Genomic research and applications in the duck (Anas platyrhynchos)

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Genomic research and applications in the duck (*Anas platyrhynchos*)

Y.H. HUANG[1,2], N. LI[1]*, D.W. BURT[2] and F. WU[1]

[1]State Key Laboratory for Agrobiotechnology, China Agricultural University, Beijing 100094, People's Republic of China; [2]Department of Genomics and Genetics, Roslin Institute (Edinburgh University), Roslin, Midlothian, EH25 9PS, UK
***Corresponding author: ninglbau@public3.bta.net.cn**

As a major natural reservoir of influenza virus and an important food source, the duck is of great biological interest, *e.g.* in the area of host-pathogen interactions. Recently, preliminary genetic and cytogenetic maps of the duck have become available, providing for the first time a glimpse at a comparative map between the duck and chicken. These genetic tools have been used to detect QTLs related to duck growth, carcass and meat quality traits. However, molecular genetic research in the duck is only in its infancy. In the future we can expect the development of new duck resources, including a high-density genetic map, detailed comparative maps with the chicken and other vertebrates - and given the pace of genomics, possibly a genome sequence. These new resources will be used to evaluate the genetic diversity of global duck breeds, to define genetic markers to increase the quantity and quality of egg and meat products, and to aid in the battle against infectious diseases, such as avian influenza.

Keywords: genomics research; applications; duck

Introduction

Besides the genomes of Chicken (Hillier *et al.*, 2004) and Zebra finch (http://genome.wustl.edu/genome.cgi?GENOME=Taeniopygia%20guttata&SECTION=reseach) there is increasing interest in the duck (Mallard) genome. In common with the chicken and other birds, the genome size of the duck is nearly one third that of mammals (Tiersch and Wachtel, 1991), which should facilitate the determination of its genome sequence in the future. Ducks belong to the order *Anseriformes*, which shared a common ancestor with *Galliformes* almost 90 million years ago (Pereira and Baker, 2006), providing greater depth of avian evolution and basic biology. This recent awareness has been stimulated by research using the duck as a model organism for the study of host interactions against infection disease, in particular avian influenza. Ducks develop no or only very mild symptoms after infection by highly pathogenic avian influenza viruses

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(Hulse-Post *et al.*, 2005; Webster, 2002) and the mechanism of this evasion is of great interest. The first genetic map and QTLs for carcass, meat quality, body weights and conformation traits in the duck were published recently (Huang *et al.*, 2006, 2007a,b). Compared with species such as humans, mice and chickens, molecular genetic research in the duck has just made its first steps. The following paper reviews the progress of genomic research and its application in the duck.

Construction of the first duck genetic linkage map

Molecular genetic maps of the duck can be used to provide insights into its genome organization and the chromosomal localization of genes, and establishes a framework for the identification and localisation of major genes associated with economically important traits (Crittenden *et al.*, 1993). High-density genetic linkage maps are now available for many livestock species, such as pigs, cattle, sheep, goats, and chicken (Archibald *et al.*, 1995; Groenen *et al.*, 2000; Ihara *et al.*, 2004; Maddox *et al.*, 2001; Vaiman *et al.*, 1996). In contrast, mapping studies in waterfowl are much less advanced. To construct saturated genetic maps the isolation of many polymorphic genetic markers, particularly microsatellite markers and single nucleotide polymorphisms, is a prerequisite. Although many chicken genetic markers are available, it is difficult to use these in the duck or other waterfowl because of poor sequence conservation between species (Huang *et al.*, 2005). Therefore waterfowl-specific genomic libraries need to be constructed. For example, in 1997, seven microsatellite loci in waterfowl were developed from spectacled eiders and greater white-fronted geese (Fields and Scribner, 1997). Following that, seven papers were published on the isolation of microsatellite loci in waterfowl (Buchholz *et al.*, 1998; Huang *et al.*, 2005; 2006; Maak *et al.*, 2000; Maak *et al.*, 2003; Paulus and Tiedemann, 2003; Stai and Hughes, 2003). To date 261 microsatellite sequences and genetic markers have been reported in waterfowl (http://www.ncbi.nlm.nih.gov/).

