A global approach of chicken genetic diversity in Taiwan combining phenotypes and molecular markers

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Agriculture, Alimentation,
Biologie, Environnement, Santé

UMR Génétique Animale
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National Chung Hsing University

國立中興大學動物科學系

博士學位論文

Thesis
to obtain the degree

DOCTEUR D'AGROPARISTECH

Field: Animal Genetics

presented and defended by

Chi-Sheng CHANG

on March 24th, 2011

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Chinese Abstract / 中文摘要

家畜遺傳資源的保存和利用對全世界人類來說是非常重要的議題。由於現代畜牧業集約生產模式下，大規模的畜產動物集中在特定幾種具有高生產性能的動物品種上，於是具有特色的地方品種在商業競爭下逐漸消失，但是這些地方動物所具有的遺傳多樣性卻是無法取代的珍貴資產。調查並保存地方品種是有效利用這些遺傳資源的重要步驟，藉由外觀性狀調查和分子標記分析可以進一步提供更多資訊做為遺傳資源的保存與利用。

本論文是在法國與臺灣合作架構下完成，並針對國立中興大學自西元 1982 年以來所保育之六種地方土雞進行遺傳資源多樣性研究調查。第一部分是應用多變量分析結合表型資料(生長與免疫反應性狀)與基因型資料(微衛星標誌、粒線體 DNA，*MC1R* 和 LEI0258 標誌)，結果顯示這種方法可將不同品種資料區分出來，除了金門和竹崎雞外，因為這兩種品系在遺傳背景具有高度相似性，但是免疫性狀和 MC1R 基因型卻有很大的不同。此外，人為選拔過程也可以在 *MC1R* 基因上觀察其控制毛色的作用。第二部分是調查台灣土雞族群內以及 2001 至 2008 年間三個世代之間主要組織相容性複合體(MHC)基因型多態性的分布情形。這是以高度變異的 LEI0258 標記之研究，以及了解它對免疫反應的影響。結果顯示六種保種族群具有 17 種 LEI0258 對偶基因，其中 7 種為新發現之對偶基因，從 2001 至 2008 年間三個世代的 LEI0258 對偶基因分布資料也顯示 MHC 基因型的多態性可以作為鑑別和監測小規模保種族群的遺傳變異情況。藉由以台灣低病原性禽流感病毒(LPAIV)攻毒後免疫反應發現金門雞具有較快的抗體力價反應，信義雞對於攻毒後感染較為敏感，在攻毒後後續疫苗接種(新城雞病、傳染性支氣管炎和傳染性華氏囊病)的免應反應，金門雞同樣有較高的抗體力價。4 種常見於品種間的 MHC 基因型對抗體力價並無影響，但是品種內 MHC 基因型效應特別可以在金門雞中發現，此外，對於金門雞和信義雞中 MHC 對偶基因的更精確的定義也是值得進行深入研究。

綜合上述研究之結論顯示，結合表型資料與基因型資料做為評估遺傳多樣性是具可行性的，此外，MHC 基因座可以做為監測遺傳多樣性與抗病能力的有效指標。

關鍵字: 雞，遺傳多樣性，分子標記，免疫反應，主要組織相容性複合體

French Abstract / Résumé Français

Titre : Une approche globale de la diversité génétique du poulet à Taiwan combinant phénotypes et marqueurs moléculaires.

La conservation et l'utilisation des ressources génétiques animales domestiques est un enjeu d'importance globale pour les sociétés humaines. Le processus de sélection des populations d'élevage mis en place par l'homme a conduit à une érosion de leur diversité génétique. Les races locales sont des ressources d'intérêt potentiel, leur caractérisation est une étape essentielle de tout programme destiné à conserver la diversité génétique. Les outils moléculaires et les caractères phénotypiques fournissent l'information nécessaire à l'évaluation du statut de la population pour éclairer la prise de décision dans un programme de conservation.

Cette thèse s'inscrit dans un programme de collaboration entre Taiwan et la France. Elle s'intéresse à la diversité génétique de six races locales de poulet à Taiwan avec deux objectifs.

Le premier objectif est de combiner les données de performances d'élevage (croissance, réponse immunitaire) et les données moléculaires (marqueurs microsatellites, ADN mitochondrial, gène *MC1R*, marqueur LEI0258 pour le CMH) pour analyser la diversité génétique de six races de poulet conservées à l'Université nationale de Chung-Hsing depuis 1982. Une méthode d'analyse multivariée est proposée pour combiner les variables continues (performances) et les fréquences alléliques (données moléculaires). L'analyse combinée discrimine clairement toutes les races à l'exception des races Ju-chi et Quemoy qui apparaissent très semblables sauf pour la réponse immunitaire. Un processus de sélection est mis en évidence sur le gène *MC1R*, contrôlant la variation de la couleur du plumage.

Le second objectif cible le polymorphisme du complexe majeur d'histocompatibilité, décrit par le marqueur hypervariable LEI0258, et ses effets sur la réponse immunitaire. Sur un total de 17 allèles, 7 allèles sont nouveaux. L'évolution des fréquences alléliques dans chaque race en conservation, a été étudiée pour trois générations entre 2001 et 2008. Les résultats suggèrent que le

polymorphisme du CMH peut être un indicateur utile pour la surveillance de la diversité génétique dans des petites populations en conservation. Une épreuve d'infection expérimentale par un virus d'influenza aviaire faiblement pathogène a montré des différences significatives entre races, avec une réponse immunitaire plus rapide pour la race Quemoy et une sensibilité relativement plus forte à l'infection pour la race Hsin-Yi. Les races différaient aussi pour la réponse secondaire aux vaccins contre la maladie de Newcastle, la bronchite infectieuse et la bursite infectieuse, avec une réponse généralement plus forte dans la race Quemoy. Seuls 4 haplotypes du CMH étaient communs à plusieurs races sur les 19 allèles présents mais ils n'avaient pas d'effet significatif. Toutefois, un effet significatif du CMH a été observé intra-race en particulier pour la race Quemoy. Il serait intéressant de caractériser plus précisément les allèles présents dans cette race ainsi que ceux présents dans la race Hsin-Yi.

En conclusion, cette thèse montre qu'il est possible de combiner des caractères de performance et des données moléculaires pour évaluer la diversité génétique et souligne l'intérêt d'un suivi spécifique du CMH pour mettre en relation la diversité génétique et la résistance aux maladies.

Mots clés : poulet, diversité génétique, marqueur moléculaire, réponse immunitaire, CMH

English Abstract

Conservation and utilization of domestic animal genetic resources is a global and important issue for human society. Animal genetic diversity has become eroded during the process of breeding and human selection. Local breeds are potentially useful genetic resources that need to be characterized as the first step of any programme aimed at the conservation of genetic diversity. Molecular tools and phenotypic traits provide information to evaluate the status of population and for conservation program decision-making.

This thesis is part of a collaborative programme between Taiwan and France, and is focusing on genetic diversity of six local chicken breeds in Taiwan. The first objective is to use phenotypic data (growth and immune responses) and molecular data (microsatellite markers, mtDNA, MC1R and LEI0258 marker of MHC) to analyze genetic diversity of six chicken breeds conserved in National Chung Hsing University since 1982. A multivariate method is proposed to combine continuous variables (performance traits) and allelic frequencies (molecular data). The combined analysis clearly discriminates all breeds except Ju-Chi and Quemoy which appear very similar except for immune response and MC1R. A selection process has been observed on the MC1R gene which controls variability of plumage colour. The second objective is focusing on chicken MHC polymorphism, as described by the hypervariable LEI0258 marker, and it is effect on immune responses. On a total of 17 LEI0258 alleles, 7 were new. The distribution of MHC genotypes in each breed under a conservation program has been studied for three generations between 2001 and 2008. The results suggest that the polymorphism at the MHC locus can serve as a useful indicator of genetic diversity for monitoring conservation of small populations. A challenge experiment with a low pathogenic avian influenza virus has shown significant differences between breeds, with a faster antibody response in the Quemoy breed and a higher sensitivity to the infection for the Hsin-Yi breed. Breeds also differed in their secondary response to vaccines against Newcastle Disease, Infectious Bronchitis, Infectious Bursal Disease, with most often a higher response in the Quemoy breed. Only four out of nineteen MHC haplotypes were shared between breeds and were found not to influence antibody response. However, a significant MHC effect could be observed

within breed, particularly for the Quemoy breed. A refined characterization of MHC alleles found in Quemoy and Hsin-Yi breeds would be interesting.

The final conclusion suggest that by combining phenotypic traits and molecular data for evaluating genetic diversity is possible and that MHC locus can be used as a useful indicator for monitoring diversity in addition to disease resistance.

Key-words : chicken, genetic diversity, molecular marker, immune response, MHC

INTRODUCTION

INTRODUCTION

Worldwide intensive livestock production is utilizing a narrow range of selected breeds. A limited number of high-output breeds is most profitable to modern and industrial production systems. During this process, genetic resources are eroded and in a risky status. The important principle for keeping genetic diversity and resources is to prepare for further needs, such as disease challenge or environment changing. Genetic resources provide a reservoir for future economic, scientific and socio-cultural opportunities.

The recommendation from FAO suggested methods to characterize and manage genetic resources and establish conservation programme. In this framework, the first aim of this study is to combine molecular data and phenotypic data, in order to provide a comprehensive view of the genetic diversity for breeds kept under a conservation programme. Since 1982, National Chung Hsing University started this conservation programme for four local breeds and two imported breeds. Previous studies showed Taiwan local chickens are considered to have better resistance against many local diseases and environmental stress (Lee 2006). The second aim of this study is to investigate the contribution of chicken MHC to the assessment of genetic diversity, as this complex locus plays a major role for the control of immune response. The LEI0258 marker has been used to identify MHC alleles and genotypes in the six local breeds which are under the conservation programme. The functional importance of MHC alleles has then been evaluated by performing a challenge test with a low pathogenic influenza virus, and by testing vaccine responses to a set of 3 common pathogenic viruses (Newcastle Disease, Infectious Bursal Disease and Infectious Bronchitis). The results are expected to improve our understanding of the genetic make-up of these breeds after long term domestication and conservation and to provide new tools for the management and the use of these breeds.

BIBLIOGRAPHY

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I. FAO's recommendation and information system

In 2007, The Food and Agriculture Organization of the United Nations (FAO) published the first global assessment report for the status and trends of animal genetic resources (AnGR), which revealed that animal genetic diversity is under threat and proposed an international framework for management and conservation of genetic resources. Animal genetic diversity is an important resource for selection and breeding in domestic livestock industries. More generally, genetically diverse populations can provide society with great options to meet future challenges such as climate change and emergence of new and virulent animal diseases. Because many breeds have unique characteristics such as disease resistance, tolerance to certain climatic conditions or special phenotypes that could contribute to meet these challenges, breed inventories, characterization and monitoring are necessary for the management of AnGR. FAO had surveyed and established a Global Database for Animal Genetic Resources on a total of 7616 livestock breeds and suggested *in vivo* and *in vitro* conversation programmes. In order to promote sustainable uses of animal genetic resources, FAO made **recommendations** to characterize and assess animal genetic resources, and defined guidelines for the Measurement of Domestic Animal Diversity (MoDAD).

FAO recommended a systematic strategy for animal genetic resources management (Fig. 1). Primary assessment (baseline survey) is a key consideration for the management of AnGR and for decision-making, which should include:

- population size and structure;
- geographical distribution;
- within-breed genetic diversity;
- the genetic connectedness of breeds when populations are found in more than one country (transboundary breeds).

The decision-making process is based upon the assessment of the genetic distinctiveness, adaptive traits, relative value for food and agriculture, historical and culture values of the breeds. Conservation methods (*in vivo*, *in vitro* or a combination of both) and levels (subnational, national, regional and international level) will be decided by breeds/population characters and relevant information.

The aim of policy decisions is to ensure AnGR are conserved for the needs of present and future generations. The ultimate aim of accessing global state of AnGR should be to use the world's wealth in the best possible way for current and future needs of the human population.

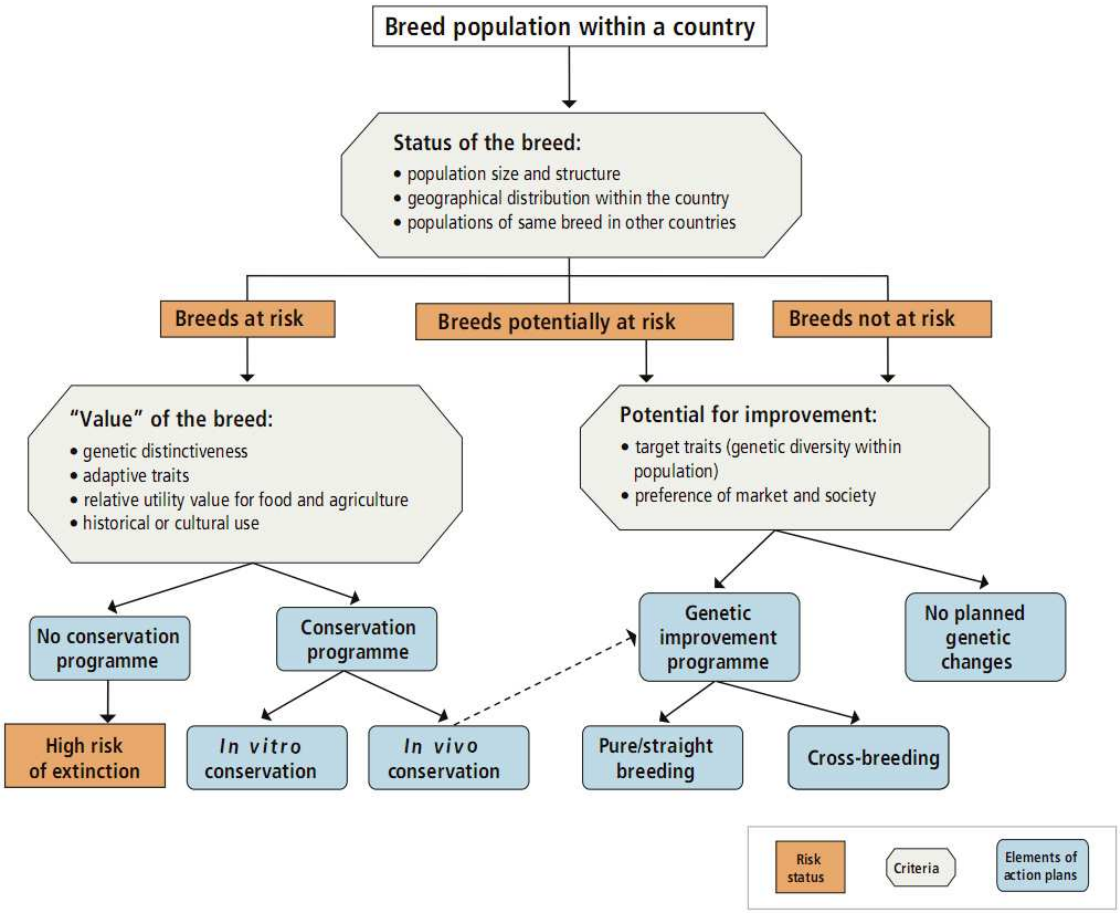

Figure 1. Information required to design management strategies (FAO 2007).

FAO also established the Domestic Animal Diversity Information System (DAD-IS, http://dad.fao.org/) for worldwide communication and information tool for

implementing strategies for AnGR. DAD-IS is the global network centre and links information from regional or countries, its objectives are to involve, coordinate and assist governments, international agencies, NGOs, training and research groups throughout the world, to achieve better management of all AnGR.

II. Characterization

The objective of characterization is to obtain a better knowledge of AnGR. The methods and tools used for characterization will depend on the current management system of a breed. When commercial or conservation farms keep regular records of pedigree, individual performance and environmental characteristics, information are rather easy to collect. In the absence of any management or conservation programme, specific surveys need to be set up. The procedure could be divided into three steps as follows.

A. Sampling

The most important and the first step for animal diversity is sample collection. Sampling may be combined with surveying and/or monitoring. FAO (2007) recommended that samples should be unrelated and representative of the populations, 30 to 50 well-chosen individuals per breed is sufficient to provide first information for breed distinctiveness and within-breed diversity. In fact, the sample size required to be representative may depend on population history; in highly inbred local populations, a lower number of individuals may be needed to describe population diversity that in widely spread population. The number of males and females sampled should be equal. Sample collection for well-defined breeds is based on herd book or pedigree record, but for indigenous populations without records, the geographic criterion is recommended for sampling (FAO 2007).

A well-chosen set of samples can be a long-lasting resource and could produce meaningful results, and initial sampling bias should be avoided from the beginning, in order to make possible future studies.

B. Molecular markers

Molecular markers are a useful tool for exploring genetic diversity, both in basic and applied researches (FAO 2007). A number of markers are available for genetic diversity studies. Whereas a few protein polymorphisms were available for the first studies of genetic diversity, the progress in molecular genetics since the 90s has provided a lot of molecular tools.

Mitochondrial DNA markers

Mitochondrial DNA (mtDNA) follows a maternal mode of inheritance, and assessing sequence polymorphism of the hypervariable segment of mtDNA can be used for phylogenetics and genetic diversity. The analysis of the mtDNA linage is used for domestication studies, to trace back ancient migration events and to detect introgression events between wild and domestic stocks (FAO 2007). Liu *et al.* (2006) revealed nine highly divergent clades by phylogenetic analysis (Fig. 2), from 834 domestic chickens (*G. g. domesticus*) and 66 Red Jungle Fowl (*G. gallus*). Seven clades contained both the RFJ and domestic chicken, and no breed-specific clade. Clades A, B, and E are distributed ubiquitously in Eurasia, but the other clades were only found in to South and South-east Asia. Clade C was distributed in Japan and Southeast China. Clades F and G were exclusive in China. Their results indicated that different clades may originate from different regions, such as Yunnan, South and Southwest China and/or surrounding areas (i.e., Vietnam, Burma, and Thailand), and the Indian subcontinent, respectively. This information also supports the theory of multiple origins in South and Southeast Asia.

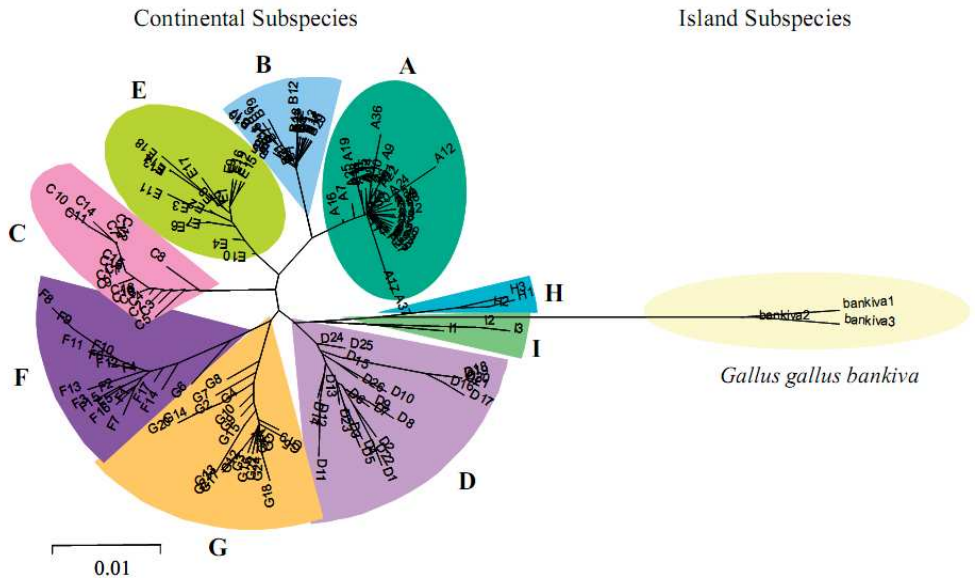

Figure 2. Highly divergent mtDNA clades from A to I, in unrooted neighbor-joining (NJ) tree in 834 domestic chickens and 66 Red Jungle Fowl (Liu *et al.* 2006).

Microsatellite markers

Microsatellites consist in tandem repeats of 2 to 4 nucleotides in nuclear DNA sequences. They became the most popular markers in livestock genetic studies during the 90s because of their high level of polymorphism. Their polymorphism is revealed after PCR amplification and estimation of fragment size by gel migration or sequencing. Fragment size estimation may strongly depend on the technics used and is not easy to standardize. Furthermore, some alleles may be amplified more efficiently than others, with the possible occurrence of 'null' alleles, i.e. alleles that cannot be amplified by PCR, leading to a wrong assessment of the genotype at a marker locus (Delany 2003).

FAO has published a list of recommended microsatellite loci to be used for diversity studies in various livestock species and recommended 25 microsatellite markers. Guidelines were also published for DNA extraction methods, so as to guarantee reliable microsatellite studies.

The criteria to select appropriate microsatellites as following (FAO 2004):

a. The microsatellite markers should be in the public domain,

b. Microsatellite loci that have been identified in mapping studies should be used, and those selected should preferably be known to be unlinked,

c. The microsatellite variants should be shown to exhibit Mendelian inheritance (highly mutable microsatellite loci may show departures from the Mendelian segregation, and would not be suitable for genetic distance analysis),

d. Each microsatellite locus should exhibit at least four alleles, There should be information on the microsatellites in a published report,

e. Microsatellite loci that can be used on several related species such as cattle, sheep and goats are preferable.

Microsatellites are considered to be neutral markers, although they may be linked to known genes with a functional effect. Marker sets were generally chosen to achieve a large genome coverage with a high level of polymorphism without any prior information about selection effects (i.e., natural or artificial). As a consequence, microsatellite genotypes can be analysed with population genetics methods. A list of microsatellites loci for chicken is in Table 1 (Hoffmann *et al.* 2004).

Table 1. List of microsatellites loci from FAO (Hoffmann *et al.* 2004)

No.	Locus Name	Chromosome	Map Position [cM (Mb)]	Primer Sequence (5'-3'), Forward and Reverse	Annealing Temperature (°C)	GenBank Accession No.	Allele Size Range (bp)
1	MCW0248	1	19 (0.58)	GTTGTTCAAAAGAAGATGCATG TTGCATTAACTGGGCACTTTC	60	G32016	205-225
2	MCW0111	1	118 (39.97)	GCTCCATGTGAAGTGGTTTA ATGTCCACTTGTCAATGATG	60	L48909	96-120
3	ADL0268	1	288 (82.96)	CTCCACCCCTCTCAGAACTA CAACTTCCCATCTACCTACT	60	G01688	102-116
4	MCW0020	1	460 (156.62)	TCTTCTTTGACATGAATTGGCA GCAAGGAAGATTTTGTACAAAATC	60	L40055	179-185
5	LEI0234	2	50 (10.72)	ATGCATCAGATTGGTATTCAA CGTGGCTGTGAACAAATATG	60	Z94837	216-364
6	MCW0206	2	104 (30.49)	ACATCTAGAATTGACTGTTCAC CTTGACAGTGATGCATTAAATG	60	AF030579	221-249
7	MCW0034	2	233 (69.66)	TGCACGCACTTACATACTTAGAGA TGTCCTTCCAATTACATTCATGGG	60	L43674	212-246
8	MCW0222	3	85 (19.35)	GCAGTTACATTGAAATGATTCC TTCTCAAAACACCTAGAAGAC	60	G31997	220-226
9	MCW0103	3	201 (67.76)	AACTGCGTTGAGAGTGAATGC TTTCCTAACTGGATGCTTCTG	64	G31956	266-270
10	MCW0016	3	247 (~90)	ATGGCGCAGAAGGCAAAGCGATAT TGGCTTCTGAAGCAGTTGCTATGG	60	L40041	162-206
11	LEI0166	3	300 (103.36)	CTCCTGCCCTTAGCTACGCA TATCCCCTGGCTGGGAGTTT	60	X85531	354-370
12	MCW0037	3	317 (106.71)	ACCGGTGCCATCAATTACCTATTA GAAAGCTCACATGACACTGCGAAA	64	L43676	154-160
13	MCW0295	4	75 (16.09)	ATCACTACAGAACACCCTCTC TATGTATGCACGCAGATATCC	60	G32051	88-106
14	LEI0094	4	153 (50.65)	GATCTCACCAGTATGAGCTGC TCTCACACTGTAACACAGTGC	60	X83246	247-287
15	MCW0284	4	167 (53.91)	CAGAGCTGGATTGGTGTCAAG GCCTTAGGAAAAACTCCTAAGG	60	G32043	235-243

Table 1. (continued)

No.	Locus Name	Chromosome	Map Position [cM (Mb)]	Primer Sequence (5'-3'), Forward and Reverse	Annealing Temperature (°C)	GenBank Accession No.	Allele Size Range (bp)
16	MCW0098	4	217 (78.89)	GGCTGCTTTGTGCTCTTCTCG CGATGGTCGTAATTCTCACGT	60	L40074	261-265
17	MCW0078	5	93 (26.44)	CCACACGGAGAGGAGAAGGTCT TAGCATATGAGTGTACTGAGCTTC	60	L43686	135-147
18	MCW0081	5	151 (45.68)	GTTGCTGAGAGCCTGGTGCAG CCTGTATGTGGAATTACTTCTC	60	L43636	112-135
19	LEI0192	6	31 (2.41)	TGCCAGAGCTTCAGTCTGT GTCATTACTGTTATGTTTATTGC	60	Z83797	244-370
20	MCW0014	6	50 (6.38)	TATTGGCTCTAGGAACTGTC GAAATGAAGGTAAGACTAGC	58	L40040	164-182
21	MCW0183	7	86 (23.42)	ATCCCAGTGTCGAGTATCCGA TGAGATTTACTGGAGCCTGCC	58	G31974	296-326
22	ADL0278	8	94 (29.24)	CCAGCAGTCTACCTTCCTAT TGTCATCCAAGAACAGTGTG	60	G01698	114-126
23	MCW0067	1	(111.68)	GCACTACTGTGTGCTGCAGTTT GAGATGTAGTTGCCACATTCCGAC	60	G31945	176-186
24	ADL0112	10	120 (20.83)	GGCTTAAGCTGACCCATTAT ATCTCAAATGTAATGCGTGC	58	G01725	120-134
25	MCW0216	13	47 (11.88)	GGGTTTTACAGGATGGGACG AGTTTCACTCCCAGGGCTCG	60	AF030586	139-149
26	MCW0104	13	74 (16.60)	TAGCACAACTCAAGCTGTGAG AGACTTGCACAGCTGTGTACC	60	L43640	190-234
27	MCW0123	14	45 (13.61)	CCACTAGAAAAGAACATCCTC GGCTGATGTAAGAAGGGATGA	60	L43645	76-100
28	MCW0330	17	41 (7.01)	TGGACCTCATCAGTCTGACAG AATGTTCTCATAGAGTTCCTGC	60	G32085	256-300
29	MCW0165	23	1 (0.59)	CAGACATGCATGCCCAGATGA GATCCAGTCCTGCAGGCTGC	60	L43663	114-118
30	MCW0069	26	47 (1.21)	GCACTCGAGAAAACTTCCTGCG ATTGCTTCAGCAAGCATGGGAGGA	60	L43684	158-176

SNPs

Single-nucleotide polymorphism (SNP) occurs throughout the genome with a high frequency, particularly in the chicken (Wong *et al.* 2004). SNPs located in non-coding regions are generally considered as neutral, but SNPs located in coding regions such as expressed sequences or regions influencing gene expression, may induce changes in protein structure or regulation. SNPs located in coding regions are generally avoided for population genetics studies that make the hypothesis of no selection.

Application of SNP genotyping can use a much higher number of markers than traditional genotyping with microsatellites. Following genome sequencing projects, millions of SNPs have been produced in several species, including the chicken with a first publication of 2.8 million SNPs (Wong *et al.* 2004) and a more recent publication with more than 7 million SNPs (Rubin *et al.* 2010).

Sets of thousands of SNPs are used to produce high density SNP chips (60k or more) providing a dense coverage of the whole genome. Genotyping technology is standardized and results are easier to compare between laboratories than they are for microsatellites. SNPs genotyping is the currently preferred approach for genome-wide association studies and will be used more and more often for diversity studies, but there is not yet a standard set of SNPs recommended by FAO.

Known genes

Genetic diversity may also be studied at the level of specific genes of particular interest. Chicken Major Histocompatibility Complex (MHC, Figure 3) is a complex locus showing a very high level of polymorphism. It plays a central role in the regulation of immune response. Effects of chicken major histocompatibility complex (MHC) on disease resistance have been reported for a long time (Bacon 1987, Lamont *et al.* 1987 & Lamont 1989). For many years, serological typing has been used to identify chicken MHC haplotypes, but this method was not easy to apply to local populations where no reference samples and no specific reagents were available.

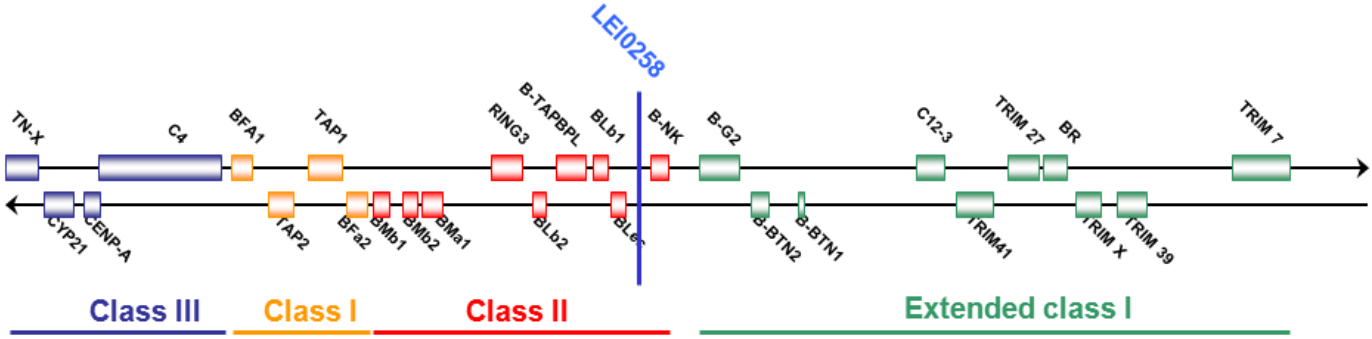

Figure 3. Map of the MHC B complex locus in the chicken.

Chicken MHC polymorphism is now easily identified by the genotyping of a hypervariable marker, LEI0258, located within the complex locus (Fulton *et al.* 2006, Table 2). Numerous new alleles have been described for LEI0258 in local breeds (Chazara *et al.* 2010) and a SNPs panel is under development for genotyping (Bed'hom & Chazara 2010).

Table 2. Polymorphisms identified within the LEI0258 alleles of defined MHC haplotypes (Fulton *et al.* 2006).

B haplotype	Consensus size (bp)	Position				R13	R12	Position					MCW0371 allele size	Unique genotype	Genebank accession number
		-61	-30-29	-28	-11			5	23-29	33	39	46			
4	182	–	–	–	A	1	2	–	Δ	A	–	–	202	*	DQ239540
15.1	193	–	–	–	–	1	3	T	Δ	–	–		200	*	DQ239512
11	193	–	–	–	–	1	3	T	Δ	–	–		201		DQ239495
61	193	–	–	–	–	1	3	T	Δ	–	–		201		DQ239547
27	193	–	–	–	–	1	3	T	Δ	–	–		205	*	DQ239538
BW3	194	–	–	–	A	1	3	–	Δ	A	–	–	203	*	DQ239561
13	205	–	–	–	–	1	4	T	Δ	–	–		202	*	DQ239501
13.2	205	–	–	–	–	1	4	–	Δ	–	–		202	*	DQ239505
17	205	–	–	–	–	1	4	–	Δ	–	–		205	*	DQ239514
BW11	205	–	–	–	–	1	4	–	Δ	–	–		206	*	DQ239560
18	247	–	Δ	–	–	1	7	–	–	–	–	A	203	*	DQ239515
15.2	249	–	–	–	–	1	7	–	–	–	T	–	206		DQ239513
22	249	–	–	–	–	1	7	–	–	–	T	–	206		DQ239531
73	249	–	–	–	–	1	7	–	–	–	T	–	206		DQ239551
15	261	–	–	–	–	1	8	–	–	–	–	A	203	*	DQ239509
2	261	–	–	–	–	1	8	–	–	–	–	A	206		DQ239523
29	261	–	–	–	–	1	8	–	–	–	–	A	206		DQ239539
11.1	295	–	Δ	A	–	1	11	–	–	–	–	–	209	*	DQ239496
5	295	–	Δ	–	–	1	11	–	–	–	–	–	209	*	DQ239541
72	307	–	Δ	A	–	1	12	–	–	–	–	–	208		DQ239550
78	307	–	Δ	A	–	1	12	–	–	–	–	–	208		DQ239555
10	309	–	–	–	–	1	12	–	–	–	T	–	205		DQ239494
24	309	–	–	–	–	1	12	–	–	–	T	–	205		DQ239533
26	309	–	–	–	–	1	12	–	–	–	T	–	205		DQ239537
76	309	–	–	–	–	1	12	–	–	–	T	–	205		DQ239554
74	321	–	–	–	–	1	13	–	–	–	T	–	202	*	DQ239552
BW4	333	–	–	–	–	1	14	–	–	–	–	A	201	*	DQ239562
14	345	–	–	–	–	1	15	–	–	–	T	–	201	*	DQ239508
130	357	–	–	–	–	1	16	–	–	–	T	–	205		DQ239506

Table 2. (continued)

B haplotype	Consensus size (bp)	Position				R13	R12	Position					MCW0371 allele size	Unique genotype	Genebank accession number
		-61	-30-29	-28	-11			5	23-29	33	39	46			
131	357	–	–	–	–	1	16	–	–	–	T	–	205		DQ239507
201	357	–	–	–	–	1	16	–	–	–	T	–	205		DQ239526
5.1	357	–	–	–	–	1	16	–	–	–	T	–	205		DQ239543
6.1	357	–	–	–	–	1	16	–	–	–	T	–	205		DQ239546
21	357	–	–	–	–	1	16	–	–	–	T	–	205		DQ239527
75	357	–	–	–	–	1	16	–	–	–	T	–	205		DQ239553
23	357	–	–	–	–	1	16	–	–	–	–	A	206	*	DQ239532
C	367	–	Δ	–	–	1	17	–	–	–	–	A	202	*	DQ239557
21.1	369	–	–	–	–	1	17	–	–	–	T	–	205		DQ239530
Q	369	–	–	–	–	1	17	–	–	–	T	–	205		DQ239558
BW1	369	–	–	–	–	1	17	–	–	–	T	–	205		DQ239559
13.1	381	–	–	–	–	1	18	–	–	–	T	–	206	*	DQ239504
1	393	–	–	–	–	1	19	–	–	–	T	–	206	*	DQ239493
8	405	–	–	–	–	1	20	–	–	–	T	–	206	*	DQ239556
62	420	–	–	–	–	16	5	–	–	–	–	–	205	*	DQ239548
6	443	–	–	–	–	15	8	–	–	–	–	–	205	*	DQ239544
12.2	474	–	–	–	–	22	3	–	–	–	–	–	205	*	DQ239499
71	474	A	–	—	22	3	–	–	–	–	–		205	*	DQ239549
12	487	–	–	–	–	23	3	–	–	–	–	–	205	*	DQ239497
12.3	513	–	–	–	–	25	3	–	–	–	–	–	205	*	DQ239500
19	539	–	–	–	–	27	3	–	–	–	–	–	205	*	DQ239516
19.1	552	–	–	–	–	28	3	–	–	–	–	–	205	*	DQ239521

C. Phenotypes

Phenotypic variation provides a basis for utilization and genetic improvement of domestic populations. Phenotypic characterization include various levels : morphological attributes, morphometrical indices, production levels and specific adaptations (Tixier-Boichard *et al*. 2008). Appearance of plumage colour, shank colour, shape and size of comb were the first traits to be used farmers for early selection and for breed definition, these traits are still useful to record because they give information on population history and can take part in a conservation program. Regarding poultry breeding, performance recording in controlled environmental conditions on large numbers of animals is used to estimate genetic parameters for a wide array of traits, such as egg production, egg quality, body weight and meat production, reproduction, feed efficiency, disease resistance, conformation and behaviour (Szwaczkowski 2003). The production environment needs also to be described, and FAO is working on a set of descriptors for that aim.

