	7	CEW	G16087

ADL0370	FAM	194-200	ACAGATATCAAACTTCCAAG	AATATCTATGCTGAAATGTG	55	3	EW
ADL0371	FAM	160-175	AAATAGGTTCTCCCAATCAC	AATCATGGAAGACTGCTTTC	55	5	EW
ADL0372	FAM	160-171	CGCCCCCGTTTACTGATTTG	GGCGCCGTTCAAGGAAGCAC	55	4	EW
ADL0373	FAM	161-174	TGTCCTTTCTCTGTCCTTCC	TAATTCCTGCACTCGTCTTG	55	3	CEW
ADL0375	FAM	113-117			50	3	CW
ADL0376	FAM	176-188	GCCCCACGGAGATGGAACAC	CCTGCCCTGCTGCTGGAACT	55	4	CEW
ADL0377	FAM	137-144	ATATTCTGGGGACATCTGTG	GTAGGGATCCGTAGTTTTTG	55	4	EW

[1] Fluorescent amidite dye attached to the 5'end of the forward primer, which allows for detection on the Applied Biosystems 373/377 DNA sequencers.

[2] Fragment length of the alleles found after PCR in our test panel according to the ABI genescan-350 standard.

[3] The optimal annealing temperature in the PCR reaction.

[4] The number of alleles found in our test panel.

[5] Polymorphism found in the different populations used; C= Compton reference population, E= East Lansing reference population and W= Wageningen resource population.

[6] Genbank accession number.

[7] Primer sequences in capital have been redesigned to improve the performance of the microsatellite marker on the automated sequencers, the other primers have been described by Khatib *et al.* (1993).

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Curriculum Vitae

Richardus Petrus Martinus Arnoldus (Richard) Crooijmans werd op 30 juni 1964 geboren te Bakel en Milheeze. In 1980 behaalde hij het MAVO diploma aan de Bernard Alfrink MAVO te Deurne. In 1982 behaalde hij het HAVO diploma aan het Peelland College te Deurne. Aansluitend studeerde hij biochemie aan de Hogere Laboratoriumschool te Oss. Vanaf 1986 werkte hij als onderzoeksmedewerker op de afdeling Radiotherapie van het Academisch Ziekenhuis Nijmegen. Vanaf juni 1990 is hij werkzaam bij de leerstoelgroep Fokkerij en Genetica van Wageningen Universiteit alwaar hij, vanaf 1995, als onderzoeker in opleiding het in dit proefschrift beschreven onderzoek verrichtte. Per 1 november 2000 is hij werkzaam als universitair docent bij bovengenoemde leerstoelgroep.