Until now, only one genetic map for the duck has been developed from a cross between two extreme Beijing duck lines by linkage analysis of 155 polymorphic microsatellite markers (Huang *et al.*, 2006). A total of 115 microsatellite markers were placed onto 19 linkage groups. The sex-averaged map spanned 1,353 cM, with an average interval distance of 15 cM (*Figure 1*). Sex-specific maps have also been constructed. The length of the male map is 1,415 cM with an average intermarker distance of 16 cM, whereas the female map is 1,388 cM, with the average intermarker spacing of 17 cM (Huang *et al.*, 2006). Assuming that the genetic maps between chicken and mallard are similar in length, then these maps only represent ~36% of the total predicted length of 3,800 cM (Groenen *et al.*, 2000).

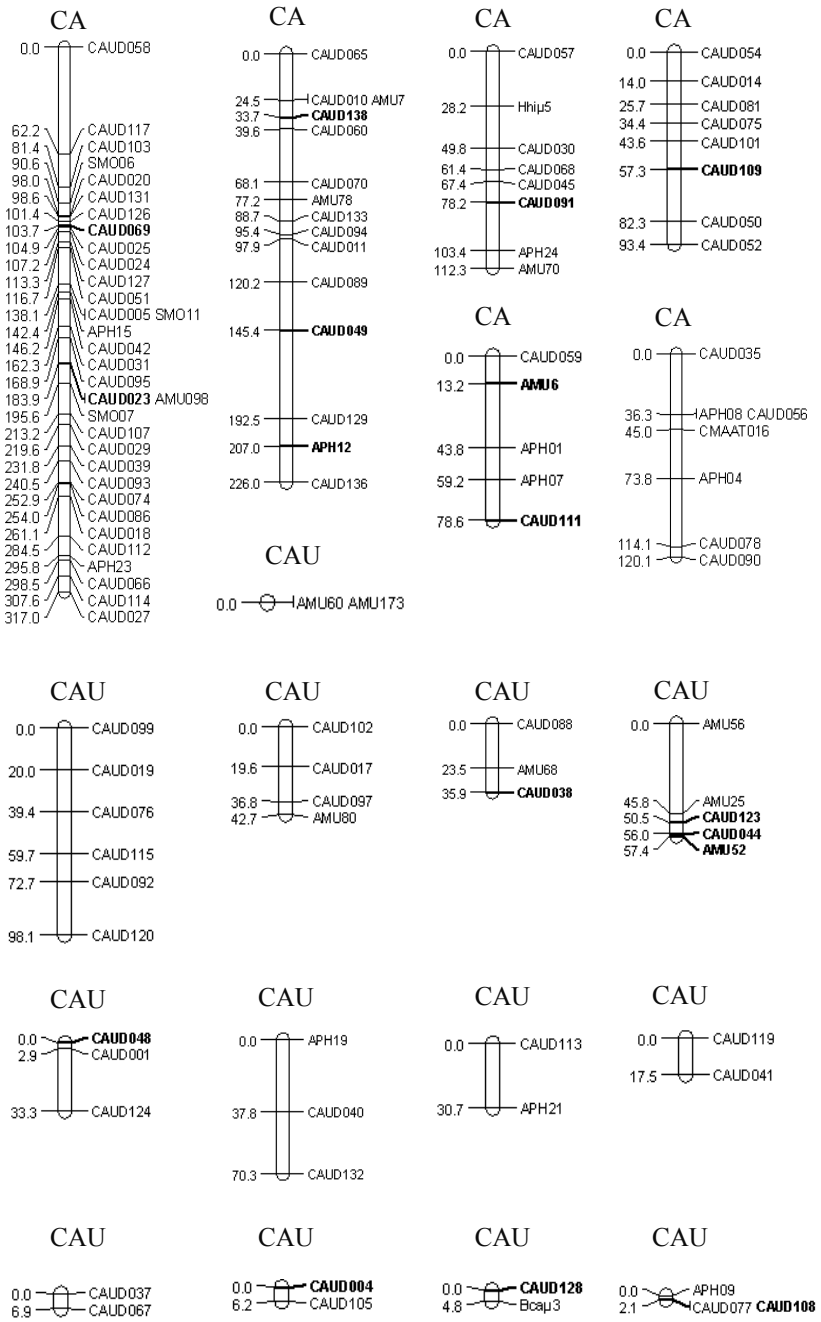

Figure 1 Sex-average map in Kosambi cM for the duck consisting of 19 linkage groups, the markers in boldface were physically assigned to duck chromosomes (APL) by FISH with chicken BAC probe (Huang *et al.*, 2006).