However, performance recording is more difficult to organise for local populations kept in village conditions for instance. Field surveys have to be set up specifically and the environment where the animals are performing needs to be described in order to document the data. The use of geographic information systems is an option currently proposed to integrate different types of data for the analysis of genetic diversity (Joost *et al.* 2010).

III. Methods of population genetics

"The science of population genetics deals with Mendel's laws and other genetic principles as they apply to entire populations of organisms" by Daniel L. Hartl & Andrew G. Clark

The main driving factors of changes in genetic diversity are the following :

- ***Artificial selection***: the process of choosing parents of the following generation on the basis of one or more heritable traits.
- ***Genetic drift***: the chance changes in allelic frequencies that result from the sampling of gametes from generation to generation, and occurs in all population; effects are particularly important in very small populations.
- ***Inbreeding***: the mating of related individuals.
- ***Inbreeding depression***: the reduction in phenotypic value due to inbreeding as compared to a normally outbreeding population.
- ***Mutations :*** spontaneous change in the DNA sequence, since they occur at a very low frequency, they are generally assumed to be negligible.
- ***Crossbreeding :*** results generally in heterosis, which is the difference between the mean performance of individuals resulting from a cross of two genetically different lines, and the average performance of the parental lines.

As a consequence, ***genetic diversity*** is the heritable variation within and between populations, determined by mutation, genetic drift, migration and selection.

In the case of DNA markers such as microsatellites, SNPs, AFLPs, mutations of a known gene, allelic frequencies and genotype frequencies are used to calculate population parameters that provide information on population structure and genetic diversity, both within-population and between populations.

A. Within population parameters

Review of the terms:

- ***Allelic frequency***: the percentage of a given allele found at a locus in a population
- ***Heterozygosity***: the sum of the frequencies of the heterozygous genotypes of the population at a particular locus.
- ***Heterozygote***: an individual having different forms of an allele at a locus on homologous parental chromosome.
- ***Homozygote***: an individual having the same form of an allele at a locus on each homologous parental chromosome.
- ***Hardy-Weinberg equilibrium***: the prediction of genotype frequencies on the basis of allele frequencies in the population, assuming a randomly mating large population without selection, migration, mutation
- ***Effective population size*** (N_e): the size of an ideal population that would have the same rate of increase in inbreeding or decrease in genetic diversity by genetic drift.

The mean number of alleles per marker locus provides a first description of gene diversity. The observed heterozygosity level is also a simple description of genetic diversity. A low heterozygosity generally indicates some inbreeding in the population. When the breeding structure and mating plans of a population are known, with random choice of males and females for reproduction, N_e can be easily calculated from the number of reproducing males M and females F :

$N_e = 4MF/ (M+F)$.

Assuming **Hardy-Weinberg equilibrium;** the frequency of genotypes can simply be calculated from the frequency of alleles, which makes possible to calculate the expected heterozygosity as the product of allelic frequencies. A low heterozygosity indicates generally a rather inbred population. The difference between observed (Hobs) and expected heterozygosity (Hexp) can be tested, and a large difference indicates that the hypotheses of a panmictic population are not met.

Wright's Fis statistics is calculated as the ratio of [(Hexp – Hobs)/Hexp] and represents the deviation from Hardy-Weinberg equilibrium. Deviations can be observed in case of an excess of heterozygotes or an excess of homozygotes, which can be the result of population fragmentation in small groups (excess of homozygotes) or recent introductions and crossbreeding (excess of heterozygotes). Such data can be very useful to understand past selection and to take decisions for further management and conservation of genetic resources.

B. Between population parameters

When a set of populations is genotyped for the same markers, parameters can be calculated to quantify the diversity between breeds and to study genetic relationships between breeds.

- ***Wright Fst*:** measures population differentiation, considering expected heterozygosity within a given population (Hexp(i)) and expected heterozygosity calculated on all animals (Hexptot) , as the ratio (Hexptot – Hexp(i))/Hexptot

- ***Genetic distance***: a calculated value based on allelic or genotype frequencies used to evaluate genetic variation and construct phylogenetic trees.

- ***Phylogenetic tree*** : describes relationships between populations assuming total separation after a given node

Tree construction is generally achieved with either the **unweighted pair-group method with arithmetic mean** (UPGMA) or **neighbour joining. A longer branch indicates a** more distant population, which often corresponds to a more inbred population. Significance of the tree 'nodes' where breeds are separated or grouped together is calculated by bootstrapping methods. Only high bootstrap values (>90%) should be considered for population classification.

- ***Multivariate Co-inertia Analysis (MCOA)***

This method makes possible the extraction of common information from separate analyses, by setting up a reference typology, and comparing each typology separately. The efficiency of a marker is assessed by its typological value (Tv), the contribution of the marker to the construction of the reference typology, which is

equal to the product of the variance (Var) multiply by the congruence with the consensus Cos^2 (i.e. the correlation between the scores of individual locus tables and the synthetic variable of the same rank) (Laloë et al. 2007).

- ***Hill and Smith Analysis***

The method of **Hill and Smith** (1976) was used to combine discrete and continuous variables to characterize breed or populations. This method is a combination of an internal correspondence analysis for discrete data, i.e. the molecular marker data (Laloë *et al.* 2002; Berthouly *et al.* 2008) and a principal component analysis for continuous variables, i.e. performance traits.

C. Bayesian methods to infer population structure

In last decade, Bayesian approaches have been widely developed (Berthouly 2008). The STRUCTURE program (Pritchard *et al.* 2001) is one of the mostly used programs for Bayesian analysis of multilocus genotypes. As in any Bayesian approach, assumptions are of two types: i) the prior distribution for unobserved quantities and ii) the likelihood function relating these unknown parameters to the observed genotypes (Berthouly 2008). The analysis of multi-locus genotypes of a set of populations makes possible to group populations by clusters and to calculate the proportion of the genome of a given individual belonging to each cluster. The main outcome of STRUCTURE is to determine the number K of distinct genetic clusters in the total sample. This method can be very useful to detect introgressions and to identify homogenous populations corresponding to a standard breed as was illustrated in the chicken (Rosenberg *et al.* 2001).

IV. Summary of results obtained on local chicken breeds in Asia

Cattle, sheep, goat, pig and chicken are the "big five" domesticated animal and distributed on a global scale (Fig. 4), chickens are the majority of the total number of avian breeds (Fig. 5). There are 17 billion chickens worldwide, nearly 50% are in Asia, 25% in Latin America and the Caribbean, 13% in Europe and the Caucasus, and 7% in Africa. The distribution of chicken breeds reported in Asia shows 93 chicken breeds (Fig. 6), traditional characterizations are based on phenotypic traits such as feather colour and other easily measured body features (FAO 2006). Recently, by molecular technology, DNA markers have proven useful in basic and applied research (FAO 2007). Microsatellites are popular markers in livestock genetic characterization studies (FAO 2007), Table 3 summarized some of results obtained on Asia local chicken breeds to access genetic diversity and population structure.

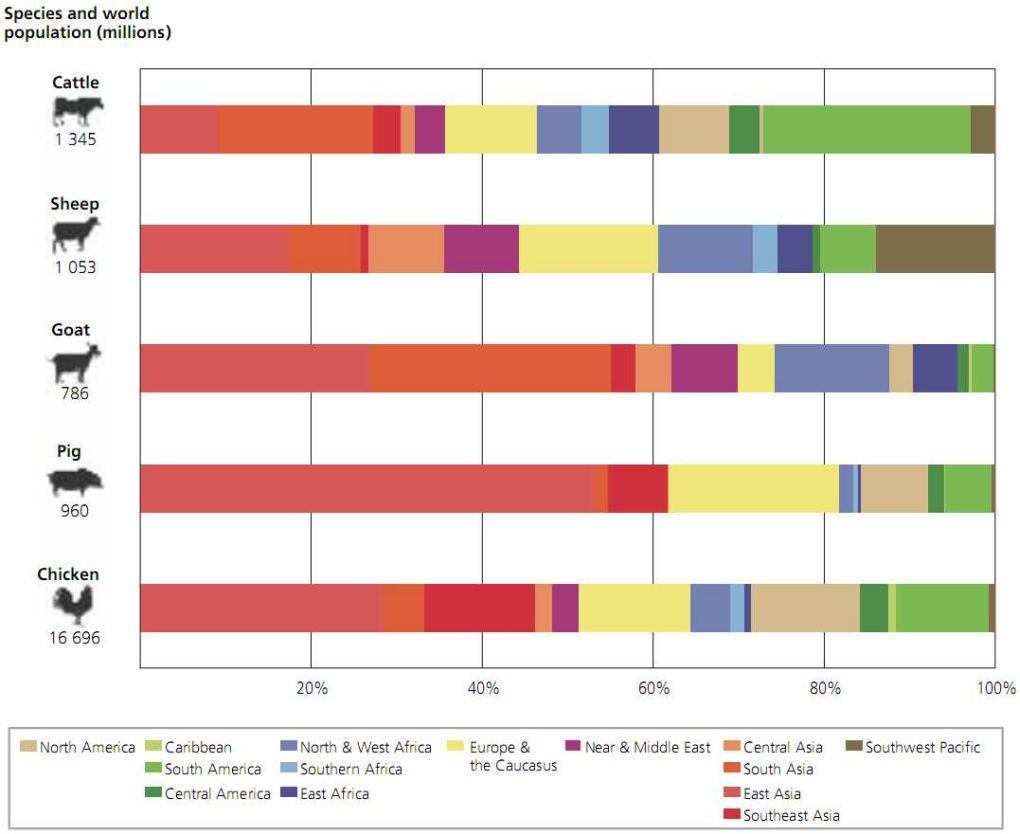

Figure 4. Regional distribution of major livestock species (FAO 2007).

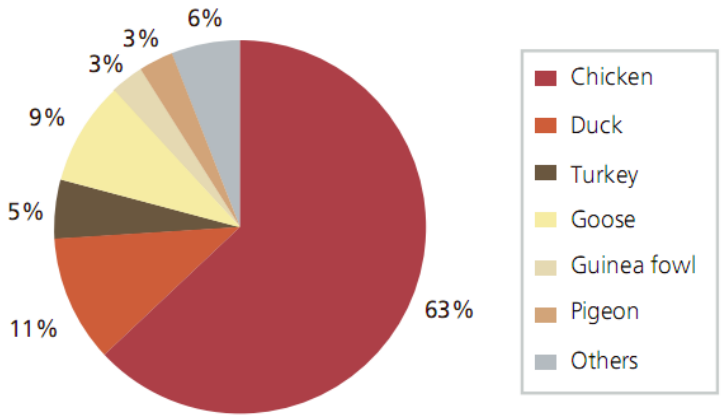

Figure 5. Distribution of the world's avian breeds by species (FAO 2007).

Avian species	Africa	Asia	Europe & the Caucasus	Latin America & the Caribbean	Near & Middle East	North America	Southwest Pacific
	percentage of countries in a region reporting breed-related information for the species						
Chicken	78	93	86	70	50	100	85
Duck (domestic)	32	61	50	33	17	0	46
Turkey	24	43	57	30	17	100	8
Goose (domestic)	16	39	61	21	17	50	8
Muscovy duck	16	39	20	18	17	0	62
Guinea fowl	28	18	11	9	8	0	0
Partridge	4	7	7	0	0	0	0
Pheasant	0	7	9	6	0	0	0
Quail	2	39	14	6	0	50	0
Peacock	0	0	0	3	0	0	0
Pigeon	10	21	9	6	17	0	15
Swallow	0	4	0	0	0	0	0
Cassowary	0	4	2	0	0	0	0
Emu	2	4	2	3	0	0	8
Ñandu	0	0	2	6	0	0	0
Ostrich	12	11	7	0	0	0	8

Figure 6. Distribution of avian species by region (FAO 2007).

Table 3. Summary of results obtained on local chicken breeds in Asia.

Reference	Number of microsatellite markers	Total Population	Asia breeds	Sample size per breed	Results*
Ponsuksili *et al.* 1999	15	12	4	8-26	H: 0.121 - 0.568
Wimmers *et al.* 2000	22	23	4	4 -20	H: 0.45 - 0.71
Hillel *et al*. 2003	22	52	3	50	H: 0.05 - 0.64
Gao *et al.* 2004	20	11	11	40	H: 0.68 - 0.74
Qu *et al*. 2004	28	4	4	31 - 32	H: 0.55 - 0.67
Qu *et al*. 2006	27	78	78	30-62	H: 0.505 - 0.678
Berthouly *et al.* 2008	22	20	6	50	H: 0.409 - 0.584
Granevitze *et al.* 2007	29	64	14	14 - 40	H: 0.05 - 0.64
Tadano *et al*. 2007	40	11	9	35 - 48	H: 0.273 - 0.523
Tadano *et al.* 2007	40	12	0	34 - 48	H: 0.295 - 0.664
Tadano *et al.* 2008	40	7	7	33 - 70	H: 0.342 - 0.505
Berthouly *et al.* 2009	18	15	7	50	H: 0.27 - 0.66
Granevitze *et al.* 2009	29	65	14	30	Genetic structure

*H means heterzygosity

Ponsuksili et al. (1999) used 15 microsatellite markers and DNA fingerprints to evaluate genetic variation of 12 populations, which contained two breeds from Taiwan (TWW & TWB), one from India (KAD) one from China (SIL) one from Indonesia (NUN) in addition to other populations from Europe or Africa (figure 7). Low bootstrap values were obtained, which did not support definitive conclusions.

Wimmers et al. (2000) applied 22 microsatellite markers to estimate the genetic diversity and distance for 23 populations, the results showed a clustering of populations according to their country of origin. Higher bootstrap values were obtained which supported the reliability of microsatellites analysis (Fig. 8).

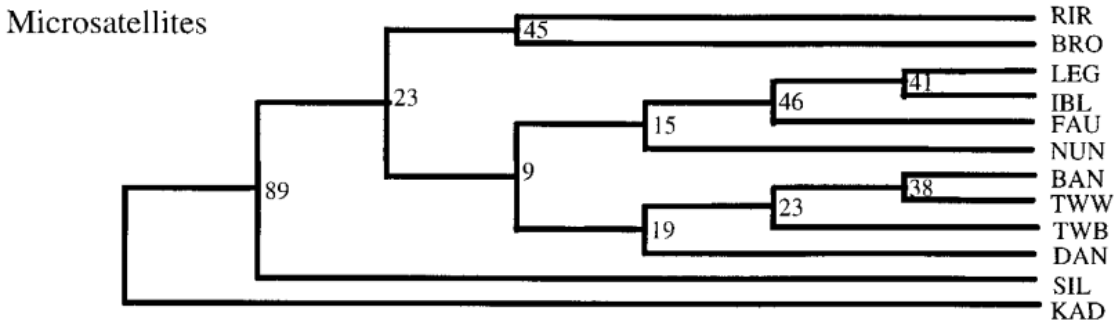

Figure 7. Genetic distance of twelve chicken populations (Ponsuksili *et al.* 1999).

Rhode Island Red layer line (RIR): Germany; Broiler male strain (BRO): Germany; White Leghorn layer line (LEG): Germany; White Leghorn inbred line ETH77 (IBL): Switzerland; Fayoumi (FAU): Egypt; Nunukan (**NUN**): Indonesia; Bankiva (BAN): Germany; Taiwan Brown-darkmeat broiler line (**TWW**): Taiwan; Taiwan White-darkmeat broiler line (**TWB**): Taiwan; Dandarawi (DAN): Egypt; Silky (SIL): China; Kadakanath (**KAD**): India.

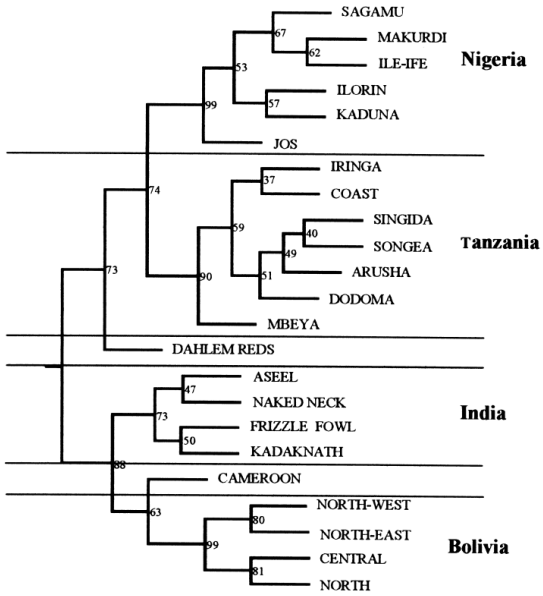

Figure 8. Genetic distance of local chicken populations from subtropical and tropical countries (Wimmers *et al*. 2000).

Gao *et al.* (2004) investigated the genetic diversity of 11 Chinese native chicken breeds, and showed Zang chicken has the highest heterozygosity (0.7432) and Langshang chicken has the lowest (0.68). Qu *et al.* surveyed four Chinese chicken populations (2004) and 78 Chinese chicken populations (2006) by microsatellite

markers, the result also showed Langshan (as Langshang) chicken has the lowest heterozygosity (0.505) and Shuanglian has the highest (0.678).

Berthouly *et al.* (2008) used 22 microsatellite markers to compare local European and Asiatic chicken breeds, including six conserved populations in National Chung Hsing University. The highest genetic diversity was found in the French breed Coucou de Rennes and the Taiwanese breed Hua-Tung. The mean expected heterozygosity of the Taiwanese breeds was .488. Hua-Tung exhibited the highest value and Nagoya the lowest. Heterozygosity was below the average for Ju-Chi and Shek-ki and above for Hsin-Yi and Quemoy. Percentage of individuals showing the highest probability of assignment to their true breed reached 100% for Hua-Tung, Quemoy and Shek-Ki, 95.7% for Hsin-Yi and laid between 90 and 95% for Juchi anbd Nagoya. Hua-tung contributed the most to aggregate diversity, due to a strong contribution to the within-breed component, followed by Nagoya who contributed mainly to the between-breeds component. Juchi contributed the least to aggregate diversity.

The neighbornet tree analysis showed a clear distinction between, on the one hand, Asiatic breeds and European breeds with an Asiatic origin, and, on the other hand, European breeds, particularly Mediterranean breeds, which did not undergo recent introgression from Asia (Figure 9).

A further study used 18 microsatellite markers to compare Vietnamese local chickens with Red Jungle Fowl, European and Taiwanese breeds (Berthouly *et al.*, 2009). The results showed the Vietnamese Ha Giang chicken population had a high genetic diversity and showed evidence of gene flow from wild jungle fowl to village chickens in this region.

More recently, a study of nine Vietnamese local chicken breeds sampled throughout the country, together with two Chinese chicken breeds, combined molecular data of 29 microsatellites, demographical data and a socio-economical survey to assess conservation potential of breeds. The highest potential was found for the Te, Dong Tao and Ac chicken breeds, whereas the lowest was observed in the Ri and Mia chicken breeds (Cuc et al., 2011).

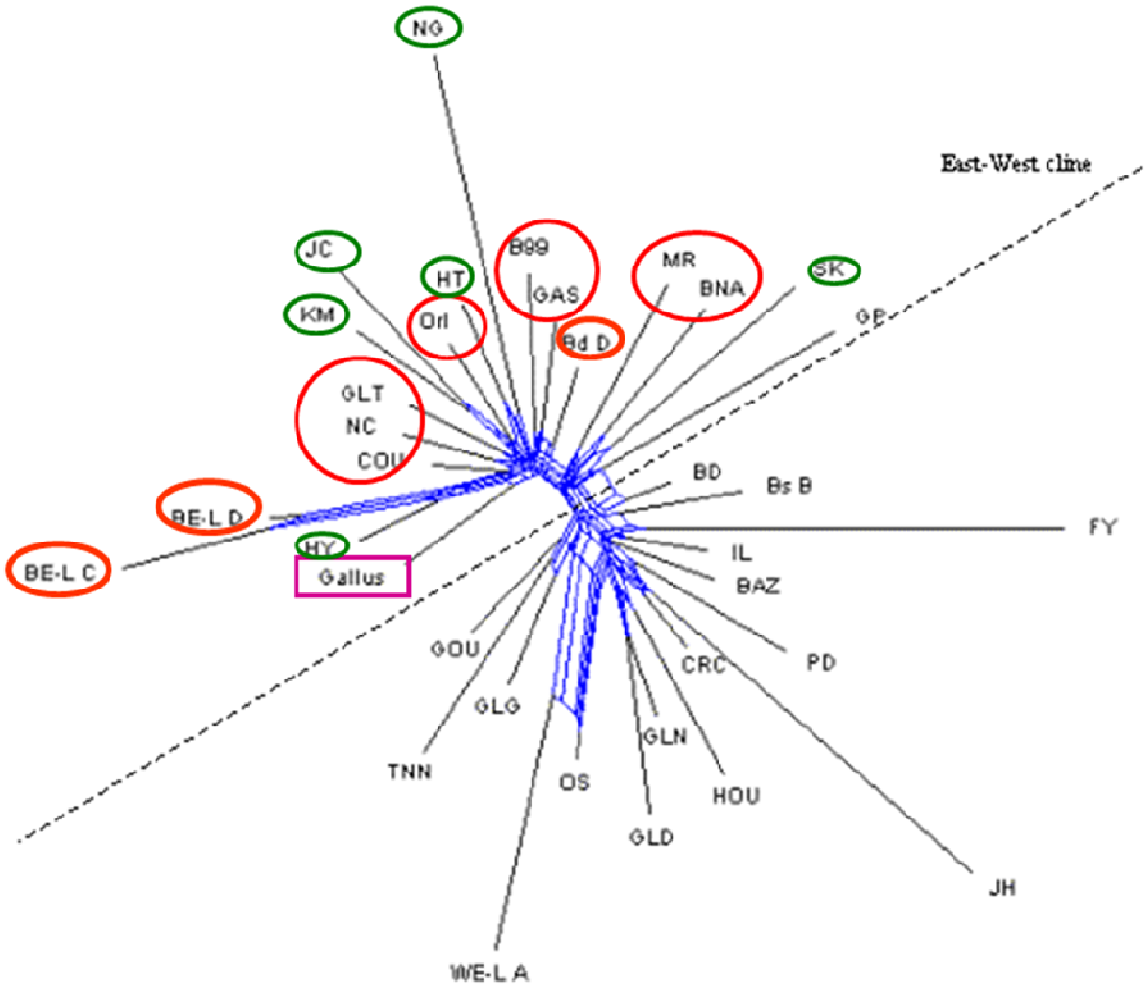

Figure 9. A neighbournet tree based on 14 microsatellite loci showing the Asiatic/European cline. (Berthouly *et al.* 2008)

Green circle: Asiatic breeds; Red : commercial lines and French breeds with an Asiatic Origin ; Purple rectangle: wild ancestor

Tadano *et al.* (2007a & 2007b) used 40 microsatellite markers for the analysis of the genetic relationships and genetic diversity of Japanese long-tailed and commercial lines. Tadano *et al.* (2008) showed that Japanese miniature breeds exhibited a strong genetic differentiation: level of heterozygosity was quite low (Table 3) and breeds were very much distant from each other (Fst from 0.336 to 0.483) and from the wild ancestor Red Jungle Fowl (Fst from 0.390 to 0.513).

MATERIALS AND METHODS

MATERIALS AND METHODS

I. Local chicken breeds in Taiwan

A. General history

Six local chicken breeds are maintained in a conservation program at NCHU since 1982 (Lee 2006; Fig. 10, Table 4 & 5). Three breeds are Taiwan local chickens, Hsin-Yi originates from an aboriginal tribe in the central mountain of Taiwan, Ju-Chi from a village in central-south of Taiwan and Hua-Tung from east of Taiwan. Quemoy comes from Quemoy Island near Fu-Jian Province of China, Shek-Ki is from the GuangDong province of China and Nagoya is from Japan.

Hsin-Yi is a medium size red feathered chicken with no special pattern, white skin, and blue shanks. Ju-Chi is a small size and Hua-Tung is a medium size, both breeds have black plumage without special pattern and black shanks. Quemoy is a small size black plumage with gold laced feather on the neck, white skin, and blue shanks. Shek-Ki has yellow skin, yellow feather and yellow shanks with medium size. Nagoya has yellow plumage with black tail, and blue shanks with medium size.

The history of local chicken breed in Taiwan can be traced back to the aborigines in the island who domesticated some jungle fowls. About 400 years ago, the Chinese immigrants came to Taiwan and brought in chickens from the southeastern China. And nearly 300 years ago, the Dutch and the Spanish had come to Taiwan for decades, and some European chickens might have been brought into Taiwan in this period. Later, Japanese ruled Taiwan from 1895 to 1945 and Rhode Island Red and White Leghorn were the earliest exotic breeds introduced to Taiwan in 1918. Also some Japanese breeds, such as Nagoya and Mikawa, and American breeds, such as Barred Plymouth Rock, were also introduced by the Japanese before 1925.

Most of chickens raised in Taiwan were the descendants of these chickens, before modern white broilers were introduced. These breeds have inhabited Taiwan for several generations and have become adapted to the environment. They have been selected by the local people, who recognized them as the native chickens of Taiwan (Lee 2006).

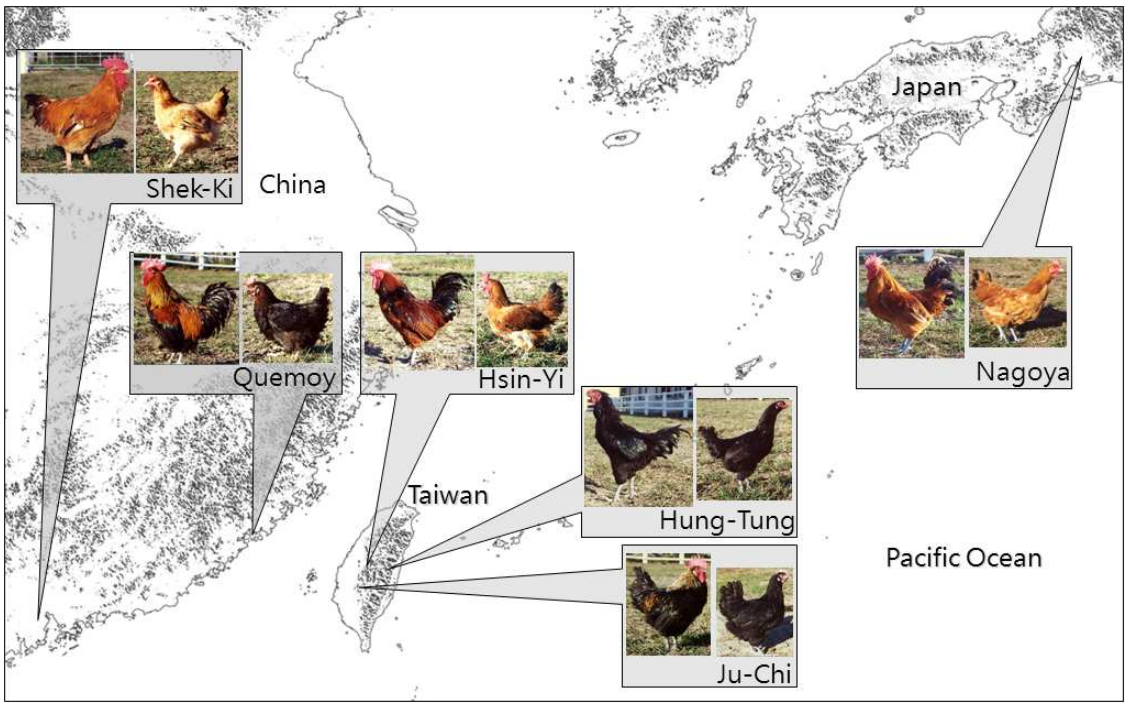

Figure 10. Six local chicken breeds are maintained in a conservation program at NCHU.

Table 4. Description of phenotype of local chicken breeds conserved in NCHU.

Breeds	Body size	Comb type	Color of			
			Skin	Shank and foot	Egg shell	Plumage and pattern
Hsin-Yi	Medium	Single	White	Blue	Pale brown	♂ Red feather with black tail ♀ Brown, red and yellow feather with some black pattern
Ju-Chi	Small	Single	White	Blue	Pale brown	♂ Black with some red and yellow feather at neck and back ♀ Black
Hua-Tung	Medium	Pea comb	White	Black	Pale brown	♂ Black feather ♀ Black feather
Quemoy	Small	Single	White	Blue	Pale brown	♂ Black with some red feather at neck and back ♀ Black feather with a few red hackles
Shek-Ki	Medium	Single	Yellow	Yellow	Pale brown	♂ Yellow feather with black tail ♀ Yellow feather with black tail
Nagoya	Medium	Single	White	Yellow	Pale brown	♂ Yellow feather with black tail ♀ Yellow feather with black tail

B. Breeds conservation program in NCHU

Conservation of chicken breeds was set up to study an array of traits, such as disease resistance or meat quality (Lee 2006). Recently, they were included in a survey of molecular diversity using a set of microsatellite markers which revealed different heterozygosity levels among them (Berthouly *et al.* 2008).

Table 5 shows the number of chickens for each breed that were used to set up the conservation programme in NCHU. Pedigree was recorded and random mating was used to produce chickens of each generation. The average generation interval varied from 1 to 2 years.

Table 5. Number of sires, dams, genetic size (N_e) and predicted increase in inbreeding per generation (ΔF).

Breeds	Sire	Dam	Ne	ΔF	Year when conservation started
Hsin-Yi	5	8	12.3	0.041	1982
Ju-Chi	1	6	3.4	0.146	1984
Hua-Tung	2	4	5.3	0.094	1990
Quemoy	1	4	3.2	0.156	1992
Nagoya	5	17	15.5	0.032	1987
Shek-Ki	N	N	N	N	1992

*Additional introductions of founders took place for Quemoy in later generations.

Table 6 shows the data on population size from 2001 to 2008, but unfortunately the data are missing in 2002 and 2003.

Table 6. Family structure, number of sires, dams and genetic size (N_e) from 2001 to 2008.

Breeds	Family structure[1]	Production years[2]					
		2001	2004	2005	2006	2007	2008
Hua-Tung	Sire	12	20	35	23	19	10
	Dam	25	45	41	43	28	38
	Ne	32	55	76	60	45	32
	Offspring	108	247	244	477	154	199
Hsin-Yi	Sire	14	18	26	25	11	19
	Dam	24	26	42	48	22	53
	Ne	35	43	64	66	29	56
	Offspring	114	102	253	384	97	298
Ju-Chi	Sire	15	18	26	22	24	19
	Dam	25	37	42	46	34	47
	Ne	38	48	64	60	56	54
	Offspring	117	251	219	471	189	286
Quemoy	Sire	17	17	26	14	19	18
	Dam	36	36	40	55	33	43
	Ne	46	46	63	45	48	51
	Offspring	116	258	242	454	155	248
Nagoya	Sire	16	16	25	22	22	19
	Dam	37	38	41	43	45	46
	Ne	45	45	62	58	59	54
	Offspring	120	164	220	324	234	233
Shek-Ki	Sire	17	14	16	19	16	14
	Dam	25	18	33	46	31	46
	Ne	40	32	43	54	42	43
	Offspring	119	85	173	311	115	171

[1]N_e mean [2]Data loss in 2002 and 2003

II. Population sampling for the thesis (years, numbers, family structure)

Table 7 shows the number of animals included in this thesis. In 2001, the sampling involved only half of the total population. The experimental protocols used for different sampling stages are listed in Table 7: samples from 2001 were used for population diversity (microsatellites, mtDNA, MC1R and LEI0258) and phenotypic data analysis (growth traits and immune responses),

Samples from generation 2007 and 2008 were used for investigating the distribution of LEI0258 locus under a non-selection situation. Samples in 2009 were used for H6N1 low-pathogenic avian influenza virus challenge experiment, and to observe the antibody response for challenge and subsequence vaccines.

Table 7. Number of animals sampled for the thesis.

Year	Hua-Tung	Ju-Chi	Quemoy	Shek-Ki	Nagoya	Hsin-Yi	Total	Use for	% of population sampled
2001	48	48	48	48	48	47	287	Microsatellites *MC1R* mtDNA LEI0258	~ 50%
2007	73	86	83	85	89	82	498	LEI0258	100%
2008	87	89	82	96	80	87	521	LEI0258	100%
2009	51	52	91	21	37	68	320	LEI0258 H6N1 LPAIV challenge Subsequence vaccines	NA[1]

[1]Specific sample for challenge experiment with planned matings for LEI0258.

III. Molecular markers

A. mtDNA

The hypervariable sequence 1 (HVS-I) of the D-loop region was amplified using the same primers (Table 8) following Liu *et al.* (2006) protocol. PCR target region is showed in Figure 11. PCR was done using 20 ng of genomic DNA, with 1 pmol of each primer, and units of HotStarTaq Master Mix Kit (Qiagen) in a final volume of 25 ul. Initial denaturation for 15 min at 95°C was followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 1 min, followed by a 10 min extension at 72°C. PCR products were sequenced by Eurofins MWG Operon, using their standard protocol for purified PCR products. The Bioedit version 7.0.9.0 (Thomas, 1999) was used to assemble sequences and identify polymorphisms. The median-joining networks (Bandelt 1999) were constructed using the program Network 4.516 (http://www.fluxus-engineering.com/sharenet_rn.htm).

Table 8. Description of the primers used in this study; PCR regions and amplification lengths, names and primer sequences.

PCR region	Length (bp)	Primer name	Primer sequence
mtDNA	600 bp	L16750	5'-AGGACTACGGCTTGAAAAGC-3'
		H547	5'-ATGTGCCTGACCGAGGAACCAG-3'
MC1R	750 bp	Mc1Co-up	5'-GAGGGCAACCAGAGCAATGC-3'
		397281-dwn	5'-TGAAGAAGCAGGTGCAGAAG-3'
MHC	200-500 bp	LEI0258-F	5'-CACGCAGCAGAACTTGGTAAGG-3'
		LEI0258-R	5'-AGCTGTGCTCAGTCCTCAGTGC-3'

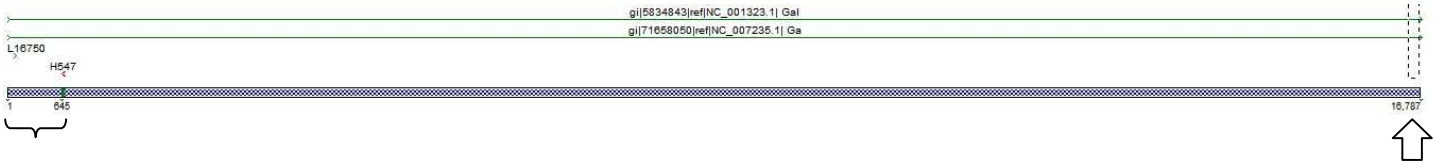

Figure 11. PCR target region of chicken mtDNA.

Figure 12. PCR target region of chicken *MC1R*.

B. Microsatellite markers

A set of 24 microsatellite markers derived from the marker set of the AvianDiv European research project was genotyped on the LABOGENA facilities at Jouy-en-Josas. The list of 24 analyzed markers is listed in Table 9.

Table 9. General characteristics of the 24 microsatellite markers for the six breeds.

Locus	Chromosome	n[1]	No. alleles	Allele size range (bp)
ADL112	10	281	3	121-127
ADL268	1	281	5	101-113
ADL278	8	283	6	110-122
LEI094	4	278	10	246-281
LEI166	3	275	6	251-261
LEI192	6	257	15	253-424
LEI228	2	278	14	163-443
LEI234	2	281	14	212-354
MCW014	6	280	5	162-183
MCW034	2	283	8	213-248
MCW037	3	280	5	150-155
MCW067	10	280	4	174-180
MCW069	26	281	7	154-174
MCW078	5	280	4	134-142
MCW081	5	276	4	109-131
MCW098	4	280	2	255-257
MCW111	1	278	5	97-111
MCW183	7	275	8	292-322
MCW206	2	278	6	220-239
MCW216	13	280	7	134-149
MCW222	3	281	4	216-222
MCW248	1	276	4	213-221
MCW295	4	271	6	85-99
MCW330	17	273	4	254-286
All loci		156		

[1] Number of successfully typed birds.

C. *MC1R*

Plumage, skin and eye color variations of the domestic fowl are used as selection criteria since the first mutant phenotypes appeared in early domesticated stocks (Smyth 1990). Plumage colour selection is not only for fancy breeding, but also as a standard for commercial poultry industry like layer production system (red plumage for brown-egg lines) or to satisfy the preferences requirement in different geographic markets (Groen 2003). The character of the genetics of cutaneous and ocular pigmentation which combined phenotypes and utility became particularly interesting for research.

Several loci control plumage or coat color in birds and mammals, such as *Extended black* (*E*), *Dominant white* (*I*), *Recessive white* (*c*) loci (Table 10; Smyth 1990, Kerje *et al.* 2003, Kerje *et al.* 2004 & Chang *et al.* 2006). *E* locus affects the distribution of black (eumelanin) and red (phaeomelanin) pigment, and Takeuchi *et al.* (1996) indicated a strong correlation between *MC1R* polymorphism and *E* locus. Further molecular studies confirmed that the gene product of the *Extended black* locus controlling plumage color in chicken was *MC1R*, which is located on chicken chromosome 11. Mutations in the *MC1R* sequence caused amino acid substitutions which were correlated with plumage color variation (Kerje *et al.* 2003 & Ling *et al.* 2003; Table 10).

PCR target region is showed in Figure 12, PCR primers for PCR are listed in Table 8. PCR was done using 20 ng of genomic DNA, with 1 pmol of each primer, and units of HotStarTaq Master Mix Kit (Qiagen) in a final volume of 25 ul. Initial denaturation for 15 min at 95°C was followed by 2 cycles of 94°C for 45 s, 66°C for 30 s, 72°C for 1 min, and 2 cycles of 94°C for 45 s, 63°C for 30 s, 72°C for 1 min, and 2 cycles of 94°C for 45 s, 60°C for 30 s, 72°C for 1 min, and 26 cycles of 94°C for 45 s, 57°C for 30 s, 72°C for 1 min, followed by a 15 min extension at 72°C. PCR products were sequenced by Eurofins MWG Operon, using their standard protocol for purified PCR products. SNPs analysis using the Staden package (Staden 1998) and defined genotype and haplotype. Phylogam tree was used Populations 1.2.30 (Langella 1999) to compute population distances and phylogenetic tree.

Table 10. Association of amino acid substitutions in *MC1R* with plumage color.

Phenotype class	Haplotypes	Breeds	Codon no.							References
			71	**92**	126	143	212	213	**215**	
Wild type	e^+	Red junglefowl	Met	Glu	Val	Thr	Ala	Cys	His	Kerje *et al.* 2003
	e^+	Brown Leghorn	Met	Glu	Val	Ala	Ala	Arg	His	Takeuchi *et al.* 1996
	e^+	Richardson's RJF	Met	Glu	-	Thr	Ala	Arg	His	Ling *et al.* 2003
	e^+	B & D RJF	Met	Glu	-	Thr	Ala	Cys	His	Ling *et al.* 2003
Extended black	E	White Leghorn	Met	Lys	Ile	Thr	Ala	Arg	His	Kerje *et al.* 2003
	E	Rock Cornish	Thr	Lys	Val	Thr	Ala	Cys	His	Takeuchi *et al.* 1996
	E	Barred Plymouth Rock	Thr	Lys	Val	Thr	Ala	Cys	His	Takeuchi *et al.* 1996
	E	Black Australorp	Thr	Lys	-	Thr	Ala	Cys	His	Ling *et al.* 2003
Black and red	E^R	ADOL line 0	Met	Lys	-	Thr	Ala	Arg	His	Ling *et al.* 2003
	E^R	Fayoumi	Met	Glu	-	Thr	Ala	Arg	His	Ling *et al.* 2003
Brown	e^{bc}	Buttercup	Thr	Lys	Val	Thr	Ala	Cys	Pro	Kerje *et al.* 2003
	e^b	Smyth Brown line	Thr	Lys	-	Thr	Ala	Cys	Pro	Ling *et al.* 2003
Recessive wheaten	e^y	Nagoya Cortin	Met	Glu	Val	Ala	Ala	Arg	His	Takeuchi *et al.* 1996
Dominant wheaten / recessive wheaten	e^{wh}/e^y	NHR, RIR, Buff Min	Met	Glu	-	Ala	Ala	Arg	His	Ling *et al.* 2003

D. LEI0258

LEI0258 marker is an atypical Variable Number of Tandem Repeat (VNTR) located within the chicken MHC region. Chicken MHC region located on chromosome 16, LEI0258 marker is a minisatellite which exhibits a good correlation with serological typing of the chicken MHC (Fulton *et al.* 2006). The structure of LEI0258 locus contain a 12 bp repeat, a 13bp repeat and other polymorphisms, and can be a criteria to determinant different types of alleles (Fig. 13 & Fig. 14). Fulton *et al.* (2006) identified LEI0258 alleles from chicken stocks (Table 8), and proposed to use marker LEI0258 as a standard procedure to identify MHC alleles in any population.

Primers for PCR are the same as those used by McConnell *et al* 1999 in Table 9. PCR was done using 20 ng of genomic DNA, with 1 pmol of each primer, and units of HotStarTaq Master Mix Kit (Qiagen) in a final volume of 25 ul. The amplification protocol included a first cycle of initial denaturation for 15 min at 95°C, followed by 35 cycles of 94°C for 45 s, 62°C for 90 s, 72°C for 60 s, with a last cycle of extension for 15 min at 72°C. Gel migration was realized with a 4% agarose gel for a first determination of allele size. Due to the complex structure of the LEI0258 sequence which contained a 13-bp and 12-bp repeat sequences and several SNP sites, sequencing is necessary to provide an unambiguous identification of alleles. Direct sequencing of PCR products was performed for homozygous genotypes (following Fulton *et al.* 2006) or after 4% agarose gel cutting and purification with QIAquick PCR Purification Kit (Qiagen) for heterozygous genotypes. Sequencing was done by Eurofins MWG Operon, using their standard protocol for purified PCR products. The Bioedit version 7.0.9.0 (Thomas 1999) was used to assemble sequences and to check the length (varying according to the number of repeats) and polymorphic sites (either single nucleotide polymorphisms or deletions) for each allele. Phylogam tree was used Populations 1.2.30 (Langella 1999) to compute population distances and phylogenetic tree.

Due to previously results showed Taiwan local chicken breeds have better disease resistance (Cheng *et al*. 1987, Fan *et al*. 1988, Cheng *et al.* 1990, Chen *et al.* 1991 and Chao & Lee 1991), and it is lack for study about investigate the relationship between Taiwanese local chickens' serotypes and immune response or more thorough experiments to carry out. Therefore, the aim of the thesis is

also to search for private LEI0258 alleles in local breeds and test their functional effect by vaccine or pathogen challenge experiment.

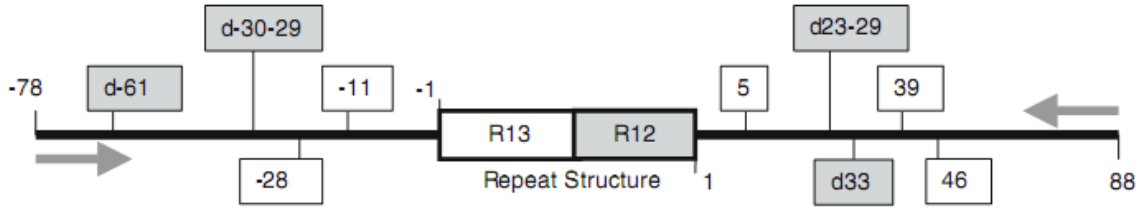

Figure 13. Schematic of the repeat structure and location of SNP and deletions within the LEI0258 Locus (Fulton *et al.* 2006).

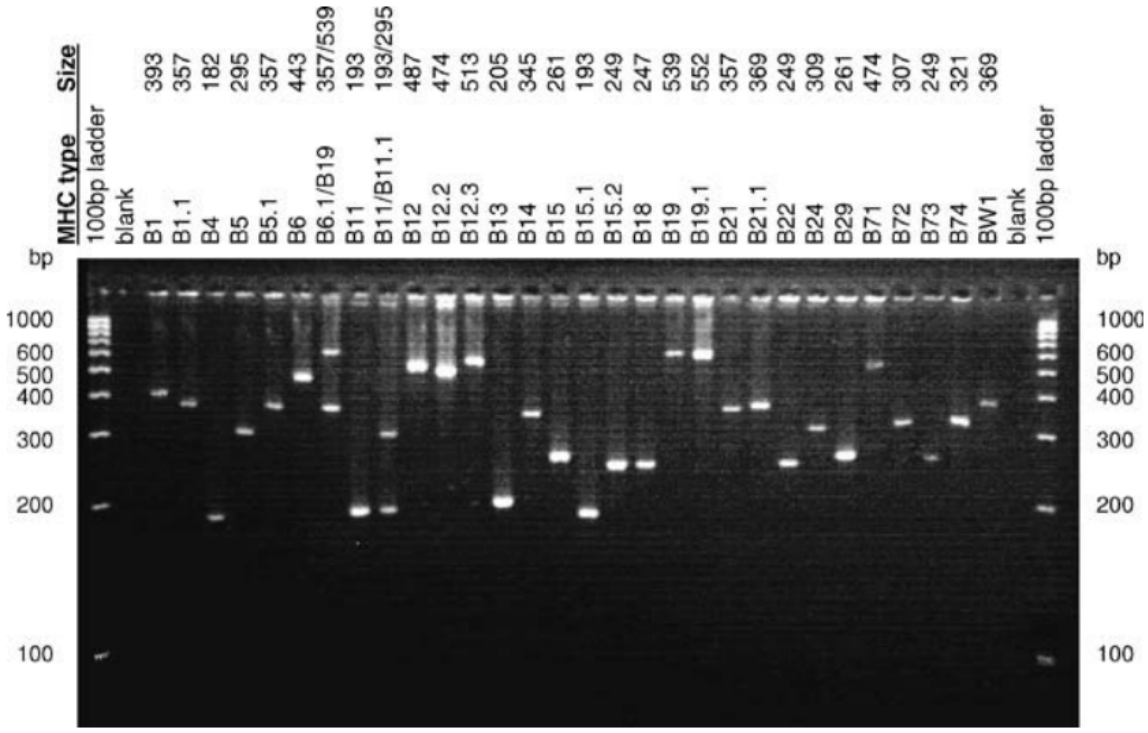

Figure 14. LEI0258 marker PCR product separation and sequence size for different chicken MHC haplotypes (Fulton *et al.* 2006).

IV. Performance traits

Growth

Body weight (BW) was measured at various frequencies depending on the experiment. For the global analysis of diversity, three measures were retained: initial body weight at hatch (BW0), body weight at the time of rapid growth (BW12) and mature body weight (BW47). For the challenge experiment, body weight was measured every week from hatch to 16 weeks of age.

Heat adaptation

Panting is generally observed when ambient temperature is above 29°C with 50% humidity (Weaver 2002). For the global analysis of diversity, panting was recorded individually on a daily basis between 24 and 27 weeks of age, in the afternoon of the hot season when the ambient temperature was higher than 30°C (highest for 36°C, lowest for 31°C and average for 33.5°C with 61% humidity). Individual panting rate was calculated as the ratio of number of days where a given chicken exhibited panting on the total number of observed days

Immune traits

Immune response was investigated for the global analysis of diversity by using two different antigens. Sheep Red Blood Cells (SRBC) are frequently used as a non-pathogenic antigen for evaluating antibody response in selection experiments, and antibody response to NDV vaccination can be used as a selection criterion for immune response (Lamont *et al.* 2003). Newcastle disease is a major poultry disease in Taiwan (Yang *et al*. 1999) and vaccination against Newcastle disease virus (NDV) is a standard procedure recommended by government sanitary authorities. All day-old chicks were vaccinated against NDV in this study. At 8 weeks of age, all chickens were vaccinated again with 0.5 mL NDV vaccine, and serum was collected 7 and 14 days later. Haemagglutination inhibition tests were used to determine antibody titers against NDV. At 11 and 13 weeks of age, chickens were injected with 0.1 mL 0.25% SRBC intravenously and serum was collected 7 days later. Antibody titers of chickens against SRBC were determined by haemagglutination tests. The antibody titer was expressed as the $\log_2$ of reciprocal of the highest dilution.

Kinetics of antibody titers was studied by calculating NDD, as the difference between anti-ND antibody titer at day 14 and anti-ND antibody titer at day 7 post-inoculation, and SRBCD, as the difference between anti-SRBC antibody titer at secondary response and anti-SRBC antibody titer at primary response

For the challenge experiment, immune response to Avian influenza and secondary immune response was measured for Infectious Bronchitis (IB), Infectious Bursal Disease (IBD) and Newcastle disease.

Anti-AI titer: Chickens received a drop with 10^7 EID_{50} of H6N1 avian influenza viruses (A/chicken/Taiwan/0825/2006, provided by Dr. Poa-Chun Chang, Graduate Institute of Microbiology and Public Health, National Chung-Hsing University, Taiwan, R.O.C.) into eye and nose. Blood samples were collected at 0, 7, 14 and 21 days post-challenge. Anti-AI titers were measured by enzyme-linked immunosorbent assay (ELISA) with commercial test kit (IDEXX Laboratories, Inc., Westbrook, ME; http://www.idexx.com/view/xhtml/en_us/livestock-poultry/poultry/avian-influenza.jsf).

Anti-IB titer: Chickens were vaccinated again with 0.5 mL Infectious Bronchitis (IB) vaccine, and serum was collected 0, 14 and 28 days later. Anti-IB titers were measured by enzyme-linked immune-sorbent assay (ELISA) with commercial test kit (IDEXX Laboratories, Inc., Westbrook, ME).

Anti-IBD titer: Chickens were vaccinated again with 0.5 mL Infectious Bursal Disease (IBD) vaccine, and serum was collected 0, 14 and 28 days later. Anti-IBD titers were measured by enzyme-linked immunosorbent assay (ELISA) with commercial test kit (IDEXX Laboratories, Inc., Westbrook, ME).

The antibody responses to ND were measured by hemagglutination inhibition test (HIT), the antibody titer was expressed as the log2 of the reciprocal of the highest dilution.

V. Methods

A. *Sequencing and sequence analysis*

PCR products of mtDNA, MC1R and LEI0258, were sequenced and sequence alignment, editing and processing were performed with software BioEdit 7.0.9.0 (Hall 1999) and Staden package (Staden *et al.* 1998).

Mitochondrial DNA and LEI0258 sequence alignment and polymorphisms identification were done by using BioEdit. Haplotypes were scored manually. The median-joining networks (Bandelt *et al.* 1999) were constructed for mitochondrial DNA data using the program Network 4.516 (http://www.fluxus-engineering.com/sharenet_rn.htm).

The length variants (number of repeats) and polymorphic sites (either single nucleotide polymorphisms or deletions) of LEI0258 have been checked for each allele. MC1R SNP analysis was done with Staden package (Staden *et al.* 1998) to define genotypes and haplotypes.

B. *Population genetics*

Allele frequencies, number of alleles, expected and observed heterozygosity were calculated by using GENETIX 4.04 (Belkhir *et al.* 2004). Genetic distances (Nei 1983) were calculated and neighbor-joining (NJ) trees were established with Populations 1.2.30 (Langella 1999).

All marker data, including microsatellites, *MC1R* and LEI0258, were merged and treated with a Multiple CO-inertia Analysis (MCOA) by using R (R Core Development Team 2006) with ADE-4 package (Chessel *et al.* 2004; Dray & Dufour 2007). This method makes possible the extraction of common information from separate analyses, by setting up a reference typology, and comparing each typology separately. The efficiency of a marker is assessed by its typological value (Tv), the contribution of the marker to the construction of the reference typology, which is equal to the product of the variance (Var) multiply by the congruence with the consensus Cos^2 (i.e. the correlation between the scores of individual locus tables and the synthetic variable of the same rank) (Laloë *et al.* 2007).

C. Analysis of performance data

Body weight, antibody titers and panting rate were analyzed by the general linear model (GLM) procedure of SAS software (SAS Institute 2008). Residual correlations between traits were calculated using the MANOVA option of the GLM procedure, considering the breed as a fixed effect in the model.

Principal component analysis (PCA) was performed with ADE-4 package from R, in order to identify the best combination of variables which explained most of the variance between breeds.

D. Combining molecular and phenotypic data

The Hill and Smith method (1976) was used to combine discrete and continuous variables to compare populations. This method is a combination of an internal correspondence analysis for discrete data, i.e. the molecular marker data (Laloë *et al.* 2002) and a principal component analysis for continuous variables, i.e. performance traits. It was implemented with functions of the ADE-4 package from R software.

E. Bayesian approach

The STRUCTURE program (Pritchard *et al.* 2001) was used to determine the number K of distinct genetic groups in the total sample of local chicken breeds on the basis of microsatellite genotypes.

F. Experimental challenge for H6N1 low pathogenic avian influenza virus

In generation 2009, chickens were produced by LEI0258 genotypes, H6N1 LPAIV challenge were performed and measured antibody titers.

G. Experimental chickens

A total of 314 chicks were hatched from 23 sires and 91 dams with full pedigree in six local breeds. Twenty-five SPF chicks were purchased from Animal Health Research Institute (Council of Agriculture, Executive Yuan R.O.C.). Day-old chicks were wing-banded and raised in floor pens until 5 weeks of age, and they were transferred to experimental cages after 6 weeks of age. Sire families were distributed between the control and the challenge group. Individual body weights were recorded weekly from hatch to 16 weeks of age.

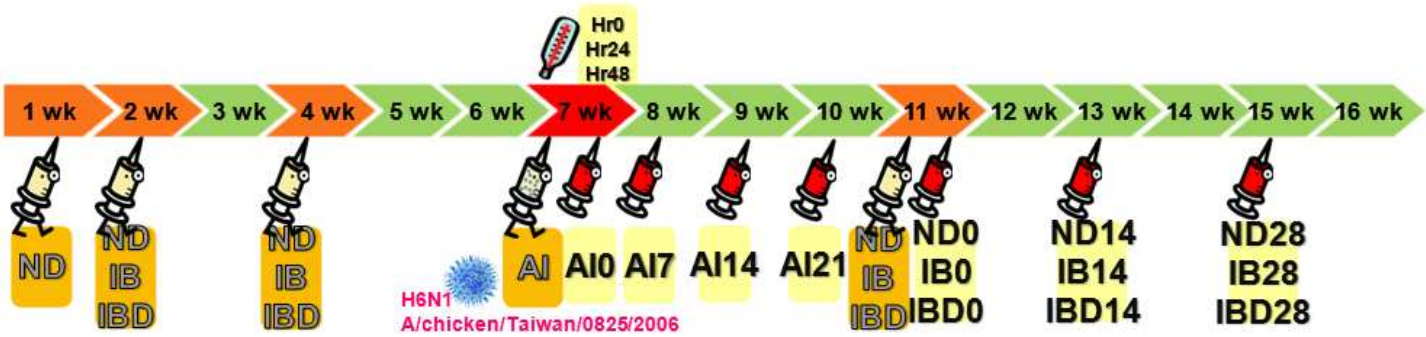

Figure 15. Experiment scheme for population in 2009.

H. Viruses

The virus strain A/chicken/Taiwan/0825/2006 (H6N1) LPAIV was isolated from a commercial broiler flock showing increased flock mortality in central Taiwan in 2006. The method used for isolation and propagation of the virus on chicken embryos was described by Lee et al. (2006). A sample of H6N1 avian influenza virus (A/chicken/Taiwan/0825/2006) was provided by Dr. Poa-Chun Chang, Graduate Institute of Microbiology and Public Health, National Chung-Hsing University, Taiwan, R.O.C.). The live virus was used for the challenge at 7 weeks of age (Fig. 15). Chickens received a drop with 10^7 EID_{50} (50% Embryo infectious Dose) into eye and nose.

I. MHC genotyping

MHC genotyping was achieved by using LEI0258 marker.

J. Mating for specific MHC genotypes of experimental chickens and management

Within each breed, sires and dams were mated according to their MHC genotype, in order to obtain the segregation of two to three alleles within each sire family (Fig. 16). A total of 23 sires and 91 dams were used to produce offspring. A batch of 24 SPF chicks was hatched at the same time. Day-old chicks were wing-banded and raised in floor pens until 5 weeks of age, and were transferred to experimental cages at 6 weeks of age. Individual body weight was recorded weekly from hatch to 16 weeks of age. Body weight gains (BWG) were calculated by periods of three successive weeks. Day-old chicks were all vaccinated against Marek's disease and ND. At two weeks of age, chicks were vaccinated against ND, IB, IBD, Fowl Pox and Avian Reovirus infection. At four weeks of age, chickens were vaccinated against ND, IB, IBD and Infectious Laryngotracheitis.

Figure 16. Example for mating for specific MHC genotypes.

*309 and 381 means LEI0258 allele size as the MHC genotype.

K. Challenge experiment and antibody responses measurement

At 6 weeks of age, sire families were distributed between the control and the challenge group, at 7 weeks of age birds from the challenge group received a drop with 10^7 EID_{50} of viruses in 20 µl via drop into eye and nose respectively. Clinical signs and mortality were recorded, rectal temperature was measured by mercury thermometer and recorded at hour 0, 24 and 48 post-challenge to monitor the body temperature (BT). Animals were in a fed state when body

temperature was recorded. Serum was collected from individual blood samples at day 0, 7, 14 and 21 post-challenge.

Individual ELISA titers of AI antibodies for each breed were measured by Avian Influenza Virus Antibody Test Kit and calculated by FlockChek software (IDEXX Lab. Inc., Maine, USA). Kinetics of antibody titers was studied by calculating the difference between different stages of post-challenge.

L. Statistical analysis

Data were analyzed by the general linear model (GLM) procedure of SAS software (SAS Institute 2008). Body weight gains, body temperature, antibody titers and differences between successive titers were analysed with the following statistical model,

$$Y_{ijkl} = \mu + \tau_i + \alpha_j + s_k + (\tau\alpha)_{ij} + e_{ijkl}$$

where Y_{ijkl} is the antibody titer of the lth animal of the ith breed, the kth sex after the jth challenge treatment, i=1, 2,…,7, j=1,2, k=1,2, l=1,2,…,338, μ is the mean, τ_i is the fixed effect of the ith breed, $\Sigma\tau_i$=0, α_j is the fixed effect of the jth challenge treatment, $\Sigma\alpha_j$=0, $(\tau\alpha)_{ij}$ is the fixed interaction effect between the ith breed and the jth challenge treatment , $\Sigma\Sigma(\tau\alpha)_{ij}$=0, and e_{ijkl} is the residual random error, $e_{ijkl} \cap N(0, \sigma_e^2)$.

All statistical analyses were conducted by using SAS software (SAS Institute, 2003).

RESULTS

RESULTS

I. Polymorphisms of LEI0258

A. Inventory of MHC alleles in Taiwan local chickens

These results were first presented at the World Poultry Conference in Brisbane 2008, (annexe 1)

A total of 17 alleles was identified according to the size of the PCR fragment among the 6 breeds (Table 11 & 12). Alleles differed mainly by the number of the R12 repeat, except for allele 443 which showed a high number of R13 repeat (table 12). A few deletions or SNPs were also observed in some cases: 2 alleles exhibited a deletion upstream of the repeats and 9 alleles exhibited a deletion downstream. The SNP A→T at position 39 downstream of the repeats was very frequent. A downstream sequence polymorphism made possible to distinguish two alleles sharing the same fragment size: 357 in Hua-Tung and 357 in Hsin-Yi, so that 18 different alleles were finally identified (Table 12). Six new LEI0258 alleles which did not match with a known B haplotype were found in the 2001 sample (Table 11). A seventh new allele was identified in Hua-Tung in 2007 (Table 14).

Table 11 shows the heterozygosity, allele frequencies and distribution for each breed in 2001. Hua-Tung had the highest number of alleles and genotypes and showed the highest heterozygosity on LEI0258 marker.

Table 11. Allele frequency and mean heterozygosity for LEI0258 marker in the six chicken breeds.

Breeds	Animal No.	Allele No.	Allele Combination	H_E	H_o	Percentage of different alleles (%) 181*	182	193	205	217*	241*	249	273*	295	309	319*	345	357	381	419*	443
Hsin-Yi	47	4	6	0.61	0.51			11		2					44						44
Ju-Chi	48	4	5	0.38	0.40				17			5			77				1		
Hua-Tung	48	7	18	0.84	0.83			6				19		22	7	11		16		19	
Quemoy	48	4	6	0.61	0.65				20			1			54				25		
Shek-Ki	48	4	8	0.73	0.71		21				14		34				31				
Nagoya	48	3	5	0.53	0.58	40						56			4						

* means new alleles

Table 12. Polymorphisms identified of LEI0258 alleles.

	Size (bp)	upstream			R13	R12	downstream					Known B haplotypes
Position		-30-29	-28	-11			5	23-29	33	39	46	
character nb		1	2	3			4	5	6	7	8	
Breeds		TT	G	G			C	ATTTTGAG	Δ	A	T	
Nagoya	181*	-	-	-	1	2	-	Δ	-	-	-	NA
Shek-Ki	182	-	-	A	1	2	-	Δ	A	-	-	4
Hua-Tung, Ju-Chi, Hsin-Yi	193	-	-	-	1	3	T	Δ	-	-	-	11, 15.1, 27, 61
Ju-Chi, Quemoy	205	-	-	-	1	4	T	Δ	-	-	-	13
Hsin-Yi	217*	-	-	-	1	5	-	Δ	-	-	-	NA
Hua-Tung	237*	-	-	-	1	6				T		NA
Shek-Ki	241*	-	-	-	1	7	-	Δ	-	-	-	NA
Nagoya	249	-	-	-	1	7				T		15.2, 22, 73
Shek-Ki	273*	-	-	-	1	9	-	-	-	-	A	NA
Hua-Tung	295	Δ	-	-	1	11	-	-	-	-	-	5
Hua-Tung, Ju-Chi, Nagoya Hsin-Yi, Quemoy	309	-	-	-	1	12	-	-	-	T	-	10, 24, 26, 76
Hua-Tung	319*	Δ	A	-	1	13	-	-	-	-	-	NA
Shek-Ki	345	-	-	-	1	15	-	-	-	T	-	14
Hua-Tung	357	-	-	-	1	16	-	-	-	-	A	23
Hsin-Yi	357	-	-	-	1	16	-	-	-	T	-	5.1, 6.1, 21, 75, 130,131, 201
Ju-Chi, Quemoy	381	-	-	-	1	18	-	-	-	T	-	13.1
Hua-Tung	419*	-	-	-	15	6	-	-	-	-	-	NA
Hsin-Yi	443	-	-	-	15	8	-	-	-	-	-	6
Gallus	369	-	-	-	1	17	-	-	-	T	-	
B19.1	552	-	-	-	28	3	-	ATTTGAGG	-	-	-	

* new alleles

B. Trends in frequencies (Tours 2010, annex 1)

1. Introduction

Following the investigation of LEI0258 allelic distribution in a sample of 50 animals per breed in year 2001, we continued to genotype LEI0258 alleles for all animals of generations born in 2007 and 2008, and combined data from three generations to analyze the possible trends in allelic frequency at the MHC locus across generations for each breed. Heterozygosity was calculated with GENETIX 4.05 (Belkhir *et al*. 2000). Hardy-Weinberg (H-W) equilibrium test was calculated with Genepop 4.0 (Raymond & Rousset. 1995).

Results were presented as an oral communication at the European Poultry Conference in Tours, 23-27 August; 2010 (annex 2)

Numbers of animals for each generation are listed in Table 13.

Table 13. Number of sires, dams, and genetic size (Ne) for each breed in each generation

Breeds	2001			2007			2008		
	sires	dams	Ne	sires	dams	Ne	sires	dams	Ne
Hsin-Yi	14	24	35	11	22	29	19	53	56
Ju-Chi	15	25	38	24	34	56	19	47	54
Hua-Tung	12	25	32	19	28	45	10	38	32
Quemoy	17	36	46	19	33	48	18	43	51
Shek-Ki	17	16	14	16	31	42	14	46	43
Nagoya	16	37	45	22	45	59	19	46	54

2. Results and discussion

A total of 17 alleles were identified for marker LEI0258, seven of them did not match with a known B haplotype. Hua-Tung exhibited three new alleles, Shek-Ki and Hsin-Yi exhibited two new alleles (with one in common) and Nagoya exhibited one new allele.

Allele frequency fluctuated slightly between generations in most breeds (Table 14). Hsin-Yi, Ju-Chi, Hua-Tung exhibited some rare alleles (frequency below 10%) whereas Quemoy, Shek-Ki and Nagoya exhibited a rather balanced

distribution of alleles. Hsin-Yi showed a marked increase in frequency of allele 193 and a marked decrease in frequency of allele 443.

Table 14. LEI0258 allele size and allele frequency according to breed and to generations

Breeds	Allele size	Allele frequency (%) 2001	2007	2008	Breeds	Allele size	Allele frequency (%) 2001	2007	2008
Hsin-Yi	193	11	38	54	Quemoy	205	20	13	13
	217[a]	2	18	9		249	1	12	20
	309	44	27	29		309	54	43	47
	357	-	1	2		381	25	31	20
	443	44	15	6	Nagoya	181[a]	40	33	38
Ju-Chi	193	-	5	7		249	56	43	42
	205	17	27	28		309	4	25	21
	249	5	1	1	Shek-Ki	182	21	26	34
	309	77	67	63		241[a]	14	24	26
	381	1	-	-		273[a]	34	25	19
Hua-Tung	193	6	14	7		345	31	25	21
	237[a]	-	16	21					
	249	19	14	20					
	295	22	14	11					
	309	7	3	1					
	319[a]	11	5	13					
	357	16	13	13					
	419[a]	19	21	14					

[a] new allele of LEI0258 marker

Figure 17 is a 3-dimension graphical representation of the frequency data given in table 14 and also shows the corresponding B haplotypes for LEI0258 alleles.

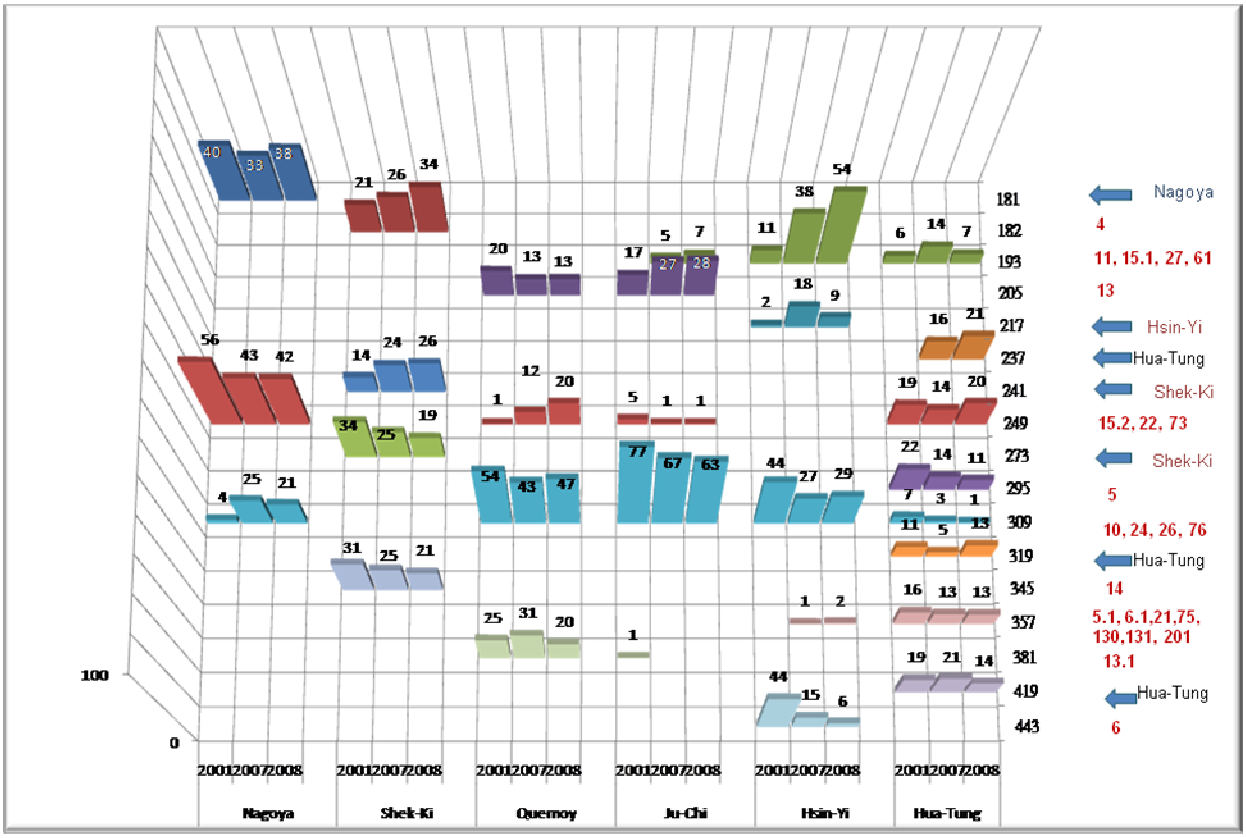

Figure 17. LEI0258 allele frequencies across three generations.