Detection and genetic mapping of Quantitative Trait Loci or QTLs

Most biological traits and common diseases have a complex pattern of inheritance, controlled by many genes and environmental factors. A chromosomal region that contains one or more genes that influence a complex trait is referred to as a QTL (Andersson, 2001). With the development of strategies for the construction of genetic maps and statistical methods for segregation analysis, many QTLs that affect a broad range of phenotypes – including growth, body composition and fertility, have already been mapped with high confidence in many livestock species (Andersson, 2001). However, the lack of genomic resources and a high-density genetic map in the duck has hampered progress in gene mapping and identification of QTL in this species. Huang *et al.* (2007a; 2007b) investigated QTLs for carcass, meat quality, body weight and conformation traits using 95 microsatellite markers distributed over 1,237 cM or ~ 33% of the duck genome (assuming total genetic length equal to the chicken 3,800 cM, Groenen *et al.*, 2000). Half-sib analysis using a multiple QTL model revealed a total of 45 QTLs for 21 traits on 10 linkage groups in a cross between four males in line 6 and 12 females in line 5 Beijing ducks (*Table 1*).

Table 1 QTL for growth, carcass and meat quality traits in the duck.

Linkage Group	Trait[1]	Position (cM)[2]	95% CI. (cM)	F value[3]	Flanking Markers[4]	Phenotypic Variation (%)
CAU1	BW0	21	257	5.35†	CAUD058-CAUD117	7.93
	BW3	298	316	3.93*	APH23-**CAUD066**	5.28
	BW6	101	183.5	5.5†	CAUD131-CAUD126	7.89
	SL	122	260.5	3.98*	CAUD051-CAUD005(SMO11)	5.29
	BMW	186	204.5	5.72†	**CAUD023**(AMU98)-SMO07	8.59
CAU2	BW0	112	139	4.62†	CAUD011-**CAUD089**	6.69
	BW1	144	70	4.91†	**CAUD089-CAUD049**	6.81
	BW3	137	109	4.82*	**CAUD089-CAUD049**	6.78
	KBL	143	186	3.98*	**CAUD089-CAUD049**	5.37
	AFW	114	139	4.65*	CAUD011-**CAUD089**	6.64
	BMW	145	173.5	4.15*	CAUD089-**CAUD049**	5.91
	CPW	39	199	3.51*	**CAUD138**-CAUD060	4.66
	HTW	201	177	4.55*	CAUD129-**APH12**	6.47
CAU3	BW4	0	112	3.48*	CAUD057-Hhiμ5	4.43
CAU4	BW7	0	120	4.11**	CAUD054-CAUD014	5.49
	BTW	59	88	3.87*	**CAUD109**-CAUD050	5.07
	AFW	21	79	4.4*	CAUD014-CAUD081	6.23
	BMW	16	82	4.62**	CAUD014-CAUD081	6.73
	CPW	0	44	12.01††	CAUD054-CAUD014	17.68
	CW	14	86.5	3.49*	CAUD054-CAUD014-CAUD081	4.52
	FW	0	41	7.32††	CAUD054-CAUD014	10.8
	LRW	42	52.5	5.16††	CAUD075-CAUD101	7.94
	NW	8	93	5.5††	CAUD054-CAUD014	7.94
	SW	23	23.5	8.84††	CAUD014-CAUD081	13.27
	TW	25	82	3.72*	CAUD014-CAUD081	4.95
	WW	6	84	5.86††	CAUD054-CAUD014	8.5
	DL	0	92	6.75††	CAUD054-CAUD014	16.67
CAU5	DL	43	78	3.16*	**AMU6**-APH01	9.09
CAU6	BW0	118	97	4.07*	CAUD078-CAUD090	5.73
	BW5	119	120	3.32*	CAUD078-CAUD090	4.16
	BW6	12	120	4.05*	CAUD035-CAUD056(APH08)	5.48
	BL	118	120	3.83*	CAUD078-CAUD090	5.03
	KBL	120	120	4.31†	CAUD078-CAUD090	5.93
	HTW	0	115	4.10*	CAUD035-CAUD056(APH08)	5.71