Inbreeding coefficient (Fis) was highest in Hsin-Yi, Quemoy and Hua-Tung in 2008, whereas Ju-Chi, Shek-Ki and Nagoya exhibited lower values (Table 15), consistent with the status of random mating. Results of H-W equilibrium test pointed out that Ju-Chi, Shek-Ki and Nagoya were under Hardy-Weinberg equilibrium at the MHC locus. However, Hua-Tung, Quemoy and Hsin-Yi were not in equilibrium for generation 2008 and Hsin-Yi was not in equilibrium also for generation 2007. The departure from equilibrium was due to an excess of homozygotes, which is quite surprising for MHC. This could be due to genetic drift with small family size and fluctuating number of sire families between generations. Hua-Tung breed had the largest number of alleles and genotype frequencies may be quite susceptible to sampling variation.

Table 15. Heterozygosity and test for Hardy-Weinberg equilibrium.

Breeds	Year	$H_{n.b.}$	$H_{obs.}$	F_{is}	P-value of H-W test	Significant
Hsin-Yi	2007	0.73	0.68	0.0649	0.0000	***
	2008	0.61	0.45	0.2699	0.0002	***
Quemoy	2008	0.69	0.56	0.1836	0.0000	***
Hua-Tung	2008	0.85	0.71	0.1631	0.0000	***

Comparing allele frequencies across the three generations showed that allele 237 was not found in the sample genotyped in 2001 for Hua-Tung, but was present in 2007 and 2008 at a moderate frequency, which could suggest recent selection. Allele 249 (matching MHC B15.2) exhibited a similar trend in Quemoy breed. Regarding Hsin-Yi breed, the important changes in allelic frequencies observed for alleles 193 and 443 could indicate short term selection pressures. These alleles can be matched to MHC B15.1 and B6, respectively. Allele 193 was also found in Ju-Chi and Hua-tung but did not exhibit any specific trend in these breeds. Allele 443 was only found in Hsin-Yi, and could have been counter-selected. In general, observed heterozygosity of LEI0258 was rather high in all breeds except Hsin-Yi, which showed a quite low value in 2008 (Table 15). Effects of MHC alleles on performance or viability deserve further studies in the Hsin-Yi breed. These changes result in a very unbalanced distribution of alleles in this breed, with one very frequent allele, 193 and one moderately frequent allele, 309, all the other alleles being rare (less than 10%). The deviation from Hardy-Weinberg equilibrium in this breed remains puzzling and could indicate the occurrence of an undescribed selection process.

Deviation from Hardy-Weinberg was not observed for microsatellite data (Berthouly et al., 2008) and appeared to be specific to LEI0258. As compared to microsatellite data, LEI0258 exhibited a higher observed heterozygosity for Hua-Tung (0.84 versus 0.58), Quemoy (0.65 versus 0.49) Shek-Ki (0.71 versus 0.42) and Nagoya (0.58 versus 0.44) breeds. However, observed heterozygosity was lower for LEI0258 in Hsin-Yi (0.51 versus 0.53) and Ju-Chi (0.40 versus 0.46) breeds. Hsin-Yi was the only breed to exhibit lower observed heterozygosity values than expected for LEI0258, which may deserve further studies on MHC function in this breed.

In conclusion, polymorphism at the MHC locus can be easily monitored with a single highly variable marker and can serve as a useful indicator to monitor genetic diversity for conservation of small populations. Mating plans could be designed in order to avoid allele loss at the MHC locus. Keeping a stable number of sire families could be recommended in order to avoid any effect of sampling on allele frequencies.

II. Combining molecular data and performance traits

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A global analysis of molecular markers and phenotypic traits in local chicken breeds in Taiwan

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Summary

Molecular and phenotypic data have been combined to characterize the genetic diversity of six local chicken breeds maintained with a long-term conservation programme. Hua-Tung, Hsin-Yi, Ju-Chi and Quemoy originated from Taiwan, Shek-Ki is from South China, and Nagoya is from Japan. Molecular tools included 24 microsatellite markers, *melanocortin 1 receptor (alpha melanocyte stimulating hormone receptor)* (*MC1R*), the *LEI0258* marker located within the major histocompatibility complex (MHC) and mitochondrial DNA. Performance was recorded on the same individuals for body weight, panting rate in summer and antibody response (antigens: Newcastle disease virus and sheep red blood cells). A multivariate method previously proposed for taxonomy was used to combine the different data sets. *Melanocortin 1 receptor (alpha melanocyte stimulating hormone receptor)* and the *MCW330* marker contributed the most to the first axis of the multiple coinertia analysis of molecular markers. *Melanocortin 1 receptor (alpha melanocyte stimulating hormone receptor)* showed evidence of selection, probably related to its effect on feather colour. The MHC exhibited a large diversity, with 16 alleles of the *LEI0258* marker. Immune response traits contributed the most to the principal component analysis of phenotypic data. Eight mitochondrial DNA haplotypes related to clades A, B, C and E were distributed across breeds and revealed an important contribution of Indian and European breeds to Ju-Chi, Quemoy and Hsin-Yi. Phenotypic data contributed less than molecular data to the combined analysis, and two markers, *LEI0258* and *LEI0228*, contributed the most. The combined analysis could clearly discriminate all breeds, except Ju-Chi, which was similar to Quemoy for many criteria, except immune response.

Keywords local chicken, microsatellites, *melanocortin 1 receptor (alpha melanocyte stimulating hormone receptor)*, major histocompatibility complex, mitochondrial DNA, immune response, body weight.

Introduction

Preserving local breeds is an important issue for the conservation of domestic animal genetic diversity (FAO 2007). There is a wide array of chicken breeds with abundant phenotypic diversity, as a result of domestication and selection. Commercial lines and industrialized livestock production systems have spread over all continents. Local breeds have low productivity but may harbour specific features as a result of adaptation to their environment. A subtropical island such as Taiwan (21°90′-25°29′N; 120°05′-122°00′E) provides an interesting framework to study genetic diversity of local chicken breeds, because of its climate and its geographic situation. Initially, native chickens were raised in Taiwan by aboriginal tribes. Historical records (Lee 2006) indicate several waves of introduction of domestic chickens from China (400 years ago), Europe (300 years ago) and Japan (100 years ago). Chicken populations were then exposed to the subtropical climate, with some selection by local farmers.

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A conservation programme was set up in 1982 for four local chicken breeds and two imported breeds at National Chung-Hsing University (NCHU) in Taiwan. These breeds were characterized for an array of traits, such as disease resistance, meat quality (Lee 2006) and molecular diversity (Chen *et al.* 2004 & Berthouly *et al.* 2008). However, the latter study mainly addressed neutral diversity, which is not sufficient to assess the global conservation potential of local breeds.

The aim of this study was to extend the diversity study to functional traits and to complete the knowledge of breed history by sequencing mitochondrial DNA (mtDNA). We added two hypervariable microsatellite markers (*LEI0228* and *LEI0192*) and two markers associated with functional information: (i) the *MC1R* gene, which is one of the main loci controlling feather colour (Takeuchi *et al.* 1996; Kerje *et al.* 2003) and (ii) the LEI0258 marker located within the MHC locus, involved in response to infectious diseases (Lamont 1989). Because individual phenotypes were also available for an array of traits, a methodological approach is proposed to combine molecular (discrete) and phenotypic (continuous) data, to provide a comprehensive view of the genetic diversity of these breeds.

Materials and methods

Experimental chickens

Six local chicken breeds have been maintained in a conservation programme at NCHU since 1982 (Lee 2006; Fig. 1). Hsin-Yi shows red feathers, white skin and blue shanks. Ju-Chi and Hua-Tung have a black plumage and black shanks. Quemoy has a black plumage with gold-laced feathers on the neck, white skin and blue shanks. Shek-Ki has yellow skin, yellow feather and yellow (the 'triple-yellow'). Nagoya has a yellow plumage with black tail and blue shanks.

The management of experimental chickens followed a routine procedure (Chao & Lee 2001). A representative set of 50 animals, 25 males and 25 females was sampled according to pedigree records. Blood was collected for DNA extraction at 40 weeks of age, and all animals sampled had individual performance records. Birds were produced in four successive hatches for the six breeds, and breeds were reared together.

Molecular markers

Microsatellite markers

A set of 24 microsatellite markers from the AvianDiv European project (Hillel *et al.* 2003; Table S1) was used to analyse within- and between-breed diversity. It included the 22 markers described previously by Berthouly *et al.* (2008) and two additional markers, *LEI0228* and *LEI0192*, which have numerous alleles (14 for each marker). Markers were genotyped with the protocol described in Berthouly *et al.* (2008).

Mitochondrial DNA

The hypervariable sequence 1 (HVS-I) of the D-loop region was amplified using the same primers (Table S2) following the protocol of Liu *et al.* (2006).

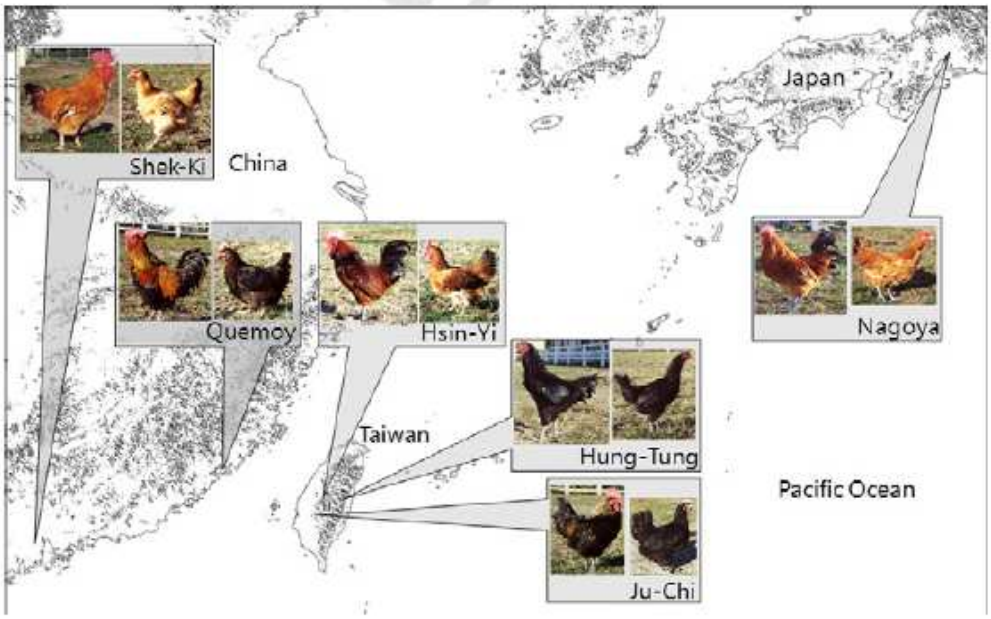

Figure 1 The six local chicken breeds under conservation at the National Chung-Hsing University with their region of origin. Hua-Tung (HT), Hsin-Yi (HY), Ju-Chi (JC) and Quemoy (QM) are from the Taiwan region, Shek-Ki (SK) is from Hong-Kong, and Nagoya (NG) is from Japan.

Melanocortin 1 receptor (alpha melanocyte stimulating hormone receptor)

The chicken *MC1R* gene-coding sequence was amplified with PCR primers (Table S2) and protocol as described by Chen *et al.* (2005).

LEI0258 marker

The *LEI0258* marker is an atypical Variable Number of Tandem Repeat that exhibits a very good correlation with serological typing of the chicken MHC (Fulton *et al.* 2006). PCR primers (Table S2) and protocol are the same as Fulton *et al.* (2006).

Performance traits

Body weight was measured every two weeks from hatch to 18 weeks of age, and again at 47 weeks of age. Three measures were kept for the analysis: initial body weight at hatch (BW0), body weight at the time of rapid growth (BW12) and mature body weight (BW47). Panting is generally observed when the ambient temperature is above 29 °C with 50% humidity (Weaver 2002). Panting was recorded individually on a daily basis between 24 and 27 weeks of age, in the afternoon of the hot season when the ambient temperature was higher than 30 °C (highest 36 °C, lowest 31 °C and average 33.5 °C with 61% humidity). Individual panting rate was calculated as the ratio of number of days where panting occurred divided by the total number of observed days.

Immune response was investigated using two different antigens. Sheep red blood cells (SRBC) are frequently used as a non-pathogenic antigen for evaluating antibody response in selection experiments, and antibody response to Newcastle disease virus (NDV) vaccination can be used as a selection criterion for immune response (Lamont *et al.* 2003). Newcastle disease is a major poultry disease in Taiwan (Yang *et al.* 1999), and vaccination against NDV is a standard procedure recommended by government sanitary authorities. All day-old chicks were vaccinated against NDV in this study. At 8 weeks of age, all chickens were vaccinated again with 0.5-ml NDV vaccine, and serum was collected 7 and 14 days later. Haemagglutination inhibition tests were used to determine antibody titres against NDV. At 11 and 13 weeks of age, chickens were injected with 0.1 ml 0.25% SRBC intravenously, and serum was collected 7 days later. Antibody titres of chickens against SRBC were determined by haemagglutination tests. The antibody titre was expressed as the $\log_2$ of the reciprocal of the highest dilution.

The kinetics of antibody titres was studied by calculating NDD as the difference between anti-ND antibody titre at day 14 and anti-ND antibody titre at day 7 post-inoculation, and SRBCD was calculated as the difference between anti-SRBC antibody titre at secondary response and anti-SRBC antibody titre at primary response.

Sequencing and sequence analysis

PCR products of mtDNA, *MC1R* and *LEI0258* were sequenced by Eurofins MWG Operon Company. Sequence alignment, editing and processing were performed with BIOEDIT 7.0.9.0 software (Hall 1999) and the STADEN package (Staden *et al.* 1998). Mitochondrial DNA and *LEI0258* sequence alignment and polymorphism identification was carried out using BIOEDIT. Haplotypes were scored manually. The median-joining networks (Bandelt *et al.* 1999) were constructed for mitochondrial DNA data using the program Network 4.516 (http://www.fluxus-engineering.com/sharenet_m.htm). The length variants (number of repeats) and polymorphic sites (either single-nucleotide polymorphisms or deletions) of *LEI0258* were checked for each allele. *MC1R* SNP analysis was carried out with the STADEN package (Staden *et al.* 1998) to define genotypes and haplotypes.

Analysis of genotypes

Allele frequencies, number of alleles and expected and observed heterozygosity were calculated using GENETIX 4.04 (Belkhir *et al.* 2004). Genetic distances (Nei *et al.* 1983) were calculated, and neighbour-joining (NJ) trees were established using POPULATIONS 1.2.30 (Langella 1999).

All marker data, including microsatellites, *MC1R* and *LEI0258*, were merged and treated with a Multiple CO-inertia Analysis (MCOA) using R (R Core Development Team 2006) with the ADE4 package (Chessel *et al.* 2004; Dray & Dufour 2007). This method makes the extraction of common information from separate analyses possible, by setting up a reference typology and comparing each typology separately. The efficiency of a marker is assessed by its typological value (T_v), which is the contribution of the marker to the construction of the reference typology, which is equal to the product of the variance (Var) multiplied by the congruence with the consensus Cos^2 (i.e. the correlation between the scores of individual locus tables and the synthetic variable of the same rank) (Laloë *et al.* 2007).

Analysis of performance data

Body weight, antibody titres and panting rate were analysed using the general linear model (GLM) procedure of SAS software (SAS Institute 2008). Breed and sex were treated as fixed effects with a breed × sex interaction. Partial correlations between body weight and panting rate were calculated for each sex after accounting for the breed effect. Principal

component analysis (PCA) was performed with the ADE-4 package from R, to identify the best combination of variables that explained most of the variance between breeds.

Combining molecular and phenotypic data

The Hill & Smith (1976) method was used to combine discrete and continuous variables to compare populations. This method is a combination of an internal correspondence analysis for discrete data, i.e. the molecular marker data (Laloë *et al.* 2002), and a PCA for continuous variables, i.e. performance traits. It was implemented with functions of the ADE-4 package from the R software.

Results

Genetic analyses of molecular markers

Ranking of breeds for heterozygosity values was similar to previous results of Berthouly *et al.* (2008) with a maximum value of 0.598 for Hua-Tung (instead of 0.584) and a minimum value of 0.412 for Shek-Ki (instead of 0.423). Hua-Tung and Ju-Chi exhibited weakly negative values for F_{IS} (−0.028 and −0.048, respectively), suggesting a slight excess of heterozygotes, whereas Shek-Ki exhibited the highest F_{IS} (0.079 as compared to 0.068 with 22 markers), which differed significantly from zero and indicated some inbreeding.

The mean Fst values ranged from 0.22 (Hua-Tung) to 0.35 (Nagoya). Shek-Ki showed the second highest mean Fst value (0.31), and mean Fst values varied from 0.24 to 0.27 for the other Taiwanese breeds (Table S3).

Melanocortin 1 receptor (alpha melanocyte stimulating hormone receptor)

There were six SNPs that defined five haplotypes, corresponding to four different protein sequences, which combined in eight genotypes (Tables 1 and S4).

Hsin-Yi and Shek-Ki shared haplotype 3 with a high frequency and were the only breeds to carry haplotype 5 at a low frequency, and they clustered together in the NJ tree (Fig. S1A). Haplotype 4 was almost fixed in Nagoya and was also found in Shek-Ki and Hsin-Yi, but at quite different frequencies, explaining the clustering of these three breeds in the NJ tree. Ju-Chi, Hua-Tung and Quemoy exhibited a high frequency of haplotype 1; Hua-Tung was the only breed to carry haplotype 2. Consequently, Ju-Chi and Quemoy grouped in the same cluster, and Hua-Tung had a basal position in the tree.

Shek-Ki was the only breed to exhibit a high heterozygosity for *MC1R* (0.578; Table S4) and a strongly negative F_{IS} (−0.313; data not shown).

LEI0258

A total of 16 alleles were found in the six breeds; six of them had never been described and were breed specific (Table 2). Hua-Tung had the highest number of alleles and the highest heterozygosity value; four alleles out of seven were private. The four alleles found in Shek-Ki were all private. The lowest heterozygosity value was found for Ju-Chi, whereas the highest F_{IS} value was observed for Hsin-Yi.

Ju-Chi, Quemoy and Hsin-Yi shared allele 309 at a rather high frequency and grouped in the same cluster of the NJ

Table 1 *MC1R* haplotypes, number of haplotypes and percentage in each breed.

	SNP position in the coding sequence						Number of haplotypes (percentage) per breed					
	212 T > A	274 G > A	427 A > G	636 G > A	637 T > C	644 A > C						
	Amino acid position											
Haplotypes	71	92	143	212	213	215	Hsin-Yi	Ju-Chi	Hua-Tung	Quemoy	Shek-Ki	Nagoya
1	C Thr	A Lys	A Thr	G Ala	T Cys	A His	1 (1%)	88 (96%)	77 (84%)	91 (99%)		
2	C Thr	A Lys	A Thr	A Ala	T Cys	A His			8 (9%)			
3	C Thr	A Lys	A Thr	G Ala	T Cys	C Pro	85 (92%)	4 (4%)	7 (7%)	1 (1%)	62 (69%)	1 (1%)
4	T Met	G Glu	G Ala	A Ala	C Arg	A His	5 (5%)				27 (30%)	93 (99%)
5 (wild type)	T Met	G Glu	A Thr	G Ala	T Cys	A His	2 (2%)				1 (1%)	

MC1R, melanocortin 1 receptor (alpha melanocyte stimulating hormone receptor).

Table 2 Allelic frequencies of the *LEI0258* marker for the six local chicken breeds.

Allele frequency							
Allele	Hisn-Yi	Ju-Chi	Hua-Tung	Quemoy	Shek-Ki	Nagoya	Corresponding B haplotype[1]
N	47	48	48	48	48	48	
181[2]						0.40	NA
182					0.21		4
193	0.11		0.06				11, 15.1, 27, 61
205		0.17		0.20			13
217[2]	0.02						NA
241[2]					0.14		NA
249		0.05	0.19	0.01		0.56	15.2, 22, 73
273[2]					0.34		NA
295			0.22				5
309	0.44	0.77	0.07	0.54		0.04	10, 24, 26, 76
319[2]			0.11				NA
345					0.31		14
357			0.16				5.1, 6.1, 21, 75
381		0.01		0.25			13.1
419[2]			0.19				NA
443	0.44						6
H_e	0.61	0.38	0.84	0.61	0.73	0.53	
H_o	0.51	0.40	0.83	0.65	0.71	0.58	
F_{IS}	0.17	−0.04	0.01	−0.06	0.03	−0.10	

[1]Corresponding B haplotypes with Fulton *et al.* (2006).
[2]Newly described private alleles.

tree. Nagoya and, to a lesser extent, Hua-Tung showed a high frequency of allele 249 and clustered together on the NJ tree; allele 295 (private) was the most frequent in Hua-Tung breed, which might contribute to the long branch length of this breed in the NJ tree. Shek-Ki was the most different, because it did not share any allele with the other breeds, and it represents an independent branch of the NJ tree (Fig. S1B).

Multivariate analysis

The first two axes of MCOA analysis with the 24 microsatellite markers explained 51.1% and 21.2% of the observed inertia, respectively. The two markers *MCW330* and *MCW034* contributed the most to the construction of the first axis for 8.6% and 8.3%, respectively. Results from MCOA analysis after addition of *MC1R* and *LEI0258* data to the microsatellite data are shown in Fig. 2. The first two axes of MCOA analysis explained 50.4% and 24% of the observed inertia, respectively. The two markers *MC1R* and *MCW330* contributed the most to the construction of the first axis with 10% and 6.7%, respectively. *LEI0258* ranked 7th of 26 markers, with a contribution of 5.2%. The first axis separated Nagoya from the set of Taiwanese–Chinese breeds. Hsin-Yi, Hua-Tung and Ju-Chi were strongly overlapping each other. The two markers *MC1R* and *LEI0228* contributed the most to the construction of the second axis, with 22.5% and 13.8%, respectively. *LEI0258* ranked 5th with a contribution of 8.1%. The second axis separated Quemoy and Shek-Ki.

Phenotypic data

Breeds differed significantly for body weight at all stages (Table 3). There was a significant breed × sex interaction ($P < 0.05$) for mature body weight, which was highest in Hua-Tung and Nagoya for males and in Nagoya and Shek-Ki for females. Ju-Chi and Quemoy exhibited the lowest body weight for both sexes, and Hsin-Yi showed intermediate values. There was a significant ($P < 0.01$) breed × sex interaction for panting rate. Hsin-Yi, Shek-Ki and Hua-Tung showed higher panting rates in males, whereas Shek-Ki and Nagoya showed the highest values in females. Quemoy exhibited a low value in males, but not in females. Ju-Chi exhibited a low panting rate for both sexes. On average, females had a higher panting rate and lower body weight than males. Partial correlation between BW47 and panting rate was moderate and positive (0.30 in females, 0.23 in males, $P < 0.01$).

Results for immunological traits are listed in Table 4. Breeds differed both in kinetics and in final titres. Quemoy exhibited a faster antibody response for both ND and SRBC, and final titres were highest in Quemoy for ND antibodies, but not for SRBC antibodies. Ju-Chi had lowest values for ND response. A negative correlation was observed between initial values (ND7 or SRBC1) and the subsequent change in

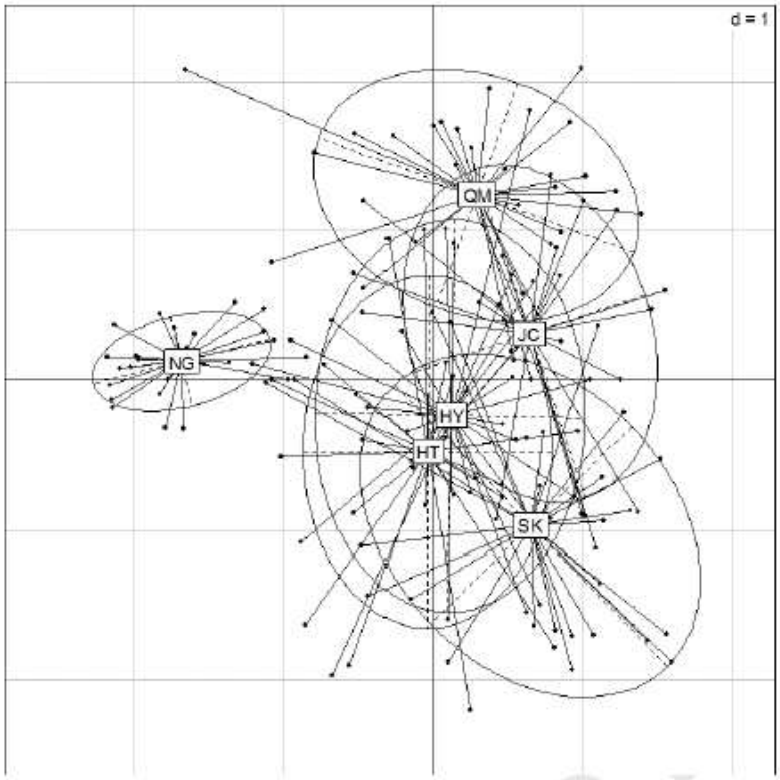

Figure 2 Position of six local breeds obtained by multiple coinertia analysis with 24 microsatellite markers, *MC1R* and *LEI0258*. HT: Hua-Tung, HY: Hsin-Yi, JC: Ju-Chi, QM: Quemoy, SK: Shek-Ki and NG: Nagoya. 10

Table 3 Least squares means of the phenotypic traits for the six local breeds.

Traits[1]	Hsin-Yi	Ju-Chi	Hua-Tung	Quemoy	Shek-Ki	Nagoya
Males						
BW0	36.2 ± 0.7^{ab}	34.8 ± 0.7^{bc}	36.7 ± 0.6^{a}	33.2 ± 0.6^{c}	35.8 ± 0.8^{ab}	34.5 ± 0.6^{bc}
BW12	1281 ± 35^{b}	1192 ± 36^{c}	1310 ± 34^{b}	1093 ± 34^{c}	1545 ± 40^{a}	1240 ± 33^{b}
BW47	2687 ± 65^{c}	2476 ± 67^{d}	3155 ± 64^{a}	2361 ± 64^{d}	2957 ± 75^{b}	3112 ± 62^{ab}
PANT	29 ± 4^{a}	13 ± 4^{bcd}	23 ± 4^{ab}	13 ± 4^{bcd}	28 ± 4^{ab}	18 ± 3^{bc}
Females						
BW0	36.5 ± 0.7^{a}	33.9 ± 0.7^{bc}	36.2 ± 0.6^{a}	32.7 ± 0.6^{c}	34.6 ± 0.7^{ab}	35.5 ± 0.6^{ab}
BW12	1032 ± 34^{a}	936 ± 32^{bc}	1049 ± 31^{a}	896 ± 31^{c}	1170 ± 33^{a}	1028 ± 30^{ab}
BW47	2013 ± 75^{b}	1492 ± 71^{c}	2022 ± 70^{b}	1522 ± 70^{c}	2197 ± 73^{ab}	2257 ± 68^{a}
PANT	56 ± 5^{bc}	39 ± 5^{d}	51 ± 5^{bcd}	60 ± 5^{bc}	76 ± 5^{a}	62 ± 5^{ab}

[1]BW0, body weight (grams) at 0 day of age; BW12, body weight (grams) at 12 weeks of age; BW47, body weight (grams) at 47 weeks of age; PANT, panting rate.

[a,b]Means ± SE within a row with no common superscript differ significantly ($P < 0.05$).

Table 4 Least squares means of the immunological traits for the six local breeds.

Trait[1]	Hsin-Yi	Ju-Chi	Hua-Tung	Quemoy	Shek-Ki	Nagoya
ND7	8.8 ± 0.3^{a}	7.4 ± 0.3^{b}	7.9 ± 0.3^{b}	8.7 ± 0.3^{a}	7.5 ± 0.3^{b}	8.1 ± 0.3^{ab}
NDD	1.3 ± 0.3^{c}	2.3 ± 0.3^{ab}	2 ± 0.3^{bc}	2.5 ± 0.3^{ab}	3.1 ± 0.4^{a}	2.1 ± 0.3^{bc}
SRBC1	4.1 ± 0.3^{ab}	2.8 ± 0.3^{c}	3.5 ± 0.3^{bc}	4.6 ± 0.3^{a}	4.3 ± 0.3^{ab}	3.6 ± 0.3^{bc}
SRBCD	0.9 ± 0.3^{abc}	1.7 ± 0.3^{a}	0.8 ± 0.3^{bc}	0.2 ± 0.3^{c}	0 ± 0.3^{c}	1.5 ± 0.3^{ab}

[a,b]Means ± SE within a row with no common superscript differ significantly ($P < 0.05$)

[1]ND7, anti-ND antibody titre at day 7 post-inoculation; NDD, difference between anti-ND antibody titre at day 14 and anti-ND antibody titre at day 7 post-inoculation; SRBC1, anti-SRBC antibody titre at primary response; SRBCD, anti-SRBC antibody titre at secondary response minus anti-SRBC antibody titre at primary response.

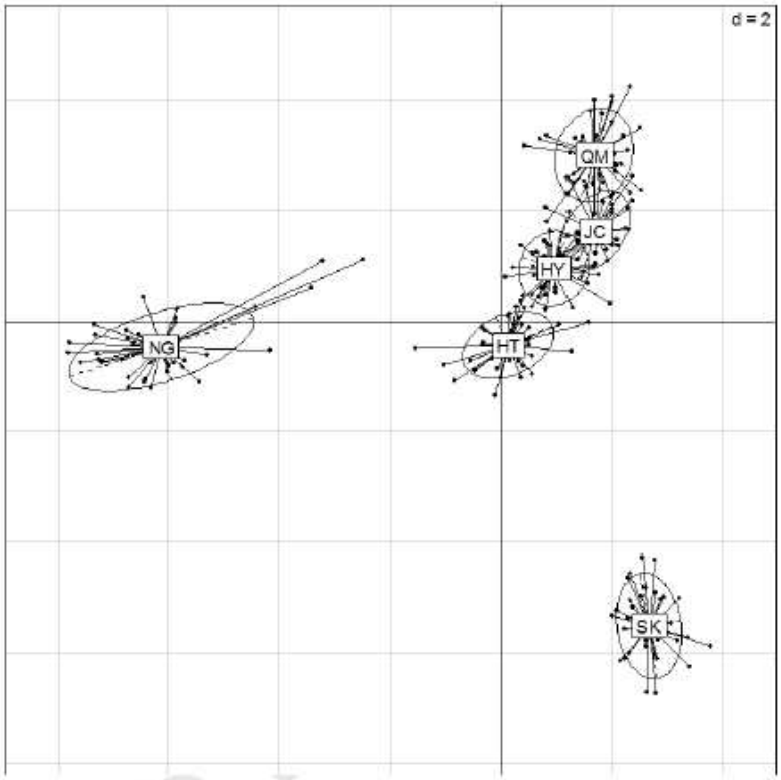

Figure 3 Position of six local breeds obtained by the Hill & Smith method for six local breeds combining molecular and phenotypic data. HT: Hua-Tung, HY: Hsin-Yi, JC: Ju-Chi, QM: Quemoy, SK: Shek-Ki and NG: Nagoya.

antibody titres ($r = -0.58$ between ND7 and NDD; $r = -0.86$ between SRBC1 and SRBCD). Correlations between body weight and immune response were not significant (data not shown).

The first two axes of PCA analysis explained 23.4% and 20.5% of the observed inertia, respectively. The two factors ND7 and SRBCD contributed the most to the construction of the first axis for 27.6% and SRBCD 24.1%, respectively; factors contributing most to the second axis were NDD and BW47 for 23.9% and 19.1%, respectively. Projection of individual data on the two axes showed a total overlap between breeds which could not be discriminated (data not shown).

Hill & Smith method for combining molecular and phenotypic data

Figure 3 shows the distribution of the six breeds using the Hill & Smith method on mixed data combining molecular and phenotypic data. The first two axes explained 9.9% and 7.2% of the observed inertia, respectively. The two markers *LEI0228* and *LEI0258* contributed the most to the construction of the first axis for 12.6% and 9.3%, respectively. *Melanocortin 1 receptor (alpha melanocyte stimulating hormone receptor)* ranked 13th and the first phenotypic factors contributing to this axis were SRBCD and SRBC1, which ranked 27 and 28th. The discriminant analysis showed that each breed made one group, except Ju-Chi and Quemoy.

Mitochondrial DNA phylogeny

There were 19 substitutions in a 497-bp mtDNA HVS-I region, and all substitutions were transitions. The eight haplotypes could be matched to previously published sequences, and the same codification as Liu *et al.* (2006) was used for further analysis and comparison.

Four clades were observed: A, B, C and E (Table 5). Three haplotypes were observed for clades A and E, while clades B and C were only represented by one haplotype each. Hsin-Yi, Ju-Chi and Quemoy were distributed in clades A and E sharing the same haplotypes. Haplotype E1 was very frequent in the Quemoy breed, followed by the Hsin-Yi breed. Nagoya was also found in clade A and E and was the only one to exhibit the A3 haplotype. Hua-Tung was in clade A and C, and Shek-Ki was in clade B and C. The median-joining network showed that clades A and E were the dominant haplogroups in Taiwan local breeds, whereas clades B and C were minor haplogroups. Among the eight haplotypes, three were breed specific and exhibited moderate to high frequencies: A2 in Hua-Tung, A3 in Nagoya and B1 in Shek-Ki.

Table 5 Identification and frequency of mitochondrial DNA haplotypes for the six breeds.

Haplotype[1]	Hsin-Yi	Ju-Chi	Hua-Tung	Quemoy	Shek-Ki	Nagoya	Total	Genbank accession number
A1	17 (35%)	34 (71%)	11 (23%)	1 (2%)		25 (54%)	88 (31%)	HQ022887
A2			27 (57%)				27 (9%)	HQ022888
A3						17 (37%)	17 (6%)	HQ022886
B1					35 (73%)		35 (12%)	HQ022881
C1			9 (19%)		13 (27%)		22 (8%)	HQ022885
E1	10 (21%)	9 (19%)		42 (88%)			61 (21%)	HQ022883
E6	19 (40%)					4 (9%)	23 (8%)	HQ022882
E9	2 (4%)	5 (10%)		5 (10%)			12 (4%)	HQ022884

[1]Mitochondrial DNA haplotypes are named after Liu *et al.* (2006).

Discussion

History of breeds

The history of multiple introductions of chickens in Taiwan from China, Europe and Japan is consistent with the finding of four clades of mtDNA. The high frequency of clade E reveals an important contribution of breeds from India or Europe, which generally exhibit a high frequency of haplotypes of the E group (Liu *et al.* 2006; Berthouly-Salazar *et al.* 2010). The Quemoy exhibited the highest frequency of E haplotypes, which is consistent with the role of the Quemoy Island as an important harbour for trading between China and Taiwan, and a likely place for introduction of exotic chickens to Taiwan hundreds years ago. The Hsin-Yi was considered the popular backyard chicken in the 1970s (Lee 2006). This breed has undergone introgression from Indian or European descent, because it also exhibited a high frequency of E haplotypes. By contrast, the Ju-Chi exhibited the highest frequency of A1 haplotypes, which suggests that this breed was influenced less by introductions of western commercial breeds and could represent a more ancient breed in Taiwan. Hua-Tung, kept for cock fighting, did not carry any E haplotypes and was the only breed from Taiwan to carry the A2 and C1 haplotypes, which can be found in China and Japan. Haplotypes A1 and C1 have been found in the Shamo breed, which is also a game bird (Liu *et al.* 2006), and A2 was found in Hinai-dori, a Japanese local breed. Thus, the Hua-Tung is a typical Asiatic breed which has probably been crossed with other breeds, as shown by its high heterozygosity level for microsatellite markers. The Shek-Ki exhibited haplotypes from haplogroups B and C, which is consistent with its origin in South China (Hong-Kong region), because B1 and C1 haplotypes have been previously described in Chinese breeds; B1 is found in the Qinyan breed from Guang-Dong province (Liu *et al.* 2006). The C haplogroup is generally found in Japanese breeds, but was not found in Nagoya. This result is consistent with previous observations of haplotypes A1 and A3 in Nagoya-dori (Liu *et al.* 2006).

Neutral markers vs. markers with a functional effect

Microsatellite markers may be considered neutral, whereas *MC1R* and MHC alleles are related to a function or a phenotype. The multivariate analysis showed that *MC1R* was the most discriminative marker and exhibited the highest typological value. This can be explained by the association of *MC1R* with feather colour, which is a phenotypic trait that farmers could easily select for, to obtain a breed with a uniform appearance. Indeed, haplotypes identified in the Taiwanese breeds could be matched with alleles at the Extension (*E*) locus: haplotypes 1 and 2 were previously associated to the extended black allele (Takeuchi *et al.* 1996; Kerje *et al.* 2003), and these haplotypes have also been found in some wild birds (Mundy 2005); haplotype 3 has been previously associated to either the buttercup allele (Kerje *et al.* 2003) or the brown allele (Ling *et al.* 2003); haplotype 4 has been previously associated to the wheaten or yellow alleles (Ling *et al.* 2003), and haplotype 5 is the wild-type allele. The plumage colour of breeds Ju-Chi, Hua-Tung and Quemoy is mostly black, which fits with their high frequency of haplotype 1. Nagoya has a yellow plumage colour, which fits with the high frequency of haplotype 4. Hsin-Yi has a red plumage, which fits with the high frequency of haplotype 3; both the buttercup and the brown alleles give a mostly red plumage and differ in down colour at hatch (Smyth 1996). Shek-Ki segregates for haplotypes 3 and 4, with a higher frequency for haplotype 3, which is consistent with its yellow plumage. Haplotype 4 is mainly present in the heterozygous state. Indeed, the yellow allele at the *E* locus is expected to have a recessive effect on plumage colour, as compared to the buttercup allele (Smyth 1996). Thus, the *MC1R* gene represents a typical case of selection that can be easily detected by the MCOA. No other marker exhibited a similar pattern. A selection signature around the *MC1R* gene has also been detected using high-density SNP genotyping on dairy cattle breeds (Flori *et al.* 2009).

The *LEI0258* and *LEI0228* markers contributed significantly to the multivariate analysis because of their high

number of alleles. Breeds generally exhibited a higher level of heterozygosity for *LEI0258* as compared to microsatellites, which may be expected, because balancing selection rather than directional selection is expected for the MHC locus. However, Ju-Chi and Hsin-Yi showed a rather low heterozygosity, with a quite high frequency of allele 309 in the Ju-Chi, suggesting some selective advantage. Hsin-Yi exhibited the largest difference between observed and expected heterozygosity and a high F_{IS} value, which was not observed with other markers. A deviation from Hardy-Weinberg equilibrium for *LEI0258* was observed in subsequent generations for this breed (Chang *et al.* 2010) and suggests some selective pressure on this locus.

Heat tolerance

A low body weight is generally associated with better heat tolerance and heat evacuation, which is consistent with the positive correlation observed here between mature body weights and panting rate. The higher panting rate of females, in spite of their lower body weight, can be because of a higher level of heat production in mature laying hens than in cockerels, as reported by Macleod *et al.* (1978). Within the group of heavy breeds (Nagoya, Shek-Ki, Hua-Tung and Hsin-Yi), Hua-Tung females showed a better heat tolerance, as shown by a lower panting rate. In the light breeds (Ju-Chi and Quemoy), Ju-Chi females showed a better heat tolerance. Thus, differences in adaptation to heat may be observed between local breeds irrespective of their body weight.

Combining phenotypic data and molecular data with a multivariate analysis

In MCOA, the differentiation of the six breeds according to 24 microsatellite markers matched their geographical distribution. Adding genotypes for *MC1R* did not greatly change the synthetic position of breeds, but increased the distance between Quemoy and Shek-Ki.

In spite of a significant effect of the breed on performance traits, the PCA analysis using all recorded traits did not discriminate between the six breeds. This could be because of the number of animals per breed, which is low for the study of quantitative variation, but sufficient for the study of molecular variation. Because immune response traits contributed the most to breed differentiation, it is recommended that such traits are included in breed characterization under experimental conditions. For instance, the Ju-Chi and Quemoy clustered together for *MC1R* and *LEI0258* and exhibited similar body weights, but differed significantly for immune traits, with a better antibody response in Quemoy. Further studies should determine whether differences in allelic frequencies at *LEI0258*, particularly for alleles 309 and 381, could be associated with a different antibody response. It is also possible that trading activity between China and Taiwan through the Quemoy Island increased the exposure of Quemoy chickens to pathogens. Natural selection as well as human selective breeding may have resulted in a stronger antibody response for Quemoy chickens.

Combining performance traits and molecular data with the Hill & Smith method increased the discrimination between Taiwanese breeds and showed that Hua-Tung, Hsin-Yi and Quemoy did not overlap with each other, but that Ju-Chi did overlap with Quemoy and Hsin-Yi.

Combining the breed history, molecular variability and phenotypic performance makes it possible to classify the six breeds according to three types. The first type corresponds to imported breeds; Shek-Ki and Nagoya were characterized by a heavy body weight and a low heterozygosity for microsatellite markers. They did not have any mtDNA haplotype in common, which is consistent with their quite different history. Thus, these two breeds illustrate the case of well-established breeds under a long-term conservation programme. Shek-Ki is particularly interesting for further studies regarding the MHC, because it does not share any *LEI0258* allele with the Taiwanese breeds. They also showed a much higher heterozygosity level for the *LEI0258* marker than for the other markers, supporting the hypothesis of a selective advantage for heterozygous genotypes at the MHC. The second type corresponds to traditional Taiwanese breeds, such as Ju-Chi and the Quemoy, which clustered together for most of the criteria used (*MC1R*, *LEI0258*, mtDNA and body weight). From the viewpoint of genetic diversity, it could be proposed that the Quemoy be given conservation priority, as it shows a higher heterozygosity level for microsatellite markers. However, these two breeds differed markedly for immune response traits, and this difference deserves further study. The third type corresponds to more recent breeds such as Hua-Tung and Hsin-Yi, which originated in the last century. Both of breeds have been selected for growth and exhibited a higher heterozygosity than the other breeds for microsatellite markers. Hsin-Yi was relatively closer to Ju-Chi and Quemoy for *LEI0258* as well as for mtDNA. The mtDNA showed the rather unique origins of Hua-Tung, which could be related to its use as a game breed in the past.

In conclusion, the present study provided a detailed characterization of six local breeds and showed that the conservation programme is successfully maintaining six populations differing in history, molecular polymorphism and performance. The multivariate analysis revealed selection at the *MC1R* locus, which is supported by the association of *MC1R* alleles with differences in feather colour. No other locus exhibited such selection in this study. The use of phenotypic data alone could not discriminate breeds efficiently, but the combination of phenotypic and molecular data in a single multivariate analysis provided a better discrimination between breeds than the multivariate analysis with molecular data alone. Regarding performance traits, panting rate revealed interesting differences between

breeds, irrespective of body weight, and antibody response identified a subset of breeds (Ju-Chi and Quemoy) where the relationships between MHC alleles and immune response should be further studied. Recording immune response traits in association with MHC genotyping could be a useful option for the characterization of local breeds in experimental conditions. The search for selection signatures for disease resistance should be carried out using a much higher density of markers in a set of populations including local breeds from different climatic regions.

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Supporting information

Additional supporting information may be found in the online version of this article.

Figure S1 A (upon)- Neighbor-joining tree analysis of the *MC1R* gene for the six local breeds. B (down)- Neighbor-joining tree of the LEI0258 marker for the six local breeds.

Table S1 General characteristics of the 24 microsatellite markers for the six breeds.

Table S2 Description of the primers used in this study. PCR regions and amplification lengths, names and primer sequences are shown.

Table S3 The semi-matrix of Fst in six local breeds.

Table S4 *MC1R* genotypes, animal number and percentage in each breed.

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B. Supplementary tables for paper 1

Table S1. General characteristics of the 24 microsatellite markers for the six breeds.

Locus	n[1]	No. alleles	Allele size range (bp)	H_O[2]
ADL112	281	3	121-127	0.35
ADL268	281	5	101-113	0.49
ADL278	283	6	110-122	0.47
LEI094	278	10	246-281	0.61
LEI166	275	6	251-261	0.49
LEI192	257	15	253-424	0.63
LEI228	278	14	163-443	0.48
LEI234	281	14	212-354	0.73
MCW014	280	5	162-183	0.41
MCW034	283	8	213-248	0.54
MCW037	280	5	150-155	0.55
MCW067	280	4	174-180	0.51
MCW069	281	7	154-174	0.55
MCW078	280	4	134-142	0.35
MCW081	276	4	109-131	0.53
MCW098	280	2	255-257	0.20
MCW111	278	5	97-111	0.58
MCW183	275	8	292-322	0.45
MCW206	278	6	220-239	0.44
MCW216	280	7	134-149	0.54
MCW222	281	4	216-222	0.43
MCW248	276	4	213-221	0.47
MCW295	271	6	85-99	0.57
MCW330	273	4	254-286	0.47
All loci		156		

[1] Number of successfully typed birds.

[2] Observed heterozygosity (Nei 1987).

Table S2. Description of the primers used in this study; PCR regions and amplification lengths, names and primer sequences.

PCR region	Length (bp)	Primer name	Primer sequence
mtDNA	600 bp	L16750	5'-AGGACTACGGCTTGAAAAGC-3'
		H547	5'-ATGTGCCTGACCGAGGAACCAG-3'
MC1R	750 bp	Mc1Co-up	5'-GAGGGCAACCAGAGCAATGC-3'
		397281-dwn	5'-TGAAGAAGCAGGTGCAGAAG-3'
MHC	200-500 bp	LEI0258-F	5'-CACGCAGCAGAACTTGGTAAGG-3'
		LEI0258-R	5'-AGCTGTGCTCAGTCCTCAGTGC-3'

Table S3. The semi-matrix of Fst in six local breeds.

	Hsin-Yi	Ju-Chi	Hua-Tung	Quemoy	Shek-Ki	Nagoya
Hsin-Yi						
Ju-Chi	0.19					
Hua-Tung	0.18	0.19				
Quemoy	0.23	0.22	0.23			
Shek-Ki	0.30	0.28	0.23	0.31		
Nagoya	0.31	0.38	0.27	0.35	0.42	
Mean Fst	0.24	0.25	0.22	0.27	0.31	0.35

Table S4. *MC1R* genotypes, animal number and percentage in each breed.

Genotypes[1]	Number of carriers (percentage) per breed					
	Hsin-Yi	Ju-Chi	Hua-Tung	Quemoy	Shek-Ki	Nagoya
1/1		42 (91%)	32 (68%)	45 (98%)		
1/2			8 (17%)			
1/3	1 (2%)	4 (9%)	5 (11%)	1 (2%)		
2/3			2 (4%)			
3/3	39 (83%)				18 (40%)	
3/5	2 (4%)				1 (2%)	
3/4	5 (11%)				25 (56%)	1 (2%)
4/4					1 (2%)	46 (98%)
Heterozygosity	0.149	0.087	0.319	0.021	0.578	0.021

[1] Genotype of *MC1R* was named according to haplotypes described in Table 1

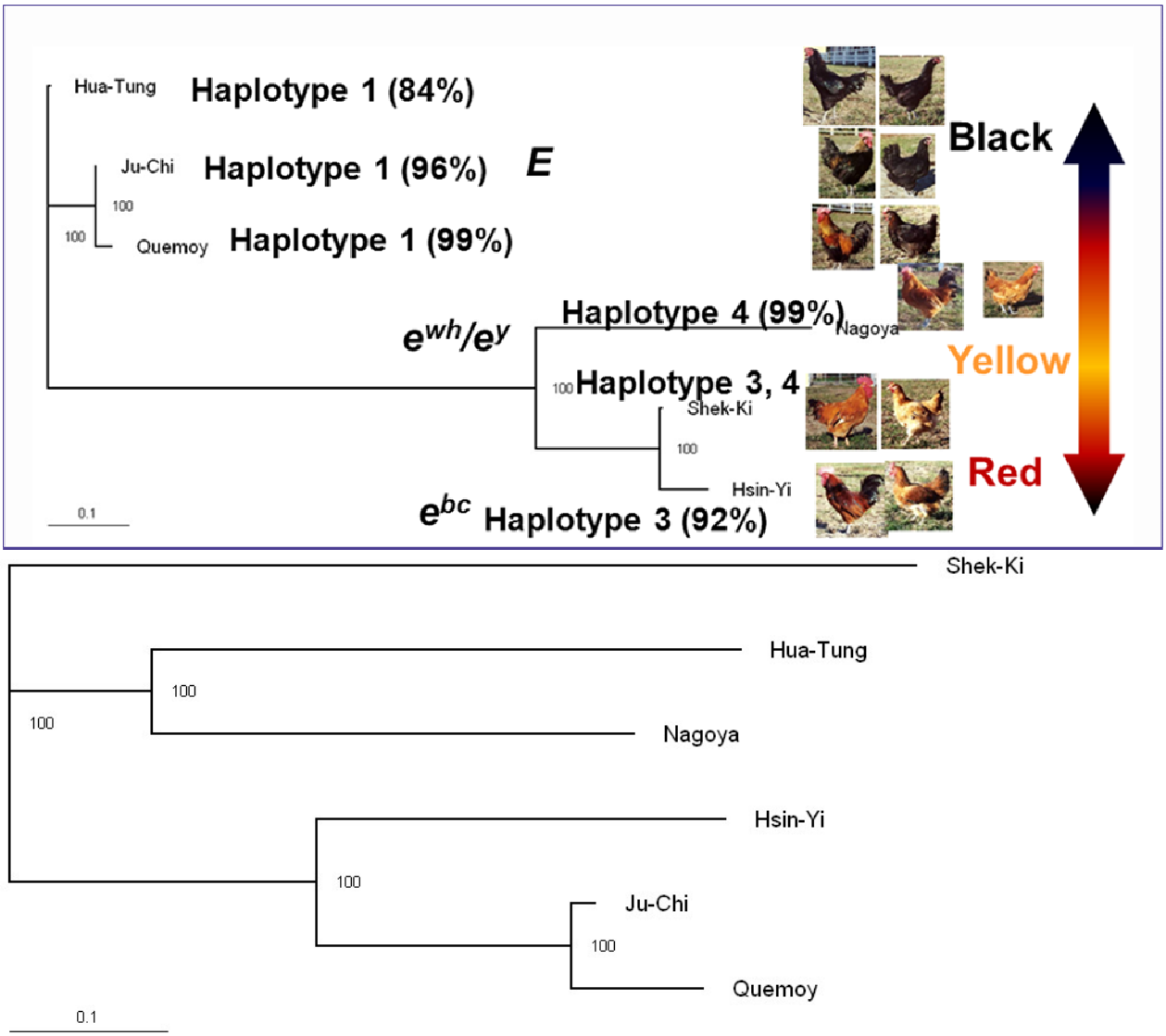

Figure S1. A (upon)- Neighbor-joining tree analysis of the *MC1R* gene for the six local breeds. B (down)- Neighbor-joining tree of the LEI0258 marker for the six local breeds.

C. Complementary data and analyses

1- Additional data on the combined analysis

The multivariate analysis revealed selection at the *MC1R* locus, which is supported by the association of *MC1R* alleles with differences in feather colour. No other locus exhibited such selection in this study as shown by the typological values (figure 18).

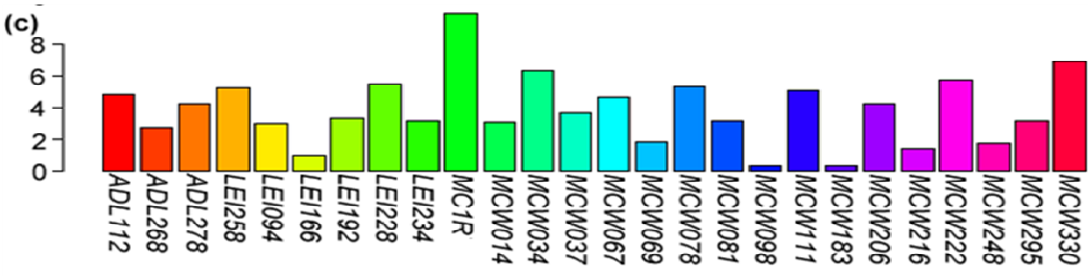

Figure 18. Typological values of the 24 microsatellite markers, *MC1R* and LEI0258 in the multivariate coinertia analysis for the six chicken breeds.

The use of phenotypic data alone could not discriminate breeds efficiently, but the combination between phenotypic and molecular data in a single multivariate analysis provided a better discrimination between breeds (Figure 19).

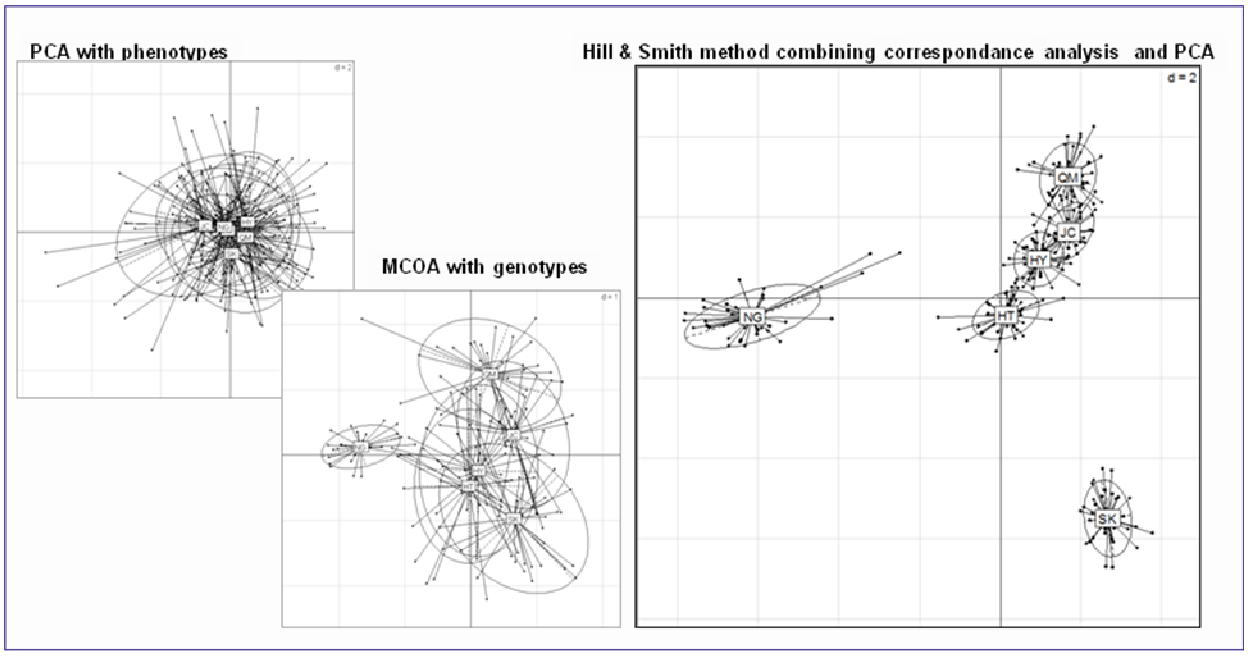

Figure 19. Comparison of principal component analysis with phenotypic traits, multivariate co-inertia analysis with molecular data, and results of combined analysis.

When the Hill and Smith analysis was restricted to the 4 Taiwan breeds, Hua-Tung and Hsin-Yi appeared clearly separated and Ju-Chi occupied a central position, rather close to Quemoy (Figure 20).

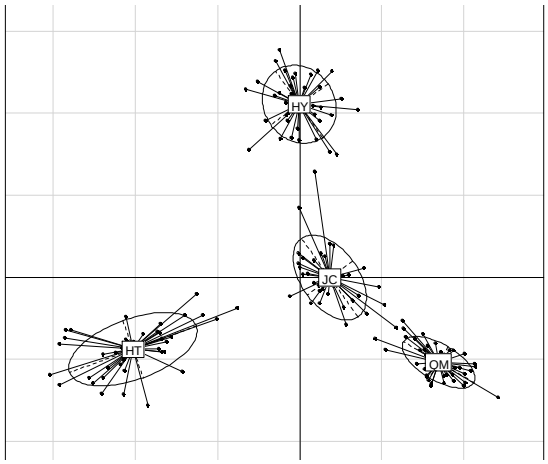

Figure 20. Position of the four Taiwan breeds obtained by Hill & Smith method. HT: Hua-Tung, HY: Hsin-YI, QM: Quemoy, JC: Ju-Chi.

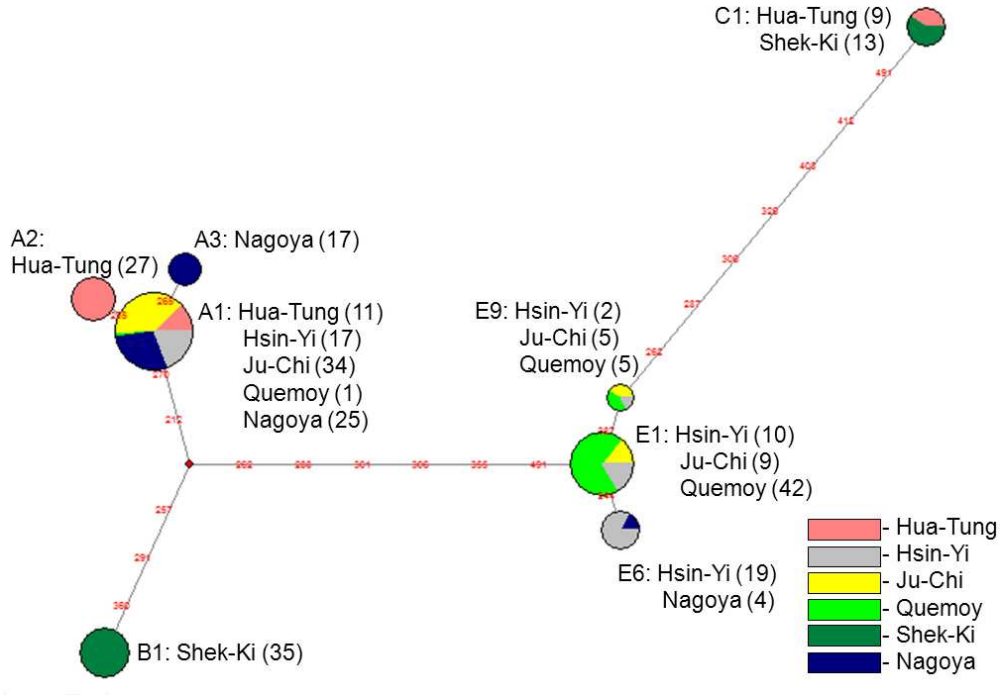

Figure 21. Network of mitochondrial DNA haplotypes for the six chicken breeds in Taiwan.

This network showed rather long branches for the Shek-Ki breed, which has clearly a distinct origin, but shares one haplotype with the highly variable Hua-Tung breed. Hsin-Yi, Ju-Chi and Quemoy shared haplotypes from the E haplogroup and haplotypes from the A haplogroup could be found in all breeds except Shek-Ki.

Regarding performance traits, panting rate revealed interesting differences between breeds that were moderately correlated to body weight (figure 22). In particular, the Nagoya breed was the heaviest for females and was as heavy as Hua-Tung and Shek-Ki, but exhibited a lower panting rate in males and ranked 4 for this trait in males.

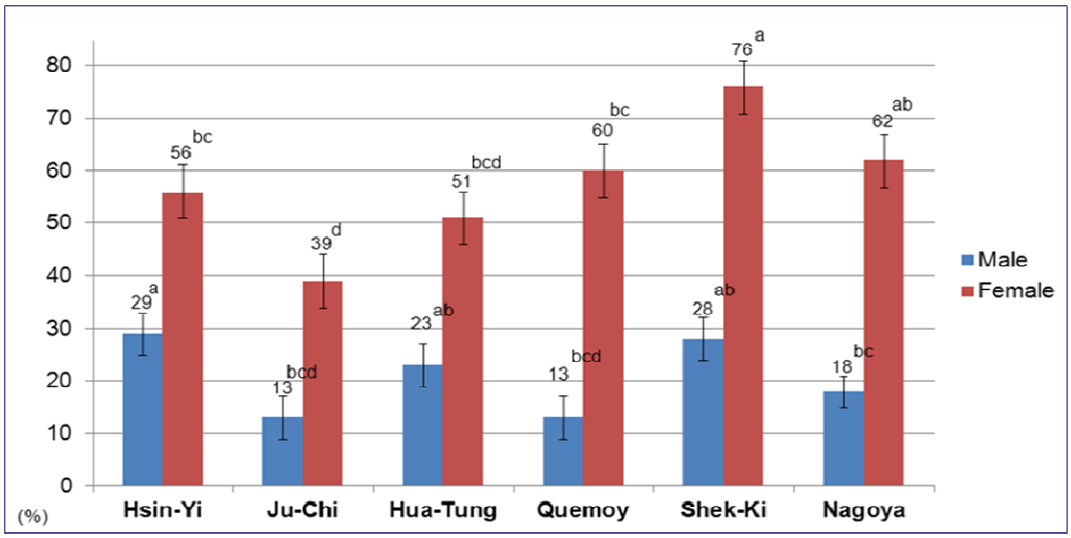

Figure 22. Panting rate observed during the hot season according to breed and sex.

2- Bayesian clustering

Results obtained by Structure clustering applied to genotypes for 25 microsatellites are shown in Figure 22. At K=2, the Taiwanese local breeds (Ju-Chi, Quemoy and Hsin-Yi; Cluster green) made one group and the imported breeds (Shek-Ki and Nagoya; Cluster red) were grouped with the game bird breed (Hua-Tung). For K=3, one cluster grouped three Taiwanese local breeds (Ju-Chi, Quemoy and Hsin-Yi; Cluster blue), one imported breed (Nagoya; Cluster Red) made its own cluster and one cluster grouped Hua-Tung and Shek-Ki. For K=4, the two traditional Taiwanese local breeds (Ju-Chi and Quemoy) still clustered together, each of the imported breeds (Shek-Ki and Nagoya) made its own cluster and the last cluster grouped the two Taiwanese breeds (Hua-Tung and Hsin-Yi; Cluster yellow). In K=5, Ju-Chi and Hsin-Yi belonged to the same cluster, and each other breed made its own cluster. The likelihood stabilized at K=6:

K value	1	2	3	4	5	6	7	8	9	10
Estimated Ln Prob of Data	-18122	-16135	-14508	-13596	-12555	-11887	-11821	-11817	-12438	-11872

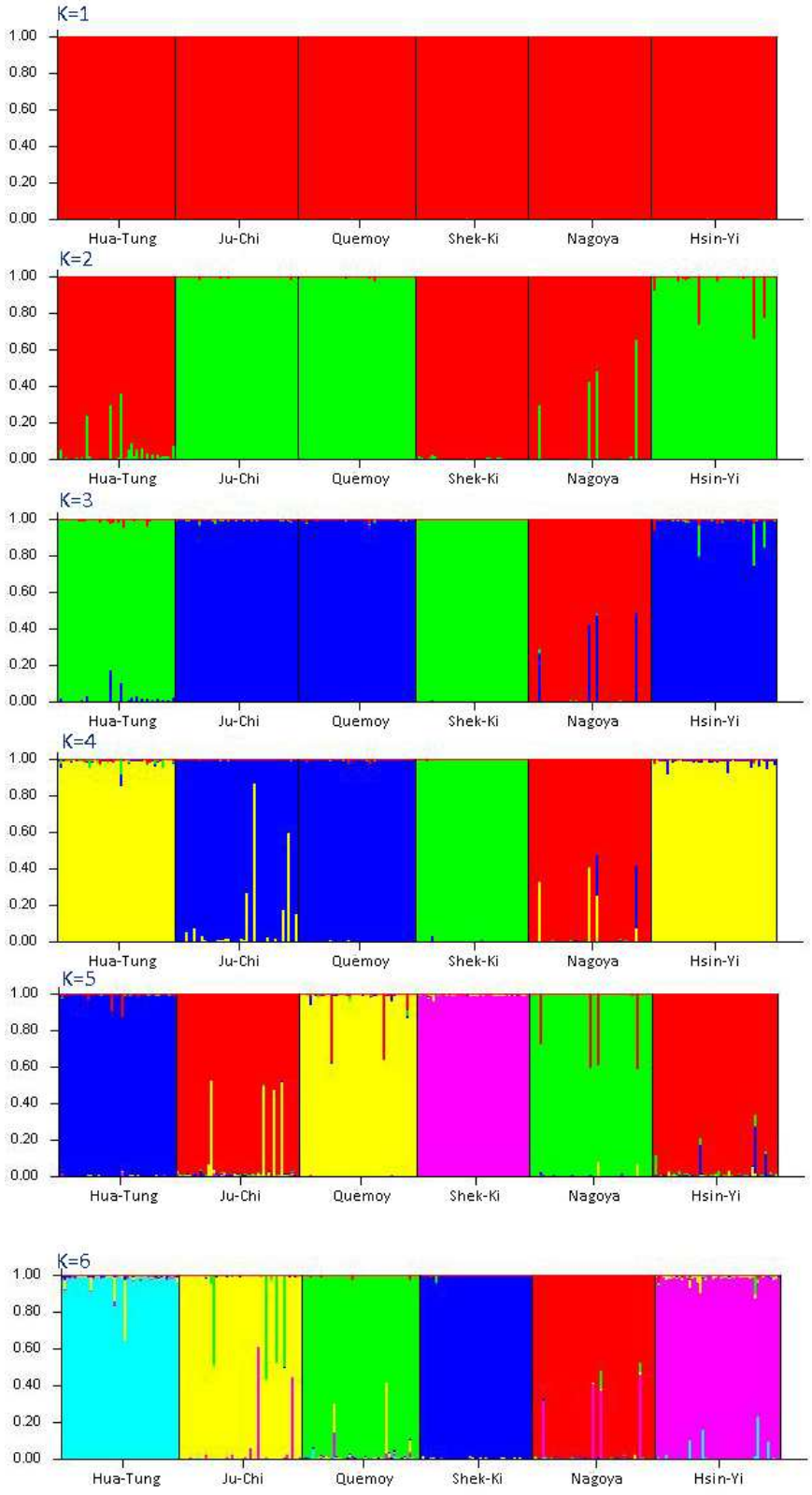

Figure 23. Clustering diagrams of the six chicken populations obtained from K=1 to 6.

III Immune response

A. AI challenge and MHC effects

1. Introduction

Influenza is a highly epidemic disease caused by viruses of the family *Orthomyxoviridae* (Swayne & Halvorson 2003, Perdue & Swayne 2005). Avian influenza (AI) viruses may be classified as high pathogenic avian influenza (HPAI) or low pathogenic avian influenza (LPAI) according to the symptoms observed (Swayne & Halvorson 2003). Both HPAI and LPAI caused huge economic losses in the world (Swayne & Halvorson 2003; Table 16). In addition, AI virus infections in poultry may be transmitted to humans, with a public health risk (Perdue & Swayne 2005).

Table 16. Examples of economic losses from HPAI and LPAI epidemics as reported in US dollars (Swayne & Halvorson 2010).

Year	Outbreak	Birds dead or culled	Cost item	Original cost	Cost in 2007 $US	Cost/farm in 2007 $US
HPAI						
1924-25	USA – Fowl Plague	Unknown	Direct losses	$ 1 M	$ 12.2 M	-
1983-84	USA – H5N2 HPAI	17 M (449 farms)	USDA eradication	$ 63 M	$ 126 M	$ 280,000
			Non-indemnified industry losses	$ 15 M	$ 30 M	$ 66,500
			Increased customer costs	$ 349 M	$ 700M	$ 1.5 M
1985	Australia – H7N7 HPAI	238,518 (1 farm)	Eradication cost	$ 1.4 M	$ 2.7 M	$ 2.7 M
1999-2000	Italy – H7N1 HPAI	13 M (413 farms)	Compensation	$ 100 M	$ 121 M	$ 298,000
			Indirect costs	$ 500 M	$ 605 M	$ 1.5 M
1997	Hong Kong – H5N1 HPAI	1.5 M	Eradication	$ 13 M	$ 17M	-
Late 2003-mid-2005	Asia – H5N1 HPAI	220 M	Losses to the poultry industries	> $ 10 B	> $ 10 B	-
LPAI						
1978	Minnesota USA - various LPAI	141 farms	Losses to the poultry industries	$ 5 M	$ 16 M	$ 113,000
1995	Minnesota USA – H9N2 LPAI	178 farms	Losses to the poultry industries	$ 6 M	$ 8.2 M	$ 46,000
1978-1995	Minnesota USA - various LPAI	1058 farms	Losses to the poultry industries	$ 22 M		$ 21,000
1995	Utah USA – H7N3 LPAI	2 M (60 farms)	Losses to the poultry industries	$ 2 M	$ 2.7 M	$ 45,000
2002	Virginia USA – H7N2 LPAI	4.7 M (197 farms)	USDA eradication	$ 81 M	$ 94 M	$ 477,000
			Losses to the poultry industries	$ 130 M	$ 150 M	$ 761,000
			State government	$ 1 M	$ 1.2 M	$ 6000

HPAI viruses have not been isolated in Taiwan so far, but LPAI viruses such as H6N1 virus have been isolated frequently. Although Taiwanese H6N1 viruses were classified as low pathogenic, 50% of layers and 30% of the native broilers carried antibodies against H6N1 viruses. Genetic and antigenic analysis showed that H6N1 viruses found in Taiwan differed from viruses circulating in Hong Kong and South-eastern China (Lee *et al.* 2006). Potential transmission to mammals was reported and bacterial secondary infections could cause economic losses, (Lee *et al.* 2006).

Antibiotics and vaccines are major tools to combat avian diseases. Effects of chicken major histocompatibility complex (MHC) on disease resistance have been reported for a long time (Bacon 1987, Lamont 1987 & Lamont 1989). For many years, serological typing has been used to identify chicken MHC haplotypes, but this method was not easy to apply to local populations where no reference samples and no specific reagents were available. Fulton *et al.* (2006) proposed to use marker LEI0258, an atypical Variable Number of Tandem Repeat (VNTR) located within the MHC, as a standard procedure to identify MHC alleles in any population.