Table 1 Continued

Linkage Group	Trait[1]	Position (cM)[2]	95% CI. (cM)	F value[3]	Flanking Markers[4]	Phenotypic Variation (%)
	LRW	119	91.5	5.35††	CAUD078-CAUD090	8.27
	CL	0	120	4.24*	CAUD035-CAUD056(APH08)	5.66
CAU7	LRW	84	78	3.63*	CAUD092-**CAUD120**	5.16
	ST	61	66	4.05*	CAUD115-CAUD092	5.62
CAU10	BW0	45	56.5	2.93*	AMU56-AMU25	3.68
	NL	51	57	3.84**	**CAUD123-CAUD044**	5.04
	SW	0	56	2.96*	AMU56-AMU25	3.68
CAU12	BW2	6	70	3.97*	APH19-CAUD040	5.26
	SG	0	70	5.09†	APH19-CAUD040	7.38
CAU13	GW	0	30	2.78*	CAUD113-APH21	3.29
	ST	0	30	3.49*	CAUD113-APH21	4.64

[1]AFW= weight of abdominal fat, BL=length of the body, BMW= weight of breast meat, BTW=width of breast, BW0=body weight of birth, BW1=body weight at 1 week of age, BW2=body weight at 2 weeks of age, BW3=body weight at 3 weeks of age, BW4=body weight at 4 weeks of age, BW5=body weight at 5 weeks of age, BW6=body weight at 6 weeks of age, BW7=body weight at 7 weeks of age, CL= cook loss of breast meat, CPW= weight of crop, CW= weight of carcass body, DL= drip loss of breast meat, FW= weight of skin fat (skin plus subcutaneous fat), GW= weight of gizzard, HTW= weight of heart, KBL=length of keel bone, LRW= weight of liver, NL=length of neck, NW= weight of neck, SG=girth of shank, SL=length of shank, ST= fat thickness in tail, SW= weight of shanks, TW= weight of thighs, WW= weight of wings.
[2]Position in the duck genetic map (Huang *et al.*, 2006);
[3]F values "*", "**", "†", and "††" represents significance at chromosome-wide suggestive (5%), chromosome-wide significant (1%), genome-wide suggestive (5%) and genome-wide significant (1%) levels, respectively.
[4]Corresponding orthologs of the markers in boldface type were found in chickens (Huang *et al.*, 2006).

Construction of a whole genome BAC library and a cytogenetic map

The full utilization of genetic maps requires knowledge of the correspondence between the genetic and the cytogenetic maps to define chromosomes and chromosome orientations. Several methods have been used to correlate genetic and cytogenetic maps (Stephens *et al.*, 2004). Fluorescence *in situ* hybridization (FISH) provides the most direct way of physically mapping DNA sequences onto chromosomes. However, FISH mapping of eukaryotic genomes depends heavily on the development of large insert genomic clones; in particular those cloned into bacterial artificial chromosome (BAC) cloning vectors. BAC libraries for several agricultural species, including cattle, sheep, pigs and chickens, have been constructed (Jeon *et al.*, 2003; Liu *et al.*, 2006a; Liu *et al.*, 2003; Zhu *et al.*, 1999). In order to provide a resource for marker development aimed at increasing the resolution of QTL and physical maps of chromosomal regions in the duck, a 10-fold genome-wide duck BAC library was constructed in the vector pIndig-5 with genomic DNA isolated from a female Beijing duck. This library comprised 84,480 clones ordered in 22 superpools contained in 10×384-well plates with an average insert size of 118-kb (Yuan *et al.*, 2006). In addition, a fosmid library of duck was also constructed (Moon and Magor, 2004) containing inserts of about 38-kb.

Recently, a preliminary cytogenetic map of the duck was constructed by FISH using duck and chicken BAC clones (Huang *et al.*, 2006; Yuan, 2007). Using chicken primers, orthologous to duck microsatellite sequences, 28 BAC clones were isolated from a chicken library (Huang *et al.*, 2006). FISH analysis detected fluorescent signals for 24 (86%) of these chicken BAC clones when cross-hybridised to duck chromosomes. Hybridisation signals were detected on duck chromosomes APL1, 2, 3, 4, 7, 8, 9, 10

and other undefined microchromosomes (*Figure 2*, *Table 2*). Nine of the 19 linkage groups were assigned to nine pairs of duck chromosomes. Two unlinked groups were placed on APL3. The map was extended further by Yuan (2007) using eight BAC clones isolated from a duck library using duck microsatellite primers and assigned to duck chromosomes (*Table 2*). In total 22 microsatellite loci from 12 genetic linkage groups have been assigned to 10 duck chromosomes.