Genetic diversity of local chickens may affect the spread of epidemics, since it has been shown that genetic heterogeneity in a population was associated with an increased probability of minor epidemics and a decreased probability of major epidemics (Springbett *et al.* 2003). Local breeds may be more resistant to diseases as reported in the case of four native Egyptian breeds for Infectious Bursal Disease and Newcastle Disease (ND) by Hassan *et al.* (2004). Since 1982, a conservation program was set up for four local chicken breeds and two imported breeds at National Chung-Hsing University (NCHU) in Taiwan. Previous studies showed that these local breeds tended to have higher resistance to coccidiosis (Fan *et al*. 1988), higher antibody responses to Newcastle disease (ND) vaccine and Sheep Red Blood Cells (SRBCs) inoculation (Chao & Lee 1991) and better resistance to Leucocytozoonosis (Chen *et al*. 1991).

This part of the thesis was aimed at comparing breeds' responses to H6N1 virus and assessing the effect of MHC genotypes on this response.

2. Results

MHC genotypes distribution

A total of 343 chickens were obtained from parents of known MHC genotypes. The number of chicks per treatment and per breed was higher than 20 (Table 17), except for the Shek-Ki, SPF and the Nagoya breeds because of hatchability problems**.**

There were 19 LEI0258 alleles which combined into 42 genotypes across the seven breeds, with five to thirteen genotypes per breed (Table 18). Six LEI0258 alleles were new, as described in Chang *et al.* (2008). Most alleles (15 out of 19) were found only within a breed.

Table 17. Sample size and mortality per breed in H6N1 LPAIV challenge experiment.

Treatment	Sex	Hsin-Yi	Ju-Chi	Quemoy	Hua-Tung	Shek-Ki	Nagoya	SPF	Total
		68	52	90	51	21	37	24	343
Control	Male	13	13	27	9	7	9	5	83
	Female	18	12	14	14	2	6	7	73
Challenge	Male	16	15	24	16	5	9	5	90
	Female	21	12	25	12	7	13	7	97
Mortality	Male	5	0	0	0	1	0	0	6
	Female	2	1	0	1	0	0	0	4
Mortality (%)		22.6	3.7	0	3.6	8.3	0	0	

Table 18. LEI0258 allele frequency in the H6N1 challenge experiment

LEI0258 alleles	Hsin-Yi	Ju-Chi	Hua-Tung	Quemoy	Shek-Ki	Nagoya	SPF	Correspondence with B haplotypes[1]
181						0.338		*
182					0.381			4
193	0.434	0.238	0.147					11, 15.1, 27, 61
205		0.346		0.144			0.354	13
217	0.132							*
237			0.186					*
241					0.214			*
249		0.960	0.196	0.194		0.527		15.2, 22, 73
261							0.250	2, 15, 29
295			0.294					5
309	0.324	0.414		0.389		0.135		10, 24, 26, 76
319			0.186					*
345					0.448			14
350							0.417	-
357			0.196					5.1, 6.1, 21, 75, 130, 131, 201
381				0.272				13.1
419			0.588					*
443	0.113							6
539							0.354	19
Number of alleles	4	4	7	4	3	3	4	
Number of genotypes	8	7	13	9	6	5	6	

* means new allele

Growth traits and mortality

Most of mortality (table 17) occurred between day 7 and 14 post-challenge. Hsin-Yi had the highest mortality (7 birds, 22.6%) dead birds carried various MHC genotypes 193/193, 193/309, 217/309 and 309/309. The distribution of LEI0258 alleles among the 7 dead animals in the challenge group of the Hsin-Yi breed did not show any deviation from the mean distribution of alleles in this breed.

A low mortality was observed in Ju-Chi (one bird with MHC 205/309), Hua-Tung (one bird with MHC 193/319) and Shek-Ki (one bird with MHC 182/345). Quemoy, Nagoya and SPF chickens showed no mortality. Growth traits are shown in Table 19; there were significant effects of breed and sex on body weight measured between 7 and 16 weeks of age, and on body weight gain (BWD) measured every three weeks. Body weight of males was significant higher than that of females. Shek-Ki showed the highest BW and SPF the lowest. BWD (7-10) and BWD (13-16) showed significant differences between control group and challenge groups: growth between 7 and 10 weeks of age was lower in the challenge group than in the control group, but the opposite was observed between 13 and 16 weeks of age, with higher growth in the challenge group, so that final body weight at 16 weeks of age did not differ between control group and challenge group.

Table 19. Least-square means of body weight and weight gain according to age, breed, and AI infection.

Source of variation	BW7	BW8	BW9	BW10	BW16	BWD(0-4)	BWD(4-7)	BWD(7-10)	BWD(10-13)	BWD(13-16)
Breeds										
Hua-Tung	540±10^{bc}	679±11^{bc}	806±14^{b}	909±14^{b}	1523±27^{a}	199±4^{b}	311±6^{b}	368±8^{ab}	281±10de	333±15^{b}
Ju-Chi	515±9^{cd}	635±11^{d}	748±13^{c}	848±14^{c}	1372±26^{c}	191±4^{b}	298±6^{bc}	332±8^{c}	285±10de	239±15^{d}
Quemoy	514±7^{d}	636±8^{d}	749±10^{c}	847±10^{c}	1418±20^{c}	199±3^{b}	290±5^{c}	333±6^{c}	315±7^{bc}	256±11^{d}
Shek-Ki	603±15^{a}	729±17^{a}	853±21^{ab}	994±22^{a}	1618±42^{a}	222±7^{a}	357±10^{a}	392±12^{a}	366±16^{a}	259±24^{cd}
Nagoya	564±11^{b}	703±13^{ab}	854±16^{a}	940±16^{ab}	1515±33^{ab}	201±5^{b}	339±8^{a}	377±9^{ab}	259±12^{e}	314±18^{bc}
SPF	370±14^{e}	492±16^{e}	589±20^{d}	688±20^{d}	1427±38^{bc}	94±6^{c}	236±9^{d}	319±11^{c}	338±14^{ab}	401±21^{a}
Hsin-Yi	551±8^{b}	659±10^{cd}	770±12^{c}	907±13^{b}	1434±24^{c}	217±4^{a}	309±5^{b}	355±7^{b}	303±9^{cd}	224±13^{d}
Sex										
Female	484±6^{b}	599±6^{b}	703±8^{b}	804±8^{b}	1312±16^{b}	179±3^{b}	277±4^{b}	321±5^{b}	267±6^{b}	241±9^{b}
Male	561±6^{a}	696±6^{a}	831±8^{a}	949±8^{a}	1633±16^{a}	199±3^{a}	334±4^{a}	387±5^{a}	346±6^{a}	338±9^{a}
AI infection										
Control	516±6	640±7	777±9	884±9	1468±17	186±3	302±4	369±5^{a}	309±6	273±10^{b}
Challenge	529±6	655±6	757±8	868±8	1477±16	192±3	309±4	339±5^{b}	304±6	306±9^{a}
Significance level (P)										
Breeds (B)	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
Sex (S)	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
AI Infection (A)	0.105	0.1312	0.0837	0.1762	0.6791	0.1613	0.1793	<.0001	0.5423	0.0149
B $\times$ S	0.7052	0.1938	0.0062	0.3505	0.2783	0.6111	0.7015	0.0937	<.0001	0.1791

[a,b,c,d] Means ± SE within the same column for a given stage with no common superscript differ significantly ($P < 0.05$).

Body temperature

There were significant effects of breed at all stages for body temperature, with the highest initial value in Shek-ki and the lowest initial value in Nagoya (Table 20). The challenge group showed a significantly higher body temperature as compared to the control group at BT0 and BT48 without any interaction between breed and challenge (Table 20). Body temperature tended to decrease 24 hours after challenge in control as well as in challenge group, but the main difference between the challenge and the control group was observed between 24h and 48h post-inoculation, since body temperature increased in the challenge group and decreased in the control.

Table 20. Least-square means of body temperature at different stages post challenge for the different breeds.

Source of variation	BT0	BT24	BT48	BTD24	BTD48
Breeds					
Hua-Tung	41.96±0.04^{bc}	41.79±0.04^{c}	41.88±0.04^{c}	-0.18±0.05^{b}	-0.09±0.06^{ab}
Ju-Chi	42.04±0.04^{b}	42.05±0.04^{a}	41.88±0.04^{c}	0.01±0.05^{a}	-0.16±0.05^{b}
Quemoy	41.94±0.03^{cd}	41.92±0.03^{b}	41.97±0.03^{bc}	-0.02±0.04^{a}	0.03±0.04^{a}
Shek-Ki	42.24±0.07^{a}	42.15±0.07^{a}	42.12±0.07^{ab}	-0.09±0.09^{ab}	-0.12±0.09^{ab}
Nagoya	41.84±0.05^{d}	41.76±0.05^{c}	41.88±0.05^{c}	-0.07±0.06^{ab}	0.05±0.06^{a}
SPF	42.11±0.06^{ab}	41.87±0.06^{bc}	42.16±0.06^{a}	-0.24±0.08^{b}	0.05±0.08^{a}
Hsin-Yi	41.97±0.04^{bc}	41.94±0.04^{b}	41.93±0.03^{c}	-0.03±0.05^{a}	-0.04±0.05^{ab}
Sex					
Female	41.95±0.03^{b}	41.9±0.03	41.94±0.02^{b}	-0.04±0.03	-0.01±0.03
Male	42.08±0.03^{a}	41.95±0.03	42.01±0.03^{a}	-0.13±0.03	-0.07±0.04
AI infection					
Control	41.97±0.03^{b}	41.89±0.03	41.84±0.03^{b}	-0.08±0.04	-0.13±0.04^{b}
Challenge	42.06±0.02^{a}	41.96±0.02	42.11±0.02^{a}	-0.1±0.03	0.05±0.03^{a}
Significance level (P)					
Breeds (B)	<.0001	<.0001	0.0001	0.0469	0.0485
Sex (S)	0.0003	0.2202	0.0357	0.0643	0.2225
B $\times$ S	0.0331	0.4287	0.6334	0.0048	0.0773
AI Infection (A)	0.0206	0.0641	<.0001	0.7404	0.0002
B $\times$ A	0.7087	0.0026	0.803	0.0113	0.6705

BT0 = body temperature at 0 hour post challenge; BT24 = body temperature at 24 hour post challenge; BT48 = body temperature at 48 hour post challenge; BTD24 = BT24 minus BT0; BTD48 = BT48 minus BT0.

^{a,b,c,d} Means ± SE within the same column for a given stage with no common superscript differ significantly ($P < 0.05$).

There was a significant interaction between breeds and challenge for body temperature 24 hours after challenge, so that there was no general effect of the challenge on body temperature at that

stage. Shek-Ki had still the highest body temperature 24h after challenge, whereas SPF exhibited the highest value 48h after challenge. Nagoya and Hua-tung had the lowest body temperatures 24 hours after challenge. Nagoya, Hua-Tung and Ju-Chi showed the lowest body temperature 48hours after challenge. Sex effect was significant for BT0 and BT48 with higher values in males but sex did not affect the change in body temperature. There was no interaction between sex and challenge.

Immune response

Some differences were observed between breeds at day 0 of challenge, particularly for Shek-Ki which showed a rather high antibody level, but there was no effect of the challenge on mean antibody values at day 0. Starting from 7 days post challenge, there were significant effects of the breed and of challenge on antibody titers (Table 21) but no effect of sex and no interaction between sex and challenge. The greater increase in antibody took place between day 7 and day 14. The interaction between breed and challenge was significant at all stages except day 0.

Quemoy had the most rapid antibody response on day 7 post-challenge (AI7; Figure 24). Other breeds showed increased antibody titers on day 14 post-challenge (AI14) but their titers remained lower than that observed in Quemoy at day 14. At day 21, antibody titers were close to their level at day 14, except for Hua-Tung which exhibited a strong increase in antibody titers between day 14 and day 21.

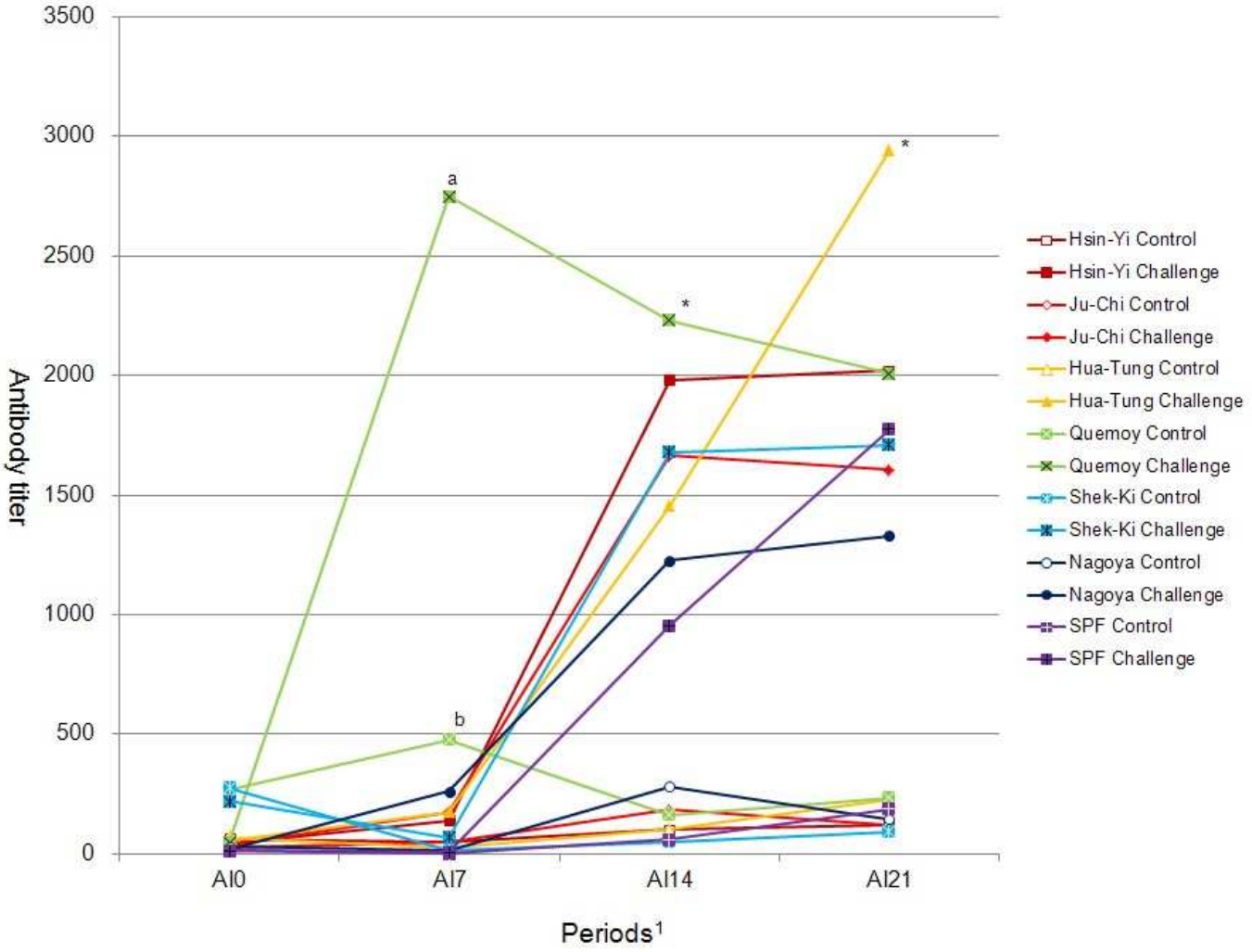

Figure 24. Antibody titers against H6N1 LPAIV on 0, 7, 14 and 28 days post-challenge in all breeds.

[a,b] within days post-challenge with no common superscript differ significantly ($P < 0.05$) in Quemoy.

* means all breeds have significantly different between control and challenge group.

Table 21. Least-square means of anti-AI antibody titer for different breeds at different stages post challenge.

Source of variation	AI0	AI7	AI14	AI21	AID7	AID14	AID21
Breeds							
Hua-Tung	60±13^{bc}	101±101^{b}	779±137^{bc}	1570±136^{a}	42±100^{b}	679±93^{bc}	739±73^{a}
Ju-Chi	32±13^{cd}	108±96^{b}	921±129^{abc}	863±130^{bc}	80±97^{b}	823±87^{ab}	-58±69^{c}
Quemoy	66±10^{b}	1566±75^{a}	1155±99^{a}	1084±102^{b}	1526±77^{a}	-411±67^{d}	-71±53^{c}
Shek-Ki	246±23^{a}	37±171^{b}	886±226^{abc}	909±233^{bc}	-209±170^{b}	848±153^{ab}	24±121^{c}
Nagoya	24±16^{cd}	205±119^{b}	683±156^{bc}	700±161^{c}	181±119^{b}	478±106^{c}	17±83^{c}
SPF	12±19^{d}	3±143^{b}	493±186^{c}	944±192^{bc}	-9±142^{b}	490±126^{b}	451±100^{b}
Hsin-Yi	51±11^{bcd}	97±85^{b}	1044±114^{ab}	1068±120^{bc}	48±85^{b}	945±77^{a}	7±62^{c}
Sex							
Female	75±8	290±61	837±80	973±82	214±61	550±55	140±43
Male	65±9	315±64	866±85	1066±88	260±64	551±58	177±46
AI Infection							
Control	76±9	95±67^{b}	118±89^{b}	144±91^{b}	18±67^{b}	23±60^{b}	27±48^{b}
Challenge	64±8	509±57^{a}	1585±77^{a}	1895±79^{a}	455±57^{a}	1078±52^{a}	290±41^{a}
Significance level (P)							
Breeds (B)	<.0001	<.0001	0.0185	0.0015	<.0001	<.0001	<.0001
Sex (S)	0.3815	0.7756	0.8087	0.4399	0.6048	0.996	0.5604
B × S	0.0539	0.9937	0.4984	0.7597	0.9899	0.2681	0.5233
AI Infection (A)	0.2919	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
B × A	0.8467	<.0001	0.0031	0.0052	<.0001	<.0001	<.0001
B × S × A	0.8859	0.9989	0.1637	0.7788	0.9924	0.0012	0.0341

AI0 = anti-AI antibody titer at 0 day post challenge; AI7 = anti-AI antibody titer at 7 days post challenge; AI14 = anti-AI antibody titer at 14 days post challenge; AI21 = anti-AI antibody titer at 21 days post challenge; AID7 = AI7 minus AI0; AID14 = AI14 minus AI7; AID21 = AI21 minus AI14.

^{a,b,c,d} Means ± SE within the same column for a given stage with no common superscript differ significantly ($P < 0.05$).

Correlations between body weight, body weight gain, body temperature, AI response

There was no significant correlation between BW and BT in both groups and both sexes, excepted in male of control group where BW7 showed a negative correlation with BT 24. There were significant negative correlations between anti-AI titers and BW in females of challenge group, and between anti-AI titers and BWG (7-10) in both sexes of challenge group. A positive correlation was observed between anti-AI titer and BWG (10-13) in female and BWG (13-16) in male.

MHC across breeds

Analysis of variance was performed with all breeds for the four MHC alleles that were shared by several breeds. This analysis did not reveal any effect of the MHC genotype across breeds. Main effects were still the breed and the AI challenge (Table 22).

MHC within breeds

Regarding private MHC alleles, analysis of MHC effect was performed within breed. Quemoy, Shek-Ki and SPF showed a significant effect of MHC genotypes on the boost of antibody titers.

Quemoy MHC genotype 381/381 showed higher antibody titers than other genotypes, and 309/309 had lower antibody titers whereas 309/381 had medium antibody titers, thus showing an additive effect (Figure 25a). When combined with allele 249, allele 309 appeared to have a negative effect. Thus, the Quemoy breed exhibits MHC effects with a positive effect of allele 381 and a negative effect of allele 309 on antibody titers.

Shek-Ki MHC genotype 345/345 (4 birds only) had higher antibody titers than 182/345 (6 birds). SPF MHC genotype 539/539 (8 birds) had higher antibody titers than 205/205 (7 birds).

Although the overall analysis in Hsin-Yi did not show an effect of genotype, there was surprising non-additive effects in this breed (Figure 25b) with a lower antibody level for the heterozygous genotype 217/309 genotype (7 birds) as compared to each homozygous (309/309 with 14 birds and 193/193 with 21 birds).

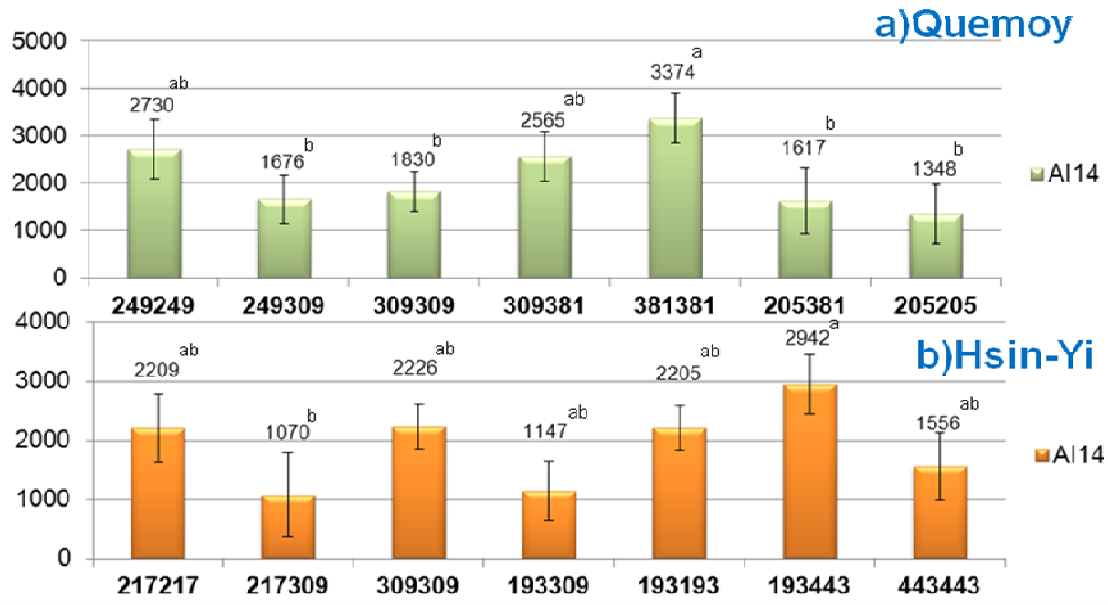

Figure 25. Antibody levels 14 days post-challenge with H6N1 according to MHC genotype. a) within the Quemoy breed. b) within the Hsin-Yi breed.

Table 22. Least-square means of anti-AI antibody titers for the main effects of breed, challenge and MHC genotype at different stages post challenge.

Source of variation	AI0	AI7	AI14	AI21
Breeds				
Hua-Tung	47±35	-166±282[b]	402±390	1504±364[a]
Ju-Chi	23±15	193±119[b]	1007±159	987±154[ab]
Quemoy	59±16	1355±126[a]	765±164	804±163[ab]
Nagoya	19±27	-271±219[b]	548±285	511±282[b]
SPF	-1±43	269±352[b]	246±454	322±451[b]
Hsin-Yi	55±19	47±148[b]	1144±201	1203±200[ab]
AI Infection				
Control	37±14	-46±111[b]	-59±154[b]	109±142[b]
Challenge	30±13	521±106[a]	1430±139[a]	1668±139[a]
MHC genotypes				
193193	31±22[ab]	303±174	575±239[ab]	806±229
193205	14±37[ab]	92±303	220±436[ab]	649±388
193309	6±27[b]	164±223	245±290[b]	679±295
205205	38±24[ab]	94±193	750±249[ab]	919±247
205309	56±25[ab]	107±206	838±272[ab]	781±268
249249	20±23[ab]	643±189	1164±244[a]	1412±243
249309	38±26[ab]	239±212	907±274[ab]	993±272
309309	66±17[a]	260±135	782±177[ab]	870±175
Significance level (P)				
Breeds (B)	0.3316	<.0001	0.2623	0.1863
AI Infection (A)	0.6379	<.0001	<.0001	<.0001
MHC genotype (M)	0.3535	0.6217	0.403	0.6872
A × M	0.9745	0.0084	0.6967	0.4638

AI0 = anti-AI antibody titer at 0 day post challenge; AI7 = anti-AI antibody titer at 7 days post challenge; AI14 = anti-AI antibody titer at 14 days post challenge; AI21 = anti-AI antibody titer at 21 days post challenge.

[a,b,c,d] Means ± SE within the same column for a given stage with no common superscript differ significantly ($P < 0.05$).

3. Discussion

General effects of the H6N1 challenge

The subtype A/chicken/Taiwan/0825/2006 H6N1 LPAIV was isolated in Nantou country located in the centre of Taiwan. The increase of antibody responses in the challenge group revealed that the H6N1 LPAIV challenge was effective. The results on body temperature are more difficult to interpret since an initial difference was observed between the challenge and the control groups, without any obvious explanation. However, it seems that body temperature increased between hour 24 and hour 48 in the challenge group only.

Results on body weight showed clearly a negative effect of the challenge on short term growth, which was compensated by an increased growth rate later on, corresponding to a recovery phase.

Breed effects

In this experiment, estimation of breed effects may be affected by limited sample size, particularly for Shek-Ki, SPF and Nagoya. As compared to the local breeds, SPF White Leghorn was the smallest breed until 13 weeks of age but reached the same body weight as Quemoy and Hsin-Yi at 16 weeks of age. This might be due to an environmental effect. All local breeds are kept in non-specific pathogen free environment, whereas SPF white leghorn chicks were introduced in the experiment room with other breeds and may have been exposed to a new ecosystem of pathogens typical of the six local breeds.

Due to the history of multiple introductions, Taiwan local chicken breeds differ by their phenotype and genetic background (Huang & Lee 2005) and differ for LEI0258 allele frequencies. Although Quemoy and Ju-Chi share many genetic features and have a similar body weight (Chang et al., 2011) they differed clearly by their immune response after H6N1 LPAIV challenge, with a faster response in Quemoy. This could be due to specific MHC alleles, such as the LEI0258 allele 381 which is more frequent in Quemoy than in Ju-Chi and was not present in the sample of Ju-Chi used for the challenge experiment. This allele was matched with B13.1 by Fulton *et al.* (2006). However, B13 was reported to have a negative effect on antibody response to Sheep Red Blood Cells in a F2 cross obtained from lines divergently selected on this antibody response (Dorshorst et al., 2010).

Shek-Ki had the highest body temperature and the heaviest body weight, and it is the only local breed showing a positive correlation between body temperature and body weight in the male

challenge group. This could suggest that these birds had already undergone some infection but there was no particular indication of an on-going infection in these birds before the experimental challenge.

Disease resistance vs. disease tolerance

According to FAO (2007) two phenomena must be distinguished in relation to the genetic management of disease, resistance and tolerance. Resistance refers to the ability of the host to resist to infection, tolerance refers to the ability of the host to limit the adverse effects of the infection that still takes place. In this experiment, local chicken breeds demonstrated different kinetics of responses to H6N1 LPAIV challenge, Quemoy showed the fastest and the highest immune response on day 7 and day 14 post-challenge with no mortality, so that Quemoy may be considered as the most resistant breed to H6N1 LPAIV challenge. Nagoya and SPF showed low antibody titers and no mortality after challenge and appeared to be tolerant to Taiwanese H6N1 LPAIV. On the other hand, Hsin-Yi showed the second highest level anti-AI antibody titer at day 14 post-challenge, but showed also the highest mortality, which occurred during 7 to 14 days post-challenge, thus Hsin-Yi was the least tolerant breed to Taiwanese H6N1 LPAIV infection. Ju-Chi, Hua-Tung and Shek-Ki showed medium to high antibody titers responses and low mortality.

B. AI challenge and vaccine response (AGAH congress 2, annex 2)

1. Introduction

This study investigated immune responses to Newcastle Disease, Infectious Bronchitis (IB) and Infectious Bursal Disease (IBD) vaccines following the H6N1 LPAIV challenge at 7 weeks of age on six local chicken breeds and SPF chicken. Vaccination against Newcastle Disease (ND), Infectious Bursal Disease (IBD) and Infectious Bronchitis (IB) was performed at 11 weeks. The anti-ND, anti-IBD and anti-IB antibody titers were measured by inhibition of hemagglutination test and ELISA at days 0, 14, 28 after vaccination.

2. Results

ND vaccine response

There was no effect of the H6N1 LPAIV challenge on anti-ND antibody titers at day 14 and 28 post-inoculation. Nagoya was the only one to show a difference of anti-ND antibody titer between the control and challenge groups at day 0, with a lower value in the challenge group. The breed effect was significant at all stages and the sex effect was not (data not shown). The Quemoy and SPF had high antibody levels from ND0 to ND28. Ju-Chi showed the lowest response to ND vaccination at day 28 post-inoculation.

IBD vaccine response

There was a significant effect of breed and no effect of the H6N1 LPAIV challenge on anti-IBD antibody titers at all stages. Interaction between breed and treatment tended to be significant at day 28 ($P < 0.05$) where the Quemoy was the only one to show significantly lower antibody titers in the challenge group (Fig. 26). There was a significant sex effect on responses at days 14 and 28 post-inoculation, and the antibody titers were higher in females than in males (data not shown). Nagoya and SPF showed no response to vaccination, but antibody titers of Nagoya were rather high at day 0. Quemoy and Hsin-Yi showed the highest antibody levels, particularly at day 28 post-inoculation. Ju-Chi, Hua-Tung and SPF showed the lowest antibody titers at all stages.

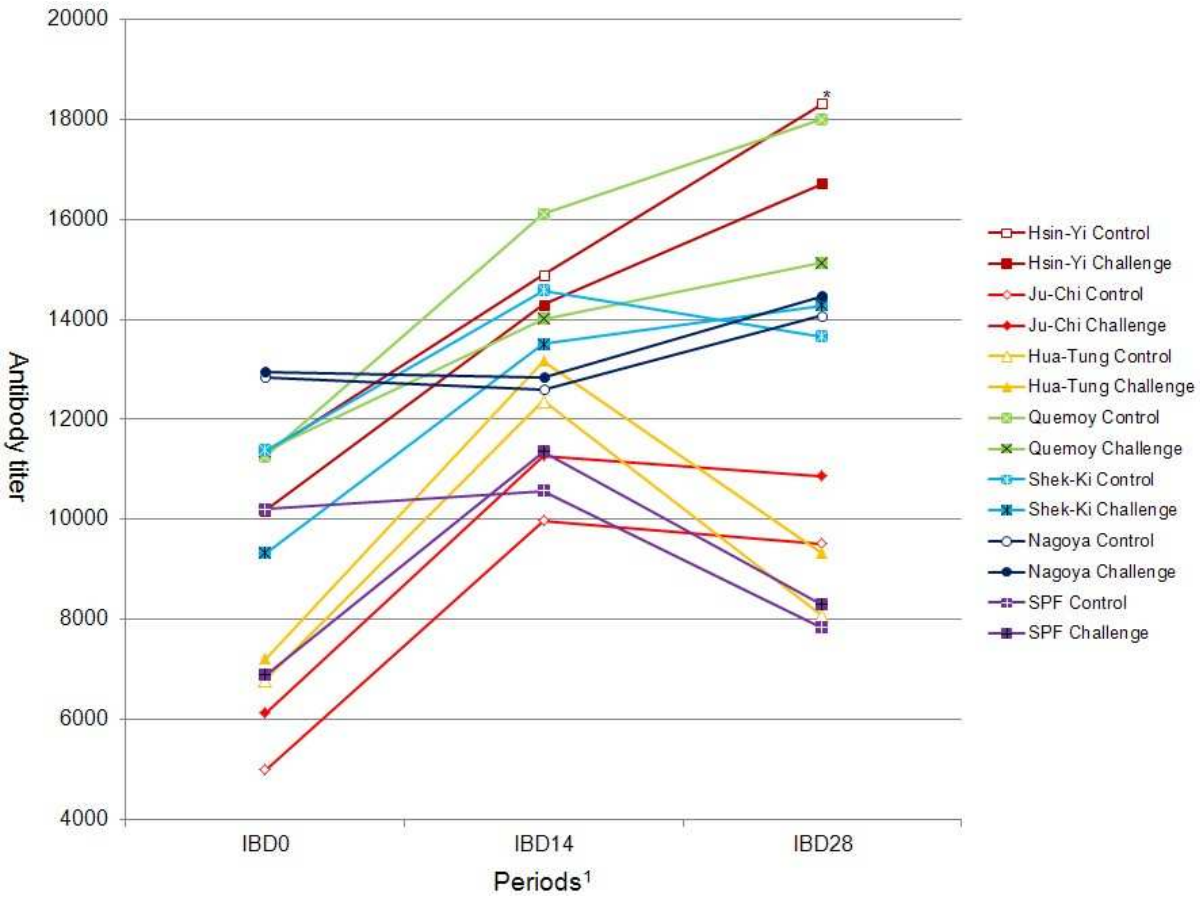

Figure 26. Antibody titers against IBD on 0, 14 and 28 days post-inoculation.

* Quemoy showed significantly lower antibody titer in the challenge group.

IB vaccine response

In contrast with the results observed for ND and IBD, the kinetics of antibody titers of IB was modified by the H6N1 LPAIV challenge (Figure 27). Interactions between breed and treatment, as well as between breed and sex, were significant for IB0. Antibody titers at day 0 were lower in the challenge group than in the control group for Ju-Chi and SPF, but did not differ between groups for the other breeds (Figure 28). The effects of breed and H6N1 challenge, without any interaction, were still observed at day 14 post-inoculation. Higher antibody levels were found in the control group for all breeds. Nagoya was the only one to exhibit a stronger response to IB vaccine in the H6N1 control group at day 14, as measured by the difference between titers at day 14 and day 0. The interaction between breed and sex was still significant at day 14 but was not observed at day 28. Breed and treatment effects were significant at day 28; antibody titers became higher in the challenge groups than in the control groups, whatever the breed. The increase in antibody titers between day 14 and day 28 was always higher in the challenge groups as compared to the control groups, this difference was highly significant in Ju-Chi and Nagoya

and tended to be significant ($P < 0.05$) in all other breeds except Shek-Ki which never showed any difference in anti-IB titers between the challenged and the control groups.

Breed comparison showed that Quemoy had the highest antibody titers for IB0. Quemoy, Hsin-Yi and Nagoya had the highest values for IB14. These three breeds had still high values for IB28, but Shek-Ki had also high values for IB28, although it exhibited low values for IB0 and IB14. Thus, this breed was characterized by a late and strong response to IB vaccine, without any significant effect of the previous H6N1 challenge.

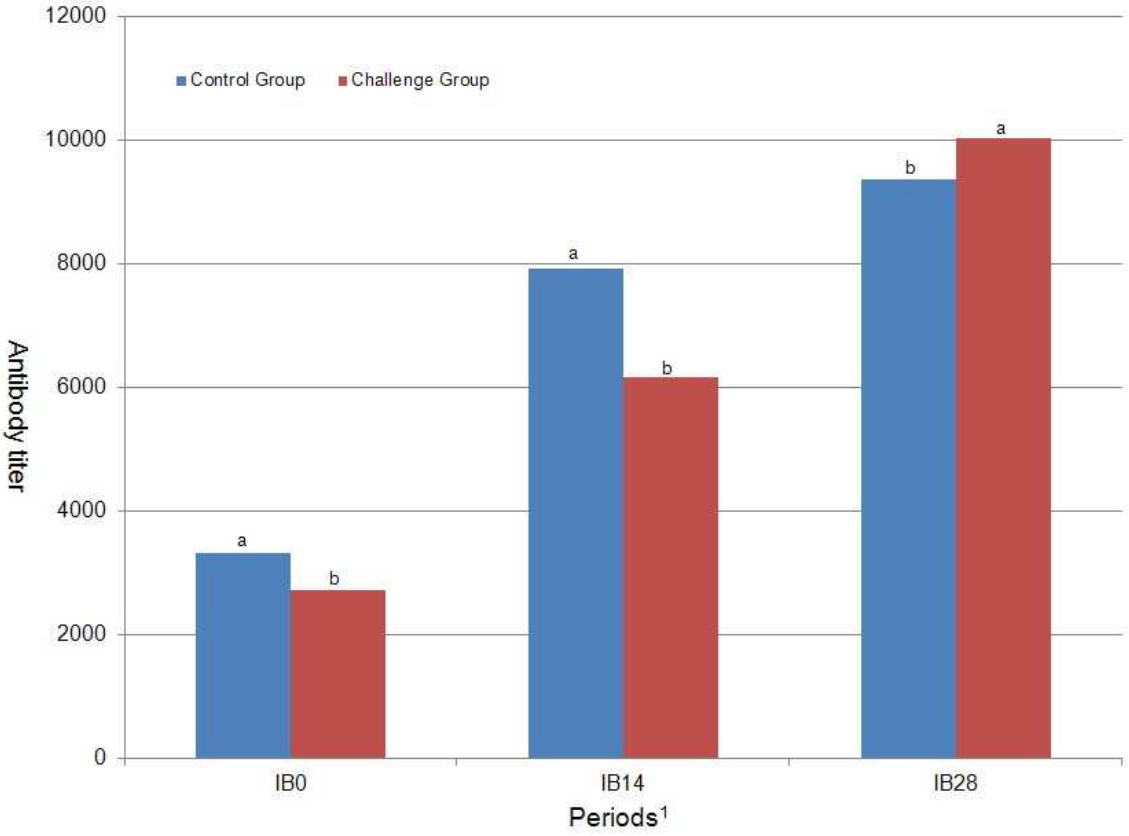

Figure 27. Antibody titers against IB on day 0, 14 and 28 post-inoculation, according to AI challenge.

[a,b] means with no common superscript differ significantly for a given stage ($P < 0.05$).

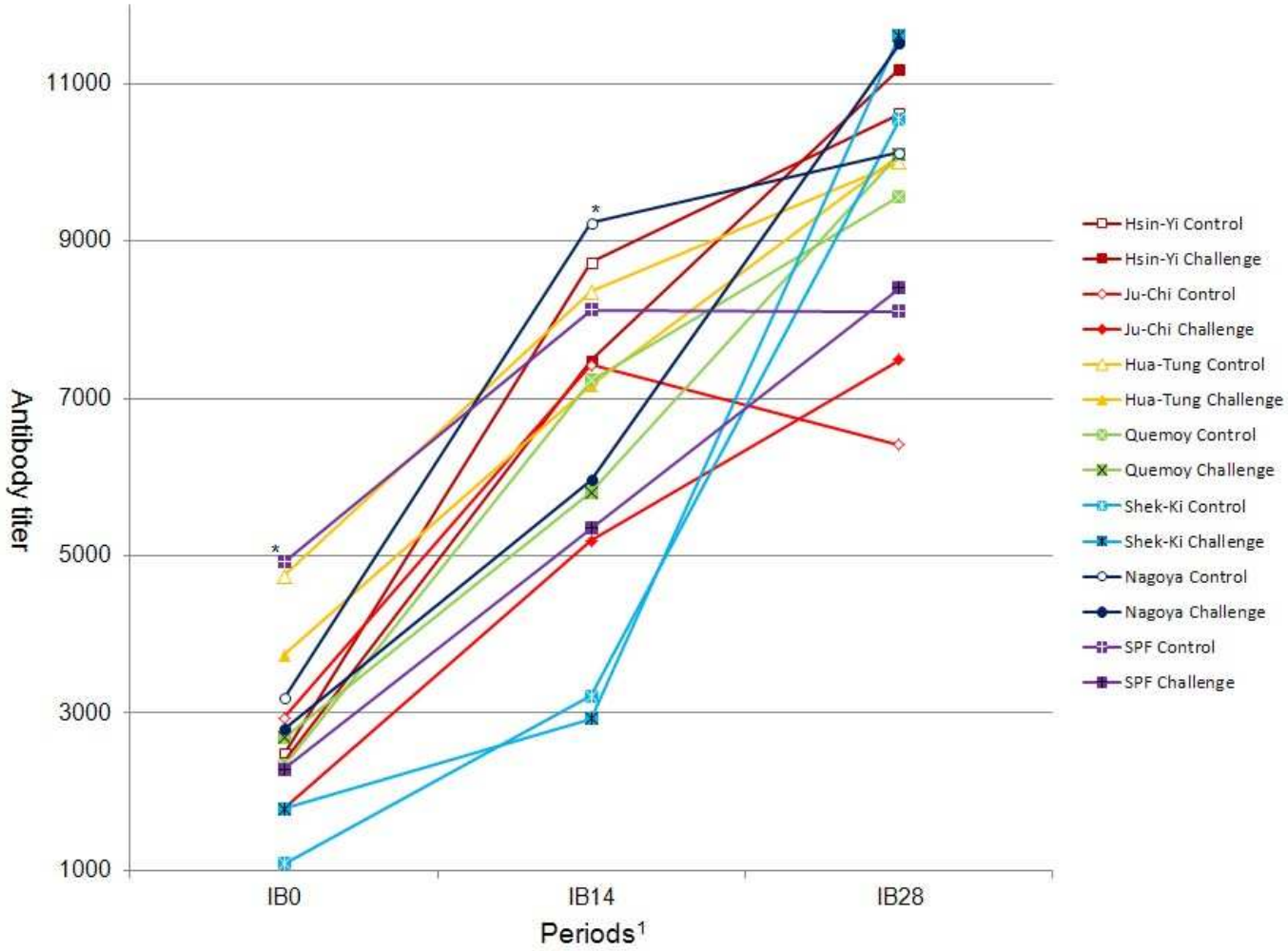

Figure 28. Antibody titers against IB on day 0, 14 and 28 post-inoculation, according to breed and AI challenge.

* Antibody titers at day 0 were lower in the challenge group than in the control group for Ju-Chi and SPF. The effects of breed and H6N1 challenge, without any interaction, were still observed at day 14 post-inoculation. Higher antibody levels were found in the control group for all breeds. Nagoya was the only one to exhibit a stronger response to IB vaccine in the H6N1 control group at day 14.

3. Discussion

Breeds effect, vaccine efficacy and duration of immunity

Antibodies were detected for ND, IB and IBD at day 0 post-inoculation because all chickens had been vaccinated for ND, IB and IBD at earlier ages. Thus, the immune response following the inoculation at 11 weeks of age may be considered as a secondary immune response. Antibody titers at day 14 post-inoculation were significantly higher than at day 0, showing vaccine efficiency, except in the case of Nagoya and SPF for IBD vaccine where no change in antibody levels was observed.

Breed significantly affected immune response. Quemoy had high and rapid responses to the three vaccines as it did for the H6N1 LPAIV challenge test, in contrast to Ju-Chi which had low immune response to vaccines and challenge test. Breed's effect on the antibody titers at day 0 revealed differences in the duration of immunity to previous vaccines. Quemoy appeared to have a better immunity than other breeds, Shek-Ki had a slow response to ND and IB, and Ju-Chi presented lowest response to IBD. The better immune response of Quemoy is consistent with previous results (Chang 2001). A negative relationship between growth rate and antibody response is generally observed, but it cannot explain the differences in immune response between Ju-Chi and Quemoy which have a similar body weight.

H6N1 LPAIV challenge effect on IB immune response

Whereas there was no effect of the H6N1 LPAIV challenge on the subsequent responses to ND and IBD vaccine at 11 weeks of age, surprisingly, the H6N1 LPAIV challenge significantly affected the kinetics of antibody levels to IB vaccine in some breeds. H6N1 LPAIV challenge had a negative effect on antibody levels to IB even before the vaccination at 11 weeks of age. Immune response to IB vaccine was delayed in H6N1 challenge groups (after 14 days). There was no final difference among the experimental groups for IB antibody titers at day 28.

The negative relationship of H6N1 LPAIV challenge and IB vaccine response could be related to the fact that both viruses target the lung tissues. Recently, Haghighat-Jahromi *et al.* (2007) showed that coinfection of H9N2 AI virus with IB live virus enhanced the virulence of H9N2 and increased the rate of mortality. In addition, Karimi-Madab *et al.* (2010) showed that IB live vaccine could be an important risk factor resulting in enhanced virulence of H9N2 LPAIV in field conditions. Although these studies were focusing on broilers and H9N2 LPAIV, the present study shows also an interaction between IB and H6N1 LPAIV infection in some local chicken

breeds. One could speculate that the type of local immune response induced by H6N1 LPAIV infection was not favourable for birds to make optimum IB-specific antibody response.

4. Conclusions

Local breeds have different immune response to H6N1 LPAIV challenge and subsequent vaccines. Differences dealt mainly with kinetics of response and with peak values. Quemoy exhibited higher antibody levels to H6N1, ND and IBD. The negative effect of the H6N1 LPAIV challenge on IB vaccine response may be related to the fact that both viruses target the lung tissues, and the type of local immune response induced by LPAIV challenge may not be favorable for birds to make optimum IB-specific antibody response.

FINAL DISCUSSION and PERSPECTIVES

FINAL DISCUSSION and PERSPECTIVES

I. Characterizing genetic diversity of a subset of local breeds

Although the number of founder animals and total population size were limited, some of the local breeds studied here exhibited a rather high level of heterozygosity for microsatellite markers, and varied greatly for their heterozygosity at the MHC complex. Clearly, heterozygosity was maintained at a rather high level for MHC in Hua-Tung, Quemoy and Shek-Ki, which was true only for Hua-Tung in the case of microsatellites. Heterozygosity at MHC may be favored by balancing selection in case of multiple pathogen pressure, where haplotype fixation would limit the immune response capacities. Originality of the Taiwanese breeds was also shown by the finding of new alleles of the LEI0258 marker.

The joint analysis of neutral markers and known genes made possible to distinguish neutral diversity and functional diversity and to identify selected loci. This was demonstrated by the study of the MC1R gene, which showed a breed-specific distribution of alleles that could be correlated to the known plumage colour. No other marker, including the MHC marker LEI0258, exhibited such a selection history.

The joint analysis of molecular and phenotypic data provided also a better discrimination between breeds than the multivariate analysis with molecular data alone. Yet, it appeared that most of the differentiation between breeds was already observed with molecular markers. Thus, adding phenotypic information for breed characterization would appear to be more useful for specific functions, such as heat tolerance or immune response. Regarding heat tolerance, panting rate revealed interesting differences between breeds, irrespective of body weight, that should be further characterized. Regarding immune response, the present study revealed that the Quemoy breed would be the most interesting breed for further studies and uses in breeding programmes. Its comparison with the Ju-Chi breed was particularly useful since both breeds appeared quite related in their genetic history but differed by MHC alleles frequency. Recording immune response traits in association with MHC genotyping could be a useful option for the characterization of local breeds in experimental conditions.

An alternative and more systematic approach for disease tolerance would consist in the search of selection signatures by using a much higher density of markers in a set of populations including local breeds from different climatic regions, as was done already with African cattle (Gautier *et al.* 2009).

II. Recommendations for the conservation program of local chicken breeds in Taiwan

The conservation program for keeping animal genetic resources is an important subject, because it is also costly to put into practice. Results of this thesis underline the usefulness to monitor the status of conservation populations. Polymorphism of LEI0258 marker in unselected populations could be a useful indicator for evaluating their genetic diversity. Monitoring MHC alleles frequencies should be recommended in order to limit drift effect at this locus. The important fluctuations of MHC alleles in the Hsin-Yi breed have not been explained, but at the same time, this breed exhibited the highest sensitivity to the challenge by the low pathogenic avian influenza virus.

Besides, a stable conservation program is also necessary, taking care of maintaining as constant as possible the numbers of sires, dams and offspring for each generation. Finally, quality of phenotype recording is also important to work on some adaptive traits, such as heat tolerance, and well-trained animal caretakers are needed.

III. Prospects for further studies

Prospects for further studies could be indicated for two parts

1. Population genetics and genetic diversity

High density panels of SNP markers are now available, that could be used for a more complete description of genome variability in these breeds in order to detect selective sweeps, such as the one detected with the *MC1R* gene. Indeed, these breeds may harbor specific alleles for adaptation to tropical environment.

Increasing the number of markers should be done together with an increase in the number of populations studied, in order to set up a large framework allowing the comparison between local tropical breeds, as well as between local breeds from various latitudes and climates.

2. MHC gene and immune response

a. A more accurate analysis of the chicken MHC region.

A 96 SNP chip has been designed at INRA, Jouy-en-Josas, by B. Bed'hom and O. Chazara, based upon the sequencing results of a set of 11 genes within the MHC and the

published sequences for part of the MHC region (Bed'hom & Chazara 2010, ISAG conference). This chip provides a more detailed analysis of the MHC complex and will make possible to identify alleles of expressed genes that are in linkage disequilibrium with the LEI0258 marker.

Preliminary results obtained with sixteen individuals and nine genotypes are shown in Table 23. SNPs mutations are generally well correlated with LEI0258 genotyping, except for Hsin-Yi 217/217, which might be due to a sample error.

Comparing alleles 309 (lower immune response to LPAIV) and 381 (higher immune response to LPAIV) for LEI0258 in Quemoy showed 26 differences among the 96 SNPs, not including the few instances where heterozygosity was observed within one of the individual. Furthermore, the analysis of SNP genotypes showed that Quemoy MHC allele 309 really matched with B24 but that Quemoy allele 381 did not match with B13.1 and Quemoy allele 205 did not match with B13 (Figure 29) which differ from the correspondence established previously by Fulton et al (2006). Interestingly, the new allele 217 found in Hsin-Yi appeared to cluster with the B13 group. Thus, more genotyping is needed for a more accurate description of MHC alleles in local chickens, and the functional consequences of these polymorphisms will require further studies.

Table 23. Genotypes of 96 MHC SNPs for nine homozygote genotypes at LEI0258

Breed	ID	LEI1	LEI2	SNPs point																																											
				2	**2**	**5**	**5**	**7**	**7**	**8**	**8**	**11**	**11**	**13**	**13**	**15**	**15**	**17**	**17**	**18**	**18**	**25**	**25**	**27**	**27**	**28**	**28**	**30**	**30**	**31**	**31**	**33**	**33**	**39**	**39**	**45**	**45**	**47**	**47**	**48**	**48**	**53**	**53**	**54**	**54**	**55**	**55**	**56**	**56**	**57**	**57**
Hua-Tung	A03	237	237	G	G	-	-	-	-	G	G	G	G	-	-	C	C	G	G	G	G	G	G	G	G	A	A	T	T	A	A	C	G	G	G	G	G	A	A	G	G	A	A	C	C	T	T	G	G	G	G
Hua-Tung	A04	237	237	G	G	-	-	-	-	G	G	G	G	-	-	C	C	G	G	G	G	G	G	G	G	A	A	T	T	A	A	C	G	G	G	G	G	A	A	G	G	A	A	C	C	T	T	G	G	G	G
Hua-Tung	A08	319	319	G	G	-	-	C	C	G	G	G	G	-	-	C	C	G	G	A	A	A	A	A	G	-	-	T	T	A	A	C	G	G	G	G	G	A	A	G	G	-	-	C	C	T	T	A	A	G	G
Hua-Tung	A11	419	419	A	A	-	-	A	A	A	A	G	G	-	-	C	C	A	G	A	A	A	A	G	G	A	A	T	T	G	G	C	C	G	G	A	A	A	A	A	A	G	G	C	C	T	T	A	A	A	A
Ju-Chi	B02	205	205	G	G	-	-	A	A	G	G	A	A	-	-	C	C	G	G	G	G	A	A	G	G	-	-	T	T	G	G	C	G	G	G	G	G	A	A	G	G	G	G	C	C	T	T	G	G	A	A
Quemoy	B07	205	205	G	G	-	-	A	A	G	G	A	A	-	-	C	C	G	G	G	G	A	A	G	G	-	-	T	T	G	G	C	G	G	G	G	G	A	A	G	G	G	G	C	C	T	T	G	G	A	A
Quemoy	B10	309	309	A	A	-	-	**A**	**A**	-	-	**G**	**G**	G	G	C	C	A	G	**A**	**A**	**A**	**A**	G	G	-	-	T	T	A	A	C	C	G	G	G	G	A	A	**A**	**A**	**G**	**G**	C	C	T	T	G	G	**A**	**A**
Quemoy	B11	309	309	A	A	-	-	**A**	**A**	-	-	**G**	**G**	G	G	C	C	A	G	**A**	**A**	**A**	**A**	G	G	-	-	T	T	A	A	C	C	G	G	G	G	A	A	**A**	**A**	**G**	**G**	C	C	T	T	G	G	**A**	**A**
Quemoy	B12	381	381	A	G	-	-	**C**	**C**	-	-	**A**	**A**	G	G	C	C	A	G	**G**	**G**	**G**	**G**	G	G	A	A	T	T	A	A	C	G	G	G	G	G	A	A	**G**	**G**	**A**	**A**	C	C	T	T	G	G	**G**	**G**
Quemoy	C01	381	381	A	G	-	-	**C**	**C**	-	-	**A**	**A**	G	G	C	C	A	G	**G**	**G**	**G**	**G**	G	G	A	A	T	T	A	A	C	G	G	G	G	G	A	A	**G**	**G**	**A**	**A**	C	C	T	T	G	G	**G**	**G**
Shek-Ki	C04	241	241	A	A	-	-	C	C	G	G	G	G	-	-	C	C	A	G	A	A	A	A	G	G	-	-	T	T	G	G	C	G	G	G	G	G	G	G	G	G	G	G	C	C	-	-	A	G	G	G
Shek-Ki	C05	241	241	A	A	-	-	C	C	G	G	G	G	-	-	C	C	A	G	A	A	A	A	G	G	-	-	T	T	G	G	C	G	G	G	G	G	G	G	G	G	G	G	C	C	-	-	-	-	G	G
Hsin-Yi	D04	217	217	G	G	-	-	C	C	A	G	A	G	G	G	C	G	-	-	A	G	A	A	A	G	A	A	T	T	G	G	C	G	G	G	G	G	A	A	G	G	A	G	C	C	T	T	A	A	G	G
Hsin-Yi	D05	217	217	G	G	-	-	C	C	A	G	A	G	G	G	C	G	-	-	G	G	A	A	A	G	-	-	T	T	G	G	C	G	G	G	G	G	A	A	G	G	A	A	C	C	T	T	A	A	G	G

Breed	ID	LEI1	LEI2	**58**	**58**	**60**	**60**	**61**	**61**	**62**	**62**	**63**	**63**	**64**	**64**	**65**	**65**	**66**	**66**	**67**	**67**	**68**	**68**	**69**	**69**	**70**	**70**	**71**	**71**	**73**	**73**	**74**	**74**	**75**	**75**	**79**	**79**	**80**	**80**	**81**	**81**	**83**	**83**	**84**	**84**	**85**	**85**	**87**	**87**	**89**	**89**
Hua-Tung	A03	237	237	G	G	A	A	A	A	A	A	G	G	A	A	G	G	C	C	A	A	A	A	G	G	A	A	G	G	A	A	A	A	G	G	G	G	G	G	A	A	A	A	G	G	A	A	G	G	A	A
Hua-Tung	A04	237	237	G	G	A	A	A	A	A	A	G	G	A	A	G	G	C	C	A	A	A	A	G	G	A	A	G	G	A	A	A	A	G	G	G	G	G	G	A	A	A	A	G	G	A	A	G	G	A	A
Hua-Tung	A08	319	319	G	G	A	A	A	A	A	A	G	G	G	G	G	G	C	C	A	A	G	G	G	G	A	A	A	A	A	A	G	G	A	A	A	A	A	A	A	A	A	A	G	G	A	A	G	G	A	A
Hua-Tung	A11	419	419	A	A	C	C	A	A	A	A	G	G	G	G	-	-	C	C	A	A	G	G	G	G	A	A	G	G	A	A	G	G	A	A	A	A	A	A	A	A	A	A	G	G	A	A	A	A	A	A
Ju-Chi	B02	205	205	A	A	C	C	-	-	G	G	A	A	G	G	G	G	C	C	G	G	G	G	G	G	A	A	G	G	A	A	G	G	A	A	G	G	G	G	A	A	A	A	G	G	A	A	G	G	C	C
Quemoy	B07	205	205	A	A	C	C	A	A	G	G	A	A	G	G	G	G	C	C	G	G	G	G	G	G	A	A	G	G	A	A	G	G	A	A	G	G	G	G	A	A	A	A	G	G	A	A	G	G	C	C
Quemoy	B10	309	309	**A**	**A**	**C**	**C**	A	A	A	A	G	G	G	G	**A**	**A**	C	C	**G**	**G**	**A**	**A**	**A**	**A**	A	A	G	G	A	A	G	G	A	A	G	G	G	G	A	A	A	A	G	G	A	A	**A**	**A**	C	C
Quemoy	B11	309	309	**A**	**A**	**C**	**C**	A	A	A	A	G	G	G	G	**A**	**A**	C	C	**G**	**G**	**A**	**A**	**A**	**A**	A	A	G	G	A	A	G	G	A	A	G	G	G	G	A	A	A	A	G	G	A	A	**A**	**A**	C	C
Quemoy	B12	381	381	**G**	**G**	**A**	**A**	A	A	A	A	G	G	G	G	**G**	**G**	C	C	**A**	**A**	**G**	**G**	**G**	**G**	A	A	G	G	A	A	G	G	A	A	G	G	G	G	A	A	A	A	G	G	A	A	**G**	**G**	C	C
Quemoy	C01	381	381	**G**	**G**	**A**	**A**	A	A	A	A	G	G	G	G	**G**	**G**	C	C	**A**	**A**	**G**	**G**	**G**	**G**	A	A	G	G	A	A	G	G	A	A	G	G	G	G	A	A	A	A	G	G	A	A	**G**	**G**	C	C
Shek-Ki	C04	241	241	G	G	A	A	A	A	A	A	G	G	A	A	G	G	C	C	G	G	A	A	A	A	A	A	G	G	A	A	G	G	A	A	G	G	G	G	A	A	A	A	G	G	G	G	G	G	A	A
Shek-Ki	C05	241	241	G	G	A	A	A	A	A	A	G	G	A	A	G	G	C	C	G	G	A	A	A	A	A	A	G	G	A	A	G	G	A	A	G	G	G	G	A	A	A	A	G	G	G	G	G	G	A	A
Hsin-Yi	D04	217	217	G	G	A	C	A	A	A	G	A	G	G	G	G	G	C	C	A	G	G	G	G	G	A	A	A	A	A	A	G	G	A	A	G	G	G	G	A	A	A	A	A	G	A	A	G	G	A	A
Hsin-Yi	D05	217	217	G	G	C	C	A	A	G	G	A	A	G	G	G	G	C	C	A	A	G	G	G	G	A	A	A	A	A	A	G	G	A	A	G	G	G	G	A	A	A	A	A	A	A	A	G	G	A	A

Table 23. (continued)

Breed	ID	LEI1	LEI2	91	91	92	92	94	94	96	96	97	97	99	99	101	101	102	102	103	103	104	104	105	105	106	106	108	108	111	111	112	112	116	116	118	118	119	119	120	120	122	122	124	124	127	127	128	128	130	130
Hua-Tung	A03	237	237	A	A	G	G	G	G	A	A	C	C	A	A	G	G	A	A	A	A	G	G	A	A	G	G	G	G	A	A	A	A	-	-	A	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
Hua-Tung	A04	237	237	A	A	G	G	G	G	A	A	C	C	A	A	G	G	A	A	A	A	G	G	A	A	G	G	G	G	A	A	A	A	-	-	A	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
Hua-Tung	A08	319	319	A	A	G	G	-	-	-	-	C	C	A	A	A	A	G	G	A	A	G	G	A	A	G	G	A	A	A	A	A	G	-	-	G	G	G	G	G	G	G	G	A	A	A	A	A	A	A	A
Hua-Tung	A11	419	419	A	A	G	G	A	A	A	A	A	A	A	A	G	G	G	G	G	G	G	G	A	A	A	A	A	G	A	G	G	G	-	-	-	-	G	G	G	G	G	G	A	A	G	G	A	A	A	A
Ju-Chi	B02	205	205	G	G	G	G	-	-	A	A	A	A	G	G	A	A	G	G	A	A	G	G	A	A	G	G	G	G	G	G	G	G	-	-	G	G	G	G	G	G	G	G	G	G	A	A	G	G	A	A
Quemoy	B07	205	205	G	G	G	G	-	-	A	A	A	A	G	G	A	A	G	G	A	A	G	G	A	A	G	G	G	G	G	G	G	G	-	-	G	G	G	G	G	G	G	G	G	G	A	A	G	G	A	A
Quemoy	B10	309	309	G	G	**A**	**A**	G	G	**A**	**A**	C	C	A	A	**G**	**G**	G	G	G	G	**A**	**A**	A	A	G	G	A	G	A	G	A	G	-	-	G	G	**G**	**G**	G	G	G	G	**A**	**A**	**A**	**A**	G	G	A	A
Quemoy	B11	309	309	G	G	**A**	**A**	G	G	**A**	**A**	C	C	A	A	**G**	**G**	G	G	G	G	**A**	**A**	A	A	G	G	A	G	A	G	A	G	-	-	G	G	**G**	**G**	G	G	G	G	**A**	**A**	**A**	**A**	G	G	A	A
Quemoy	B12	381	381	G	G	**G**	**G**	G	G	**C**	**C**	C	C	A	A	**A**	**A**	G	G	G	G	**G**	**G**	A	A	G	G	G	G	G	G	A	G	-	-	G	G	**A**	**A**	G	G	G	G	**G**	**G**	**G**	**G**	G	G	A	A
Quemoy	C01	381	381	G	G	**G**	**G**	G	G	**C**	**C**	C	C	A	A	**A**	**A**	G	G	G	G	**G**	**G**	A	A	G	G	G	G	G	G	A	G	-	-	G	G	**A**	**A**	G	G	G	G	**G**	**G**	**G**	**G**	G	G	A	A
Shek-Ki	C04	241	241	G	G	G	G	G	G	A	A	C	C	G	G	A	A	G	G	G	G	G	G	A	A	G	G	G	G	G	G	A	G	-	-	A	G	G	G	G	G	G	G	G	G	A	A	G	G	G	G
Shek-Ki	C05	241	241	G	G	G	G	G	G	A	A	C	C	G	G	A	A	G	G	G	G	G	G	A	A	G	G	G	G	G	G	A	G	-	-	A	G	G	G	G	G	G	G	G	G	A	A	G	G	G	G
Hsin-Yi	D04	217	217	A	G	G	G	A	A	C	C	A	A	A	A	A	G	A	G	G	G	G	G	A	A	A	G	G	G	G	G	G	G	-	-	G	G	A	G	A	G	A	G	G	G	G	G	G	G	A	A
Hsin-Yi	D05	217	217	A	A	G	G	A	A	C	C	A	A	A	A	G	G	G	G	G	G	G	G	A	A	A	A	G	G	G	G	A	G	-	-	G	G	G	G	G	G	G	G	-	-	G	G	G	G	A	A

				132	132	133	133	134	134	137	137	138	138	139	139	142	142	144	144	150	150	151	151	154	154	155	155	156	156	157	157	160	160	161	161	167	167	168	168	169	169	170	170	171	171	173	173	174	174	178	178
Hua-Tung	A03	237	237	G	G	A	A	G	G	G	G	C	C	G	G	C	C	G	G	G	G	C	C	-	-	G	G	G	G	A	A	G	G	A	A	C	C	G	G	A	A	G	G	A	A	G	G	A	A	C	C
Hua-Tung	A04	237	237	G	G	A	A	G	G	G	G	C	C	G	G	C	C	G	G	G	G	C	C	-	-	G	G	G	G	A	A	G	G	A	A	C	C	G	G	A	A	G	G	A	A	G	G	A	A	C	C
Hua-Tung	A08	319	319	G	G	G	G	C	C	G	G	C	C	G	G	C	C	G	G	G	G	C	C	-	-	G	G	G	G	A	A	G	G	A	A	C	C	G	G	A	A	G	G	A	A	G	G	G	G	C	C
Hua-Tung	A11	419	419	A	A	G	G	C	C	G	G	G	G	G	G	A	A	A	A	G	G	C	C	-	-	G	G	G	G	A	A	G	G	A	A	C	C	G	G	G	G	A	A	G	G	G	G	G	G	C	C
Ju-Chi	B02	205	205	G	G	G	G	C	C	A	A	C	C	G	G	C	C	G	G	A	A	C	C	-	-	G	G	G	G	G	G	G	G	G	G	C	C	G	G	A	A	A	A	G	G	G	G	G	G	C	C
Quemoy	B07	205	205	G	G	G	G	C	C	A	A	C	C	G	G	C	C	G	G	A	A	C	C	-	-	G	G	G	G	G	G	G	G	G	G	C	C	G	G	A	A	A	A	G	G	G	G	G	G	C	C
Quemoy	B10	309	309	G	G	G	G	G	G	G	G	**G**	**G**	G	G	C	C	G	G	**A**	**A**	C	C	-	-	G	G	G	G	A	A	G	G	A	A	C	C	G	G	**G**	**G**	A	A	G	G	**G**	**G**	**A**	**A**	C	C
Quemoy	B11	309	309	G	G	G	G	G	G	G	G	**G**	**G**	G	G	C	C	G	G	**A**	**A**	C	C	-	-	G	G	G	G	A	A	G	G	A	A	C	C	G	G	**G**	**G**	A	A	G	G	**G**	**G**	**A**	**A**	C	C
Quemoy	B12	381	381	G	G	G	G	G	G	G	G	**C**	**C**	G	G	C	C	G	G	**G**	**G**	C	C	-	-	G	G	G	G	A	A	G	G	A	A	C	C	G	G	**A**	**A**	A	A	G	G	**G**	**G**	C	C
Quemoy	C01	381	381	G	G	G	G	G	G	G	G	**C**	**C**	G	G	C	C	G	G	**G**	**G**	C	C	-	-	G	G	G	G	A	A	G	G	A	A	C	C	G	G	**A**	**A**	A	A	G	G	**G**	**G**	C	C
Shek-Ki	C04	241	241	G	G	G	G	G	G	G	G	C	C	G	G	C	C	G	G	A	A	C	C	-	-	G	G	G	G	A	A	A	A	A	A	C	C	G	G	A	A	A	A	A	A	G	G	G	G	C	C
Shek-Ki	C05	241	241	G	G	G	G	G	G	G	G	C	C	G	G	C	C	G	G	A	A	C	C	-	-	G	G	G	G	A	A	A	A	A	A	C	C	G	G	A	A	A	A	A	A	G	G	G	G	C	C
Hsin-Yi	D04	217	217	G	G	G	G	C	G	A	G	C	G	G	G	C	C	A	G	G	G	C	C	-	-	G	G	G	G	G	G	G	G	A	A	C	C	A	A	A	G	A	G	A	G	A	G	A	G	C	C
Hsin-Yi	D05	217	217	G	G	G	G	G	G	A	A	G	G	G	G	C	C	A	G	G	G	C	C	-	-	G	G	G	G	G	G	G	G	A	A	C	C	A	A	A	A	G	G	A	A	G	G	A	A	C	C

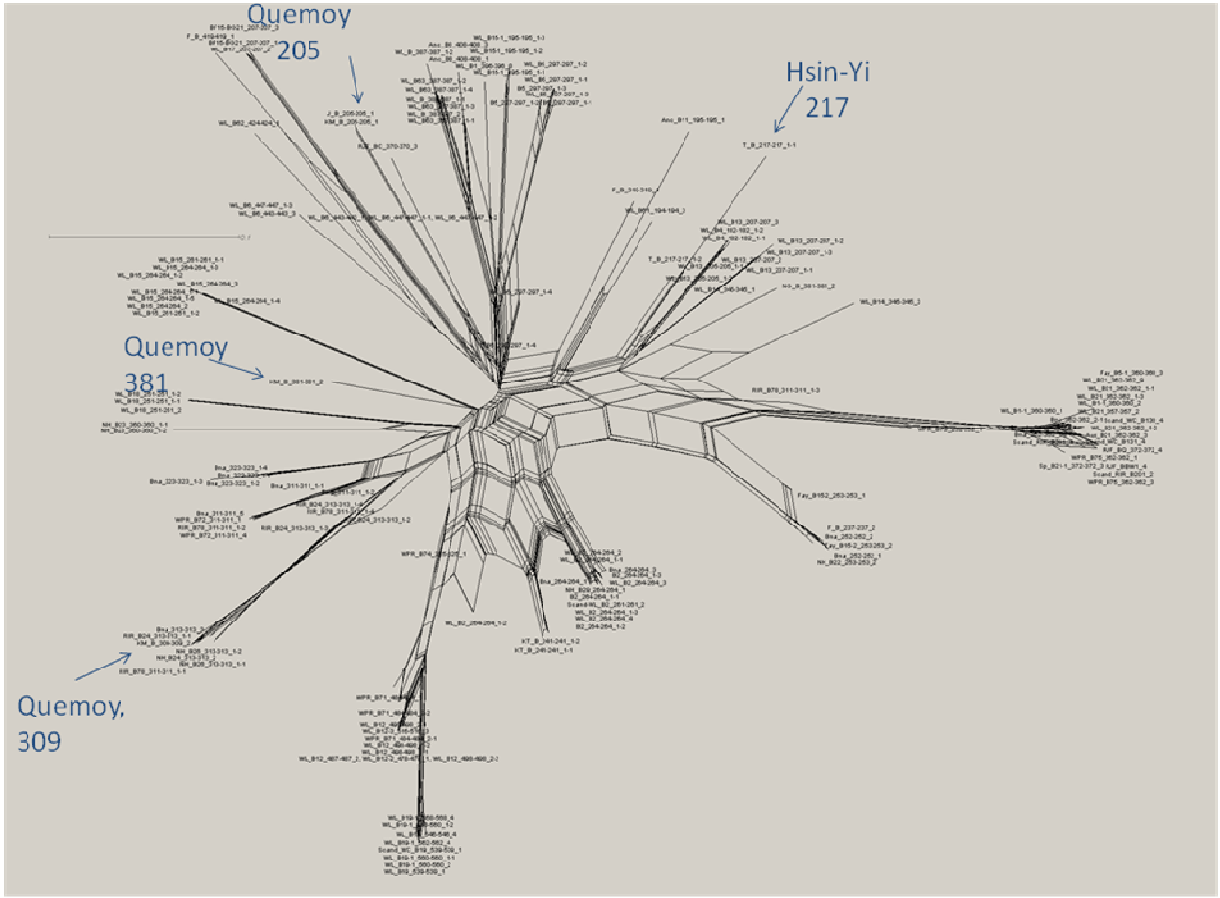

Figure 29. Neighbornet of a sample of Taiwan chickens genotyped with a 96 SNP chip dedicated to MHC.