Table 2 The positions of 32 duck microsatellite loci in duck genetic map, physical localizations in the pair of chromosomes in chicken and duck.

Locus	Linkage Group[1]	GGA[2]	APL[3]	Locus	Linkage Group[1]	GGA[2]	APL[3]
AMU006	CAU5	5	5	CAUD09	CAU3	3	3
AMU052	CAU10	10	10	CAUD108	CAU18	4	micro
AMU174	—	10	10	CAUD109	CAU4	4	4
AMU182	—	1	1	CAUD110	—	12	micro
APH12	CAU2	2	2	CAUD111	CAU5	5	5
APH20	—	8	8	CAUD123	CAU10	10	10
CAUD004	CAU16	26	micro	CAUD128	CAU17	3	3
CAUD021	—	Un	4	CAUD138	CAU2	2	2
CAUD022	—	7	7	CAUD027	CAU1	Un	1
CAUD023	CAU1	1	1	CAUD052	CAU4	Un	micro
CAUD038	CAU9	9	9	CAUD065	CAU2	Un	2
CAUD044	CAU10	10	10	CAUD088	CAU9	Un	micro
CAUD048	CAU11	11	micro	AMU056	CAU10	Un	micro
CAUD049	CAU2	2	2	AMU060	CAU19	Un	5
CAUD069	CAU1	1	1	AMU068	CAU9	Un	micro
CAUD080	—	10	10	AMU173	CAU19	Un	5

[1]"—" represents markers that were unlinked with anyone; [2]GGA pair of chicken chromosomes, duck markers were assigned to chicken chromosomes by NCBI Blast against the chicken genome sequence using the corresponding flanking sequence, "Un" indicates that the position in the chicken genome is uncertain; [3]APL pair of duck chromosomes, the duck markers were assigned to duck chromosomes by FISH. (Huang *et al.*, 2006; Yuan, 2007).

Identification and genetic mapping of duck immune genes

Ducks and chickens have different susceptibilities to avian influenza viruses. Ducks are often asymptomatic carriers of influenza virus strains that are lethal to chickens (Hulse-Post *et al.*, 2005; Webster, 2002). A comparison of the genetic makeup between chicken and duck may provide important insights into the molecular basis for this difference in susceptibility.

The release of the full genome sequence of human (Austin, 2004) and model species, including the chicken (Hillier *et al.*, 2004), has accelerated the identification of genes using bioinformatics approaches. There are about 500 genes relevant to innate immunity that are cloned or described in humans and chickens. The sequence and genomic location of genes in these other species has been exploited to clone immune-related genes in duck, such as those coding for B cell activating factor (BAFF), interleukin-2 (IL-2), CD40 ligand (CD54) and interferon gamma (Guan *et al.*, 2007; Zhou *et al.*, 2005; Fischer *et al.*, 2007; Schultz and Chisari, 1999).

High-throughput gene expression arrays are important tools used to understand the avian innate host response to infection by bacterial and viral pathogens. Keeler *et al.*

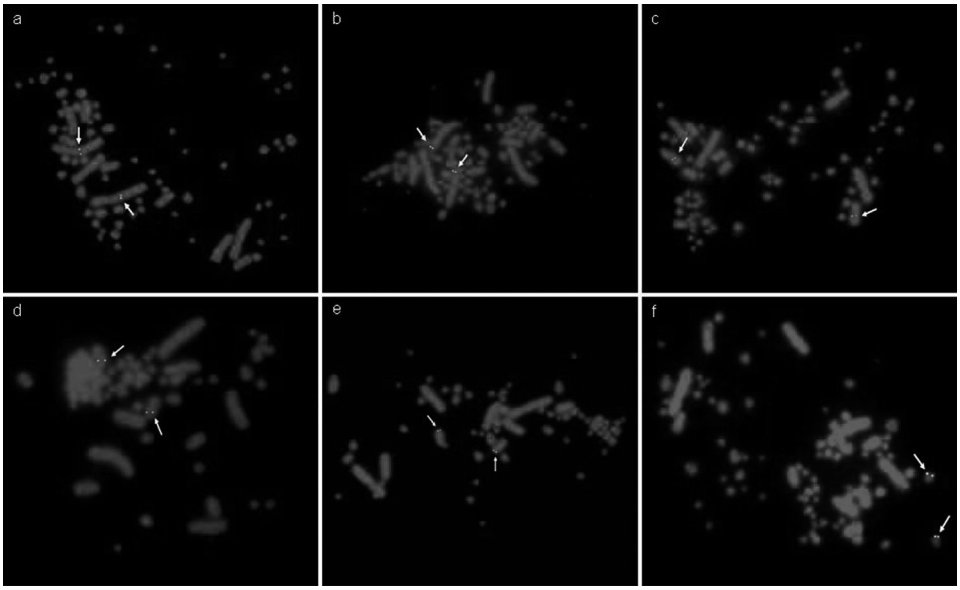

Figure 2 FISH mapping of six orthologous loci to duck chromosomes (APL) with chicken BAC probes: (a) CAUD023, (b) CAUD138, (c) CAUD091, (d) CAUD109, (e) AMU6, (f) CAUD044 (Huang *et al.*, 2006).

(2007) tested a chicken cDNA microarray of 4,959 elements targeted towards avian innate immune genes for cross hybridization towards duck mRNA isolated from spleen. Almost one-third (1,763) of the gene probes cross hybridized to the duck spleen-derived mRNA. These results suggested that, in a limited way, chicken microarrays can be used to monitor host responses to both bacterial and avian viral pathogens in duck. The limitations of using cross hybridisation between chicken genome resources and the duck are common in the field of genome research. An efficient and cost effective solution to isolate species-specific resources has been to sequence large numbers of cDNA from the species of interest to generate 1000's of partial expressed sequenced tags (ESTs). For example, Xia *et al.* (2007) have sequenced 3,168 clones from a spleen cDNA library of a Beijing duck, and identified 208 genes relevant to the duck immune system (Xia *et al.*, 2007). These EST sequences represent a new resource to facilitate studies on the duck immune responses and applications in vaccine development. They also represent a rich-source of duck-specific sequences for gene mapping and genetic map development. Recently, MacDonald *et al.* (2007) have used a targeted approach to clone specific immune-related genes from the duck genome involved in antiviral immune responses, including the immunoglobulin locus, the MHC class I genomic region, the leukocyte receptor complex genes, lectin-like immunoreceptors and Toll-like receptors.

Evaluation of genetic diversity

Farm animal genetic resource diversity has allowed both the development and the sustainability of livestock production in nearly all agro-ecological zones (http://www.sgrp.cgiar.org/CurrentSGRPInitiatives/Platform.htm). The genetic diversity of farm animals, such as cattle, pig, sheep and chicken has been evaluated using microsatellite

and mitochondrial markers (Freeman *et al.*, 2006; Larson *et al.*, 2005; Peter *et al.*, 2007; Romanov and Weigend, 2001). With the development of genetic markers and a genetic map in the duck, it is now possible to estimate genetic diversity in this species. Using the preliminary genetic map of the duck (Huang *et al.*, 2006) 15 microsatellite markers distributed over 14 linkage groups (CAUD024, CAUD060, CAUD050, CAUD115, CAUD091, CAUD038, CAUD097, CAUD001, CAUD040, CAUD056, CAUD041, CAUD044, CADUD078, CAUD067 and CAUD004) were selected as a standard panel for genetic diversity analysis by the Food and Agriculture Organization (FAO) of the United Nations in the 29th international conference on animal genetics in Japan. Liu *et al.* (2006b) investigated the genetic relationship among Chinese indigenous ducks with 806 individuals from 26 breeds using the above standard microsatellite panel. Ahmadi *et al.* (2007) estimated the genetic diversity in Beijing and Muscovy ducks with 6 polymorphic microsatellite markers. Wu *et al.* (unpublished) have evaluated the genetic diversity of 15 lines of Beijing duck with 18 microsatellite loci (including the 15 from the standard microsatellite panel). Observed heterozygosity ranged from 0.094 to 0.904; polymorphism information content (PIC) 0.195 to 0.967; and the genetic distances 0.001 and 0.265.