The position of a subset of Quemoy and Hsin-Yi samples is shown according to the size of LEI0258 allele.

b. Applied chicken microarray for analyzing the effect of MHC genotypes and gene expression.

Monitoring gene expression patterns by transcriptome analysis would provide more insight into the mechanisms explaining the differential immune response between carriers of 381 and 309 alleles of the Quemoy breed. A pilot study was undertaken with the Affymetrix generic chip for chicken. A subset of 5 to 6 animals from each homozygous genotype was challenged at 7 weeks of age with the same strain of H6N1 LPAIV used in this thesis. Lung and immune tissues (bursa, spleen) were sampled 9 days after inoculation and RNA was extracted.

Preliminary results showed a differential hybridization between the two genotypes for a subset of MHC genes. The most probable explanation would be that the allelic sequences of one of the two alleles differed so much from the sequences used to make the chip that hybridization did not occur properly. This would not be surprising considering the large number of differences revealed by the SNP genotyping between alleles 381 and 309.

As a consequence, this means that a transcriptomic analysis of immune response genes between MHC genotypes should be done by RNA resequencing instead of micro-array hybridization.

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ANNEX 1

Chang C.S., Tixier-Boichard M., Charaza O., Lee Y.P., Chen C.F. & Bed'hom B. (2010) Monitoring polymorphism at the major histocompatibility complex level in local chicken breeds under a conservation program in Taiwan. *XIIIth European Poultry Conference*, 23-27 August 2010, Tours, France.

Monitoring Polymorphism at the Major Histocompatibility Complex level in Local Chicken Breeds under a Conservation Program in Taiwan

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MHC polymorphism in local chickens

Summary

National Chung-Hsing University has a conservation program for six local chicken breeds: Hsin-Yi, Ju-Chi, Hua-Tung, Quemoy (Taiwan), Shek-Ki (China), and Nagoya (Japan). The polymorphism at the MHC (Major Histocompatibility Complex) locus was investigated with the LEI0258 marker in three generations of each breed, between 2001 and 2008. We described 18 alleles for this marker, of which 12 are line-specific and 8 are new. The mean number of alleles per line varied from 3 to 8. Hua-Tung always had the highest observed heterozygosity (0.89 in 2007), Ju-Chi had the lowest heterozygosity in 2001 and 2007 (respectively 0.40 and 0.42) and Hsin-Yi in 2008 (0.45). Whereas Ju-Chi, Nagoya and Shek-Ki were under Hardy-Weinberg equilibrium at the MHC locus, Hua-Tung and Hsin-Yi were not ($P<0.05$). and the Quemoy breed was not in 2008. The departure from equilibrium was due to an excess of homozygotes, which could be due to genetic drift with small family size. In addition, we observed clear changes in allelic frequencies for the Hsin-Yi breed that could indicate short term selection pressures. In conclusion, polymorphism at the MHC locus can serve as a useful indicator of genetic diversity for monitoring conservation of small populations.

Keywords: local chicken, Major Histocompatibility Complex, heterozygosity, Hardy-Weinberg equilibrium

Introduction

A conservation program was set up in 1982 for 4 local chicken breeds and 2 imported breeds at National Chung-Hsing University. Three of them represent Taiwan local chickens: Hsin-Yi was sampled from an aboriginal tribe in the central mountain of Taiwan, Ju-Chi was sampled from a village in central-south of Taiwan and Hua-Tung from east of Taiwan. Quemoy was sampled from Quemoy Island near Fu-Jian Province of China. Shek-Ki originated from

Kwang-Tung Province of South China and Nagoya from Japan. The LEI0258 marker exhibits a good correlation with serological typing of the chicken MHC (Fulton et al., 2006). New LEI0258 alleles have been reported in Taiwan local breeds, and allele frequency was calculated for the generation born in 2001 (Chang, 2008), In this study, we genotyped LEI0258 for generations born in 2007 and 2008, in order to analyse possible trends in allelic frequency at the MHC locus across generations for each breed. Any trend could be due either to drift or to natural selection since breeds are under conservation.

Material and Methods

Experimental chickens

Lines are bred randomly with a full pedigree system and a number of 13 to 19 sires per line per generation. Blood samples were collected from breeders in 2001, 2007 and 2008. Numbers of animals for each generation are listed in Table 1.

Table 1. Number of sires, dams, and genetic size (Ne) for each breed in each generation

Breeds	2001			2007			2008		
	sires	dams	Ne	sires	dams	Ne	sires	dams	Ne
Hsin-Yi	14	24	35	11	22	29	19	53	56
Ju-Chi	15	25	38	24	34	56	19	47	54
Hua-Tung	12	25	32	19	28	45	10	38	32
Quemoy	17	36	46	19	33	48	18	43	51
Shek-Ki	17	16	14	16	31	42	14	46	43
Nagoya	16	37	45	22	45	59	19	46	54

MHC genotyping

PCR primers for LEI0258 are CACGCAGCAGAACTTGGTAAGG forward and AGCTGTGCTCAGTCCTCAGTGC reverse. PCR was done using 20 ng of genomic DNA, with 1 pmol of each primer, and 0.5 units of HotStarTaq Master Mix Kit (Qiagen) in a final volume of 25 µl. The amplification protocol included a first cycle of initial denaturation for 15 min at 95°C , followed by 35 cycles of 94°C for 45 s, 62°C for 90 s, 72°C for 60 s, with a last cycle of extension for 15 min at 72°C. Gel migration was realized with a 4% agarose gel for a first determination of allele size. Due to the complex structure of the LEI0258 sequence which contains a 13-bp and a 12-bp repeat motif, and several flanking SNP sites, sequencing is necessary to provide an unambiguous identification of alleles. Direct sequencing of PCR products was performed for homozygous genotypes (following Fulton *et al.*, 2006) or after agarose gel cutting and purification with QIAquick PCR Purification Kit (Qiagen) for heterozygous genotypes. Sequencing was done by Eurofins MWG Operon, using their standard protocol for purified PCR products. The BioEdit version 7.0.9.0 (Hall, 1999) was

used to assemble sequences, and to check the length and polymorphic sites (either single nucleotide polymorphisms or deletions) for each allele.

Statistical analysis

Heterozygosity was calculated with GENETIX 4.05 (Belkhir et al., 2000). Hardy-Weinberg (H-W) equilibrium test was calculated with Genepop 4.0 (Raymond and Rousset., 1995).

Results and discussion

A total of 17 alleles were identified for marker LEI0258, seven of them were new and did not match with a known B haplotype. Hua-Tung exhibited three new alleles, Shek-Ki and Hsin-Yi exhibited two new alleles (with one in common) and Nagoya exhibited one new allele.

Allele frequency fluctuated slightly between generations in most breeds (Table 2). Hsin-Yi, Ju-Chi, Hua-Tung and Shek-Ki exhibited some rare alleles (frequency below 10%) whereas Quemoy and Nagoya exhibited a rather balanced distribution of alleles. Hsin-Yi showed a marked increase in frequency of allele 193 and a marked decrease in frequency of allele 443.

Table 2. LEI0258 allele size, frequency and corresponding B haplotypes.

Breeds	Allele size	Allele frequency (%) 2001	2007	2008	Breeds	Allele size	Allele frequency (%) 2001	2007	2008
Hsin-Yi	193	11	38	54	Quemoy	205	20	13	13
	217[a]	2	18	9		249	1	12	20
	273[a]	-	1	-		309	54	43	47
	309	44	27	29		381	25	31	20
	345	-	1	-	Nagoya	181	40	33	38
	357	-	1	2		249	56	43	42
	443	44	15	6		309	4	25	21
Ju-Chi	193	-	5	7	Shek-Ki	182	21	26	34
	205	17	27	28		241	14	24	26
	249	5	1	1		273	34	25	19
	309	77	67	63		309	-	-	1
	381	1	-	-		345	31	25	21
Hua-Tung	193	6	14	7					
	237	-	16	21					
	249	19	14	20					
	295	22	14	11					
	309	7	3	1					
	319	11	5	13					
	357	16	13	13					
	419	19	21	14					

[a] new allele of LEI0258 marker

Table 3. Heterozygosity and test for Hardy-Weinberg equilibrium.

Breeds	Year	Hn.b.	Hobs.	F_{is}	P-value of H-W test	Significant
Hsin-Yi	2001	0.61	0.51	0.1688	0.0466	*
	2007	0.73	0.68	0.0649	0.0000	***
	2008	0.61	0.45	0.2699	0.0002	***
Quemoy	2001	0.61	0.65	-0.0566	0.7236	
	2007	0.69	0.71	-0.0365	0.9269	
	2008	0.69	0.56	0.1836	0.0000	***
Hua-Tung	2001	0.84	0.83	0.0126	0.0080	*
	2007	0.86	0.89	-0.0355	0.0114	*
	2008	0.85	0.71	0.1631	0.0000	***

Hua-Tung had always the highest observed heterozygosity and Ju-Chi had the lowest, except in 2008 where the lowest heterozygosity value was observed in Hsin-Yi.

Inbreeding coefficient (Fis) was highest in Hsin-Yi, Quemoy and Hua-Tung in 2008, whereas Ju-Chi, Shek-Ki and Nagoya exhibited lower values, consistent with the status of random mating. Results of H-W equilibrium test pointed out that Ju-Chi, Shek-Ki and Nagoya were under Hardy-Weinberg equilibrium at the MHC locus. However, Hua-Tung and Hsin-Yi were not in equilibrium ($P<0.05$) for all three generations, and Quemoy was not in equilibrium for generation 2008 only. The departure from equilibrium was due to an excess of homozygotes, which is quite surprising for MHC. This could be due to genetic drift with small family size and fluctuating number of sire families between generations. Hua-tung breed had the largest number of alleles and genotype frequencies may be quite susceptible to sampling variation.

One of the new alleles not described in 2001 (allele 237) was found in Hua-Tung at a moderate frequency in 2007-2008, which could suggest recent selection. Allele 249 (matching MHC B15.2) exhibited a similar trend in Quemoy breed. Regarding Hsin-Yi breed, the important changes in allelic frequencies observed for alleles 193 and 443 could indicate short term selection pressures. These alleles can be matched to MHC B15.1 and B6, respectively. Allele 193 was also found in Ju-Chi and Hua-tung but did not exhibit any specific trend in these breeds. Allele 443 was only found in Hsin-Yi, and could have been counter-selected. Effects of MHC alleles on performance or viability deserve further studies in the Hsin-Yi breed.

In conclusion, polymorphism at the MHC locus can be easily monitored with a single highly variable marker and can serve as a useful indicator in monitoring genetic diversity for conservation of small populations. Mating plans could be designed in order to avoid allele loss at the MHC locus. Keeping a stable number of sire families could be recommended in order to avoid any effect of sampling on allele frequencies.

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ANNEX 2

Chang C.S., Tixier-Boichard M., Charaza O., Lee Y.P., Chen C.F., Chang P.C., Chen J.W. & Bed'hom B. (2011) Different immune responses to three different vaccines following H6N1 low pathogenic avian influenza virus challenge in Taiwanese local chicken breeds. BMC Proceedings, 5(Suppl 4):S33. Paper presented at the *Animal Genomics for Animal Health International Symposium*, 31 May – 2 June 2010, Paris, France.

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Different immune responses to three different vaccines following H6N1 low pathogenic avian influenza virus challenge in Taiwanese local chicken breeds

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Abstract

Background: H6N1 low pathogenic avian influenza virus (LPAIV) are frequently isolated in Taiwan and lead to significant economic losses, either directly or indirectly through association with other infectious diseases. This study investigates immune responses to three different vaccines following a H6N1 challenge in different local breeds.

Methods: Experimental animals were sampled from six local chicken breeds maintained at the National Chung-Hsing University, namely Hsin-Yi, Ju-Chi, Hua-Tung (Taiwan), Quemoy (Quemoy Island), Shek-Ki (China), Nagoya (Japan) and a specific pathogen free (SPF) White Leghorn line. A total number of 338 chickens have been distributed between a control and a challenge group, H6N1 challenge was performed at 7 weeks of age; vaccination against Newcastle Disease (ND), Infectious Bursal Disease (IBD) and Infectious Bronchitis (IB) was performed at 11 weeks. The anti-H6N1 LPAIV antibody titers were measured by ELISA at days 0, 7, 14 and 21 after challenge, and the anti-ND, anti-IBD and anti-IB antibody titers were measured by inhibition of hemagglutination test and ELISA at days 0, 14, 28 after vaccination.

Results: There was no effect of the H6N1 LPAIV challenge at 7 weeks of age on the subsequent responses to ND and IBD vaccine at 11 weeks of age, but, surprisingly, the H6N1 LPAIV challenge significantly affected antibody levels to IB vaccine in some breeds, since IB0 and IB14 antibody titers were lower in the challenge groups. However, there was no significant difference in IB28 antibody titers among the experimental groups.

Conclusions: Local breeds have different immune response to H6N1 LPAIV challenge and subsequent vaccines. Differences dealt mainly with kinetics of response and with peak values. Quemoy exhibited higher antibody levels to H6N1, ND and IBD. The negative effect of the H6N1 LPAIV challenge on IB vaccine response may be related to the fact that both viruses target the lung tissues, and the type of local immune response induced by LPAIV challenge may not be favourable for birds to make optimum IB-specific antibody response.

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Background

Since 1982, National Chung-Hsing University is maintaining six local chicken breeds: Hsin-Yi, Ju-Chi and Hua-Tung were collected from small villages in Taiwan, Quemoy was collected from Quemoy Island near China, Shek-Ki was from China, and Nagoya was from Japan [1]. In previous studies, Quemoy had significantly higher antibody titers against Newcastle Disease (ND) after vaccination than other local breeds, Shek-Ki, Hua-Tung and Ju-Chi had lower anti-ND antibody titers [2]. Thus, immune response was shown to differ within this set of local chicken breeds.

H6N1 Low pathogenic Avian Influenza Virus (LPAIV) is frequently isolated in Taiwan and lead to significant economic losses, either directly or indirectly through association with other infectious diseases [3]. This study investigated immune responses to ND, Infectious Bronchitis (IB) and Infectious Bursal Disease (IBD) vaccines following a H6N1 LPAIV challenge on six local chicken breeds and SPF chicken.

Methods

Experimental chickens

In this study we used the six local breeds and added an SPF White Leghorn as a control genotype.

A total of 314 chicks were hatched from 23 sires and 91 dams with full pedigree in six local breeds. Twenty-five SPF chicks were purchased from Animal Health Research Institute (Council of Agriculture, Executive Yuan R.O.C.). Day-old chicks were wing-banded and raised in floor pens until 5 weeks of age, and they were transferred to experimental cages after 6 weeks of age. Sire families were distributed between the control and the challenge group. Individual body weights were recorded weekly from hatch to 16 weeks of age.

Vaccination program and challenge

Day-old chicks were all vaccinated against Marek's disease and ND. At two weeks of age, chicks were vaccinated against ND, IB, IBD, Fowl Pox and Avian Reovirus infection. At four weeks of age, chickens were vaccinated against ND, IB, IBD and Infectious Laryngotracheitis. The H6N1 LPAIV (A/chicken/Taiwan/0825/2006) challenge was performed at 7 weeks of age, birds from the challenge group received a drop with 10^7 EID_{50} of viruses into eye and nose. Blood samples were collected at days 0, 7, 14 and 21 post-challenge. Chickens health condition and mortality were recorded and monitored. At 11 weeks of age, all chickens, from both challenge and control groups, were vaccinated again against ND, IB and IBD, blood samples were collected at days 0, 14 and 28 post-inoculation. Sera from blood samples were collected and stored in -20 ℃ refrigerator.

Immune response measurement

The antibody responses to H6N1 LPAIV, IBD and IB were measured by enzyme-linked immunosorbent assay (ELISA) with commercial test kit (IDEXX Laboratories, Inc., Westbrook, ME), the antibody titer calculation was performed according to IDEXX's formula. The antibody responses to ND were measured by hemagglutination inhibition test (HIT), the antibody titer was expressed as the log2 of the reciprocal of the highest dilution. Each measure was defined by the virus name (AI, ND, IBD or IB) and the day of sampling after inoculation (either days 0, 7, 14, 21 or 28). In addition, response to IB vaccine was calculated by the difference in antibody titers between stages, i.e. day 0 to day 14, day 14 to day 28 and day 0 to day 28.

Statistical analysis

Antibody titers and differences between successive titers were analysed with the following statistical model,

$$Y_{ijkl} = \mu + \tau_i + \alpha_j + s_k + (\tau\alpha)_{ij} + e_{ijkl}$$

where Y_{ijkl} is the antibody titer of the lth animal of the ith breed, the kth sex after the jth challenge treatment, i=1, 2,...,7, j=1,2, k=1,2, l=1,2,...,338, μ is the mean, τ_i is the fixed effect of the ith breed, $\Sigma\tau_i$=0, α_j is the fixed effect of the jth challenge treatment, $\Sigma\alpha_j$=0, $(\tau\alpha)_{ij}$ is the fixed interaction effect between the ith breed and the jth challenge treatment , $\Sigma\Sigma(\tau\alpha)_{ij}$=0, and e_{ijkl} is the residual random error, $e_{ijkl} \cap N(0, \sigma_e^2)$.

All statistical analyses were conducted by using SAS software (SAS Institute).

Results

H6N1 LPAIV challenge

There was no mortality for Quemoy, Nagoya and SPF chickens (Table 1). Ju-Chi, Hua-Tung and Shek-Ki exhibited a low mortality (one bird each), and Hsin-Yi had the highest mortality: 7 birds (22.6%, see Table 1). Most of mortality occurred between day 7 and 14 post-challenge. The analysis of variance showed a significant effect of breed on all antibody titers, no effect of sex, and a significant effect of the challenge with a significant breed with challenge interaction on antibody titers from day 7 (Fig 1). This interaction was due to differential response between breeds: Quemoy had the highest antibody titer on day 7 and 14 post-challenge, Hua-Tung showed the highest antibody titer on day 21 post-challenge, Nagoya and SPF showed lower antibody titer on day 14 and 21 post-challenge. Quemoy was the only breed to exhibit a significant antibody titer at day 7 post-challenge. The anti-AI antibody titers were significantly higher for all breeds at days 14 and 21 post-challenge.

ND vaccine response

There was no effect of the H6N1 LPAIV challenge on anti-ND antibody titers at day 14 and 28 post-inoculation (Fig 2). Nagoya was the only one to show a difference of anti-ND antibody titer between the control and

Table 1 Sample size and mortality per breed in H6N1 challenge experiment

	Hsin-Yi	Ju-Chi	Hua-Tung	Quemoy	Shek-Ki	Nagoya	SPF
Control group	31	25	23	42	9	15	12
Challenge group	31	27	28	49	12	22	12
Total size	62	52	51	91	21	37	24
Mortality*	7	1	1	0	1	0	0
Mortality rate (%)	22.6	3.7	3.6	0	8.3	0	0

* Mortality was calculated within challenge group.

challenge groups at day 0, with a lower value in the challenge group. The breed effect was significant at all stages and the sex effect was not (data not shown). The Quemoy and SPF had high antibody levels from ND0 to ND28. Ju-Chi showed the lowest response to ND vaccination at day 28 post-inoculation.

IBD vaccine response

There was a significant effect of breed and no effect of the H6N1 LPAIV challenge on anti-IBD antibody titers at all stages. Interaction between breed and treatment tended to be significant at day 28 ($P < 0.05$) where the Quemoy was the only one to show significantly lower antibody titers in the challenge group (Fig 3). There was a significant sex effect on responses at days 14 and 28 post-inoculation, and the antibody titers were higher in females than in males (data not shown). Nagoya and SPF showed no response to vaccination, but antibody titers of Nagoya were rather high at day 0. Quemoy and Hsin-Yi showed the highest antibody levels, particularly at day 28 post-inoculation. Ju-Chi, Hua-Tung and SPF showed the lowest antibody titers at all stages.

IB vaccine response

In contrast with the results observed for ND and IBD, the kinetics of antibody titers of IB was modified by the H6N1 LPAIV challenge (Fig 4). Interactions between breed and treatment, as well as between breed and sex, were significant for IB0. Antibody titers at day 0 were lower in the challenge group than in the control group for Ju-Chi and SPF, but did not differ between groups for the other breeds (Fig 5). The

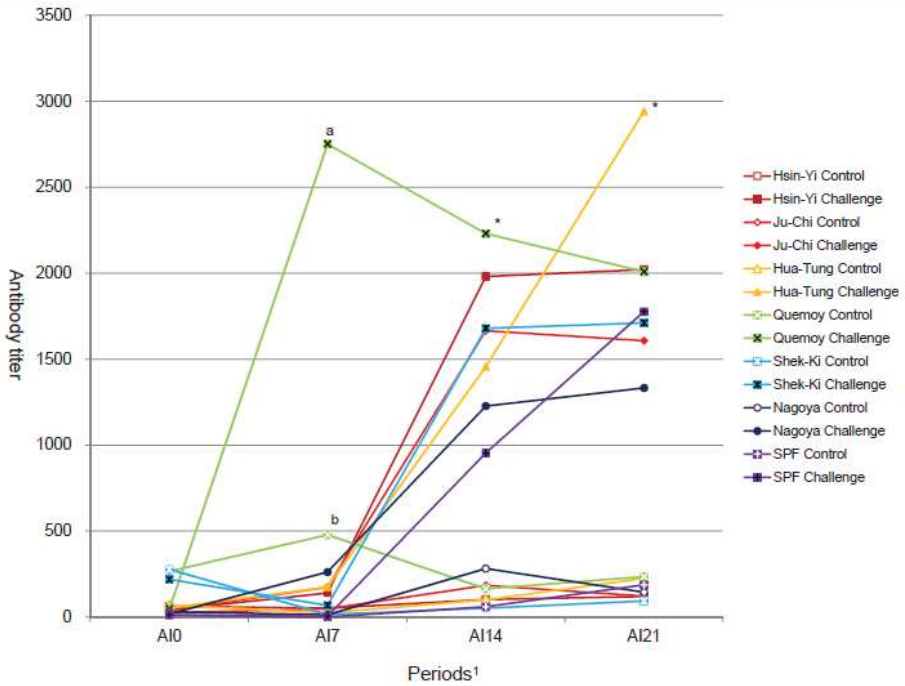

Figure 1 Antibody titers against H6N1 LPAIV on 0, 7, 14 and 28 days post-challenge. [a,b] within days post-challenge with no common superscript differ significantly ($P < 0.05$) in Quemoy. *means all breeds have significantly different between control and challenge group.

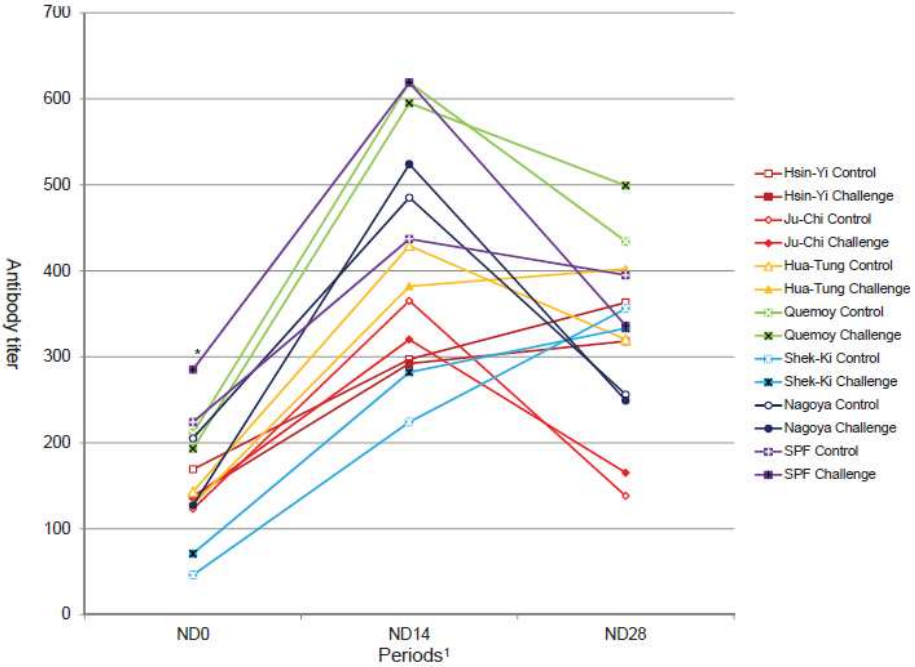

Figure 2 Antibody titers against ND on 0, 14 and 28 days post-inoculation. * Nagoya showed a difference of anti-ND antibody titer between the control and challenge groups at day 0, with a lower value in the challenge group.

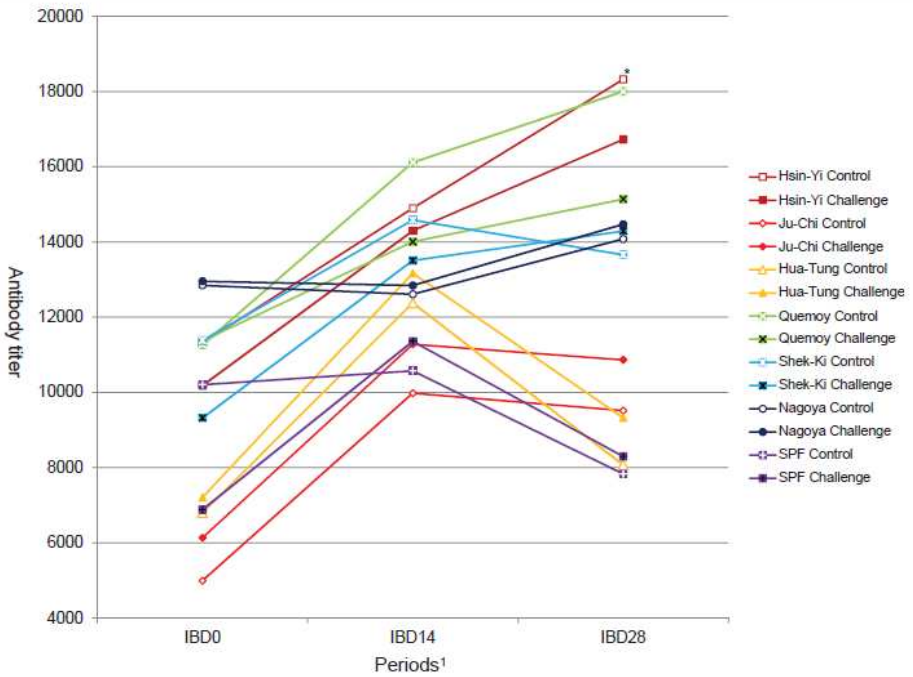

Figure 3 Antibody titers against IBD on 0, 14 and 28 days post-inoculation. * Quemoy showed significantly lower antibody titer in the challenge group.

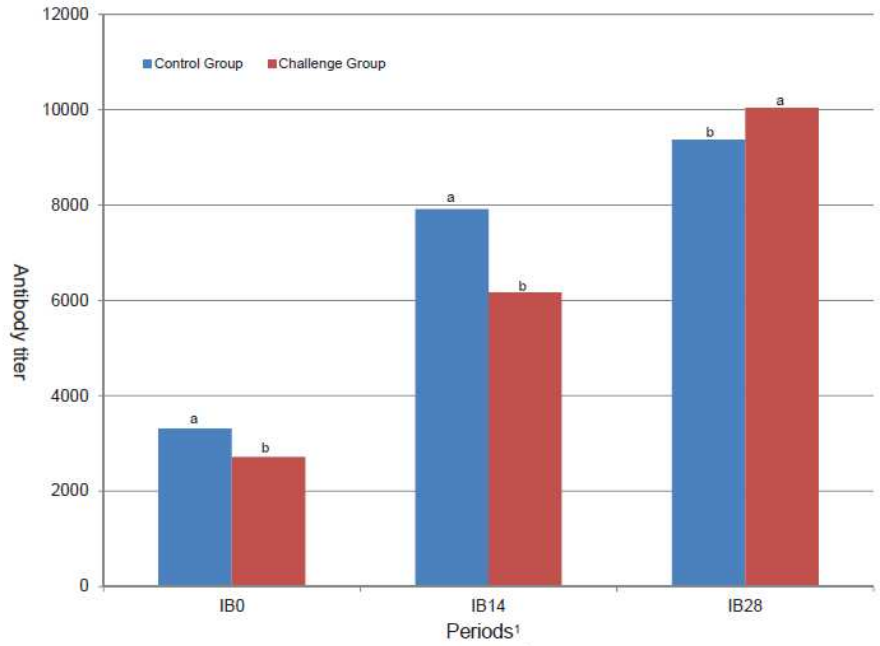

Figure 4 Antibody titers against IB on 0, 14 and 28 days post-inoculation. [a,b] within days post-inoculation with no common superscript differ significantly ($P < 0.05$).

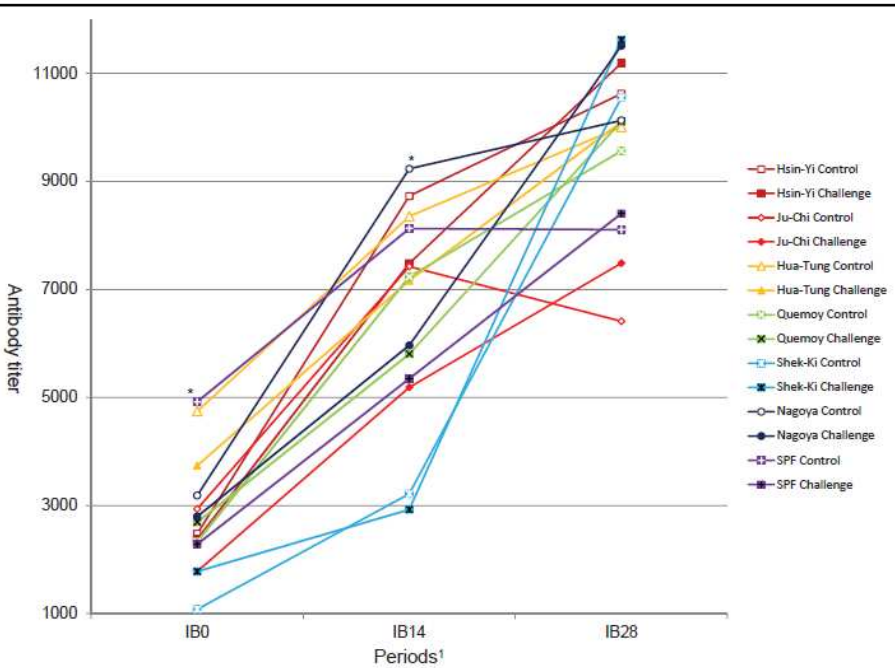

Figure 5 Antibody titers against IB on 0, 14 and 28 days post-inoculation. * Antibody titers at day 0 were lower in the challenge group than in the control group for Ju-Chi and SPF. The effects of breed and H6N1 challenge, without any interaction, were still observed at day 14 post-inoculation. Higher antibody levels were found in the control group for all breeds. Nagoya was the only one to exhibit a stronger response to IB vaccine in the H6N1 control group at day 14.

effects of breed and H6N1 challenge, without any interaction, were still observed at day 14 post-inoculation. Higher antibody levels were found in the control group for all breeds. Nagoya was the only one to exhibit a stronger response to IB vaccine in the H6N1 control group at day 14, as measured by the difference between titers at day 14 and day 0 (Table 2). The interaction between breed and sex was still significant at day 14 but was not observed at day 28. Breed and treatment effects were significant at day 28; antibody titers became higher in the challenge groups than in the control groups, whatever the breed. The increase in antibody titers between day 14 and day 28 was always higher in the challenge groups as compared to the control groups, this difference was highly significant in Ju-Chi and Nagoya (Table 2) and tended to be significant ($P < 0.05$) in all other breeds except Shek-Ki which never showed any difference in anti-IB titers between the challenged and the control groups.

Breed comparison showed that Quemoy had the highest antibody titers for IB0. Quemoy, Hsin-Yi and Nagoya had the highest values for IB14. These three breeds had still high values for IB28, but Shek-Ki had also high values for IB28, although it exhibited low values for IB0 and IB14. Thus, this breed was characterized by a late and strong response to IB vaccine, without any significant effect of the previous H6N1 challenge.

Table 2 Effects of H6N1 challenge on antibody titers in different periods across breeds

Breed	Period*	Control	Challenge
Hsin-Yi	IB14-IB0	6253 ± 499	5106 ± 499
	IB28-IB0	8119 ± 609	8769 ± 609
	IB28-IB14	1852 ± 726	3722 ± 738
Ju-Chi	IB14-IB0	4598 ± 553	3374 ± 541
	IB28-IB0	3474 ± 682[b]	5720 ± 608[a]
	IB28-IB14	-946 ± 564[b]	2408 ± 564[a]
Hua-Tung	IB14-IB0	4818 ± 877	3175 ± 784
	IB28-IB0	7149 ± 604	7461 ± 540
	IB28-IB14	2331 ± 766	4303 ± 699
Quemoy	IB14-IB0	3633 ± 508	3424 ± 469
	IB28-IB0	5261 ± 447	6362 ± 413
	IB28-IB14	1663 ± 502	2935 ± 463
Shek-Ki	IB14-IB0	2050 ± 687	1235 ± 615
	IB28-IB0	9464 ± 623	9809 ± 623
	IB28-IB14	7148 ± 938	8516 ± 884
Nagoya	IB14-IB0	6117 ± 548[a]	3165 ± 437[b]
	IB28-IB0	7038 ± 748	8718 ± 642
	IB28-IB14	580 ± 844[b]	5433 ± 695[a]
SPF	IB14-IB0	3205 ± 679	3063 ± 679
	IB28-IB0	3186 ± 962[b]	6118 ± 962[a]
	IB28-IB14	-19 ± 1107	3055 ± 1107

*IB14-IB0 represents the difference between IB14 and IB0, IB28-IB0 the difference between IB28 and IB0 and IB28-IB14 the difference between IB28 subtract IB14.

[ab] Means ± SE within a breed for a given stage with no common superscript differ significantly ($P < 0.05$).

Discussion and conclusions

H6N1 LPAIV challenge effect

Mortality data and the increased antibody titers of challenge group at day 14 post-challenge showed that the challenge test had been effective. Estimation of breed effects may be affected by limited sample size, particularly for Shek-Ki, SPF and Nagoya. However, Quemoy had the largest sample size, and can be identified as the most resistant breed since it did not show any mortality after the challenge, and exhibited the most rapid immune response at day 7 post-challenge. At the opposite, Hsin-Yi can be identified as the most susceptible breed, with a rather high mortality (7 birds out of 26) in spite of the use of a low pathogenic virus.

Breeds effect, vaccine efficacy and duration of immunity

Antibodies were detected for ND, IB and IBD at day 0 post-inoculation because all chickens had been vaccinated for ND, IB and IBD at earlier ages. Thus, the immune response following the inoculation at 11 weeks of age may be considered as a secondary immune response. Antibody titers at day 14 post-inoculation were significantly higher than at day 0, showing vaccine efficiency, except in the case of Nagoya and SPF for IBD vaccine