Prospects for Marker-Assisted-Selection and Genome-Wide-Selection

Marker-assisted-selection (MAS) is a method with great potential providing accurate selection for specific DNA sequence variants associated with desirable effects on quantitative traits. Potential benefits from MAS are greatest for traits that have low heritability, are difficult or expensive to measure, and are genetically correlated with undesirable traits. However, the application of MAS for genetic improvement relies on the level of precision at which a QTL has been identified, which in turn requires high-resolution maps of genetic markers of great utility. Using a preliminary genetic map of the duck, QTL have been mapped in the Beijing duck (Huang *et al.*, 2007a, b). Huang *et al. (*unpublished data) have evaluated QTL related to growth, carcass and meat quality traits by MAS in the Beijing duck. The efficiency of MAS in this experiment will be evaluated in the next few years.

As most economic traits are influenced by many genes, tracking a small number of these through DNA markers will only explain a small proportion of the genetic variance. In addition, individual genes are likely to have small effects and so a large amount of data is needed to accurately estimate their effects (Goddard and Hayes, 2007). To increase the accuracy of estimated breeding values (EBV), Meuwissen *et al.* (2001) proposed a variant of MAS called genomic-wide-selection (GWS), in which genetic markers covering the whole genome are used so that all QTL are in linkage disequilibrium with at least one marker. The major limitation to the implementation of GWS has been the large number of markers required and the cost of genotyping these markers. As a result of rapid rate of progress in the sequencing of livestock species and dramatic developments in SNP genotyping technology, the implementation of GWS in the breeding of duck in the future will be feasible.

Comparative analysis between chicken and duck genomes

The chicken genome has been sequenced (Hillier *et al.*, 2004) and continues to be improved (Burt, 2006). The chicken genome also serves as a model for other avian species, as a rich source of functional and structural information (see www.ensembl.org

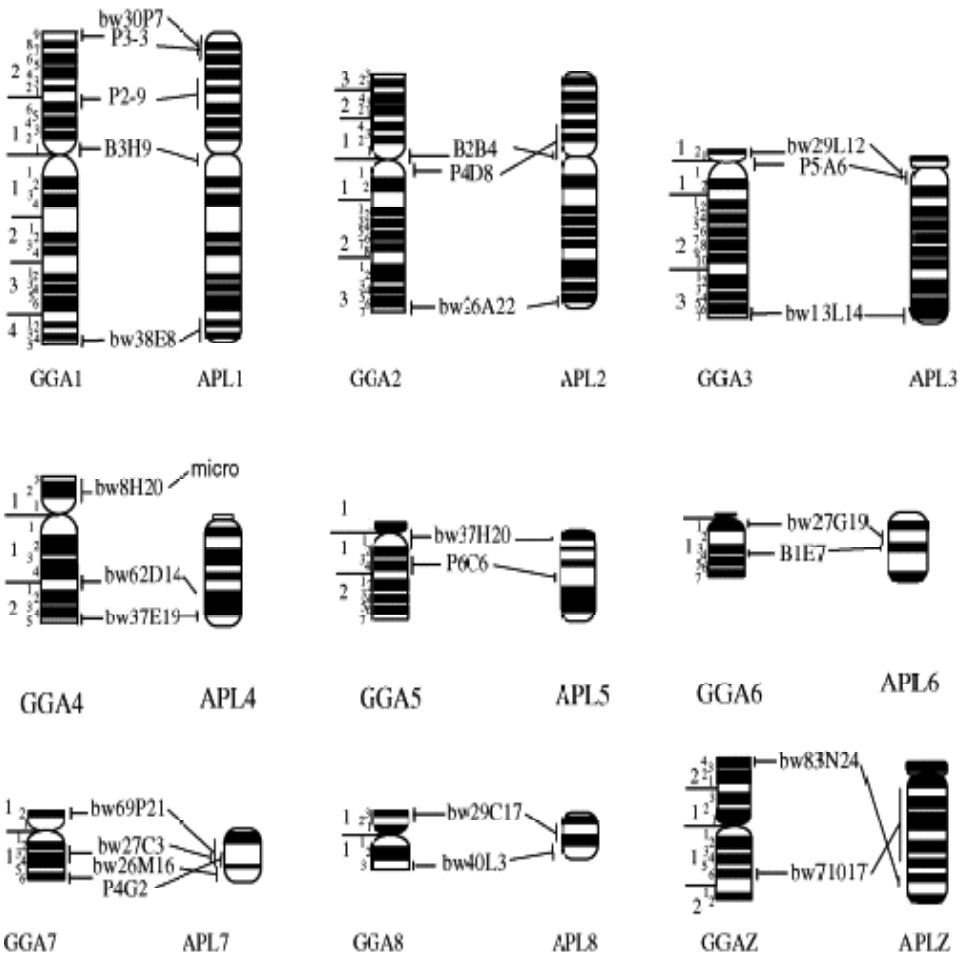

Figure 3 Comparative cytogenetic locations of chicken BAC clones on G-banded karyotypes of chicken (GGA) and duck (APL) macrochromosomes (Fillon *et al.*, 2007).

or www.ncbi.nlm.nih.gov). Comparative analyses with the chicken genome therefore might prove to be one of the most efficient ways of understanding the genetic basis of phenotypic variation in the duck. Until we have the full genome sequence of the duck, comparative analysis of chicken and duck genomes will be based on the comparison of genetic and physical gene maps.

The first attempt to make cross-species comparative genetic maps was to map duck genetic markers on the chicken genetic map (Huang *et al.*, 2005). Thirty-five duck-specific primers were used to screen for homologous loci in the chicken. Only two markers (6%) produced specific products, which sequence analysis showed to be homologous to the original duck sequences. To estimate how many duck microsatellite loci had the potential to cross hybridize to the chicken, Huang *et al.* (2006) compared 240 flanking sequences of duck microsatellite DNA against the chicken genome sequence. Only 20% of microsatellite loci were conserved in both species confirming that this approach had limited utility in making comparative maps

between chicken and duck. Huang *et al.* (2006) assigned 24 chicken BAC clones to duck chromosomes by heterologous FISH mapping. Most of the BAC clones were assigned to the same orthologous chromosomes in duck and chicken. One exception was a chicken BAC clone that mapped to GGA4 but was located on a duck microchromosomes (Huang *et al.*, 2006). This represented one of the few cross differences between the duck and chicken karyotypes and likely to be the product of a centromere fusion (Griffin *et al.*, 2007). Strong conservation of synteny between chicken and duck has been revealed by Fillon *et al.* (2007) and Lin (2007) using specific chicken BAC clones (*Figure 3*). In addition, striking homologies with chromosomes 1 to 9 of *Galliformes* and *Anseriformes* were demonstrated by comparative chromosome painting (Zoo-FISH) (Guttenbach *et al.*, 2003).

Future directions of duck genome research

Significant progress has been made in duck genomics, with the construction of genetic and cytogenetic maps, detection of QTLs, evaluation of genetic diversity and the discovery of many immune genes. However, as this review demonstrates genome research in this species is only in its infancy. Only a preliminary genetic linkage map composed of 19 linkage groups has been developed in the duck. Although the average marker interval is 15 cM, 27% of this map still has regions where the distance between two adjacent markers is considerably greater than 20 cM (*Figure 1*). Among the 45 QTLs mapped so far, 89% have 95% confidence interval ranging from 50 to 316 cM. Targeting regions for developing high-resolution genetic maps is now feasible using integrated cytogenetic and genetic maps (Yuan *et al.*, 2006). Development of high-density maps using SNPs and other genetic markers is now a priority, which will be facilitated by the availability of a genome sequence. So far more than 3,000 ESTs have been isolated, generating 208 ESTs relevant to immune processed in the ducks. A priority should be to sequence more ESTs from diverse tissues to facilitate the creation of gene maps and gene expression studies, for example in host-pathogen research. The use of chicken cDNA microarrays for gene expression studies with duck mRNAs is of limited value, with only one-third of genes showing specific cross-hybridisation. The increase in duck EST sequences will facilitate the construction of duck-specific oligonucleotide microarrays. This will be necessary to make any headway in understanding the differences in host responses to avian influenza and duck hepatitis by ducks and other poultry. Increasing efforts need to be put into comparative genomic research between chicken and duck (Fillon *et al.*, 2007; Huang *et al.*, 2006; Lin, 2007). Although there is strong conservation of synteny between these species, our knowledge of gene order at the macro and micro level is very limited. It is to be expected that this situation will change in the near future with the use of duck BAC clones and multicolour FISH.

Finally, a BAC-based physical map and genome sequence should be a priority for further genome research in the duck. They are important for our understanding of genome structure and organization of ducks and other birds. These resources facilitate position-based cloning of economically important genes, as well as the exploitation of existing genomic information from map-rich species using comparative mapping. Such possibilities are not too ambitious given the rapid rate of progress in the sequencing and analysis of genomes in the 21st century.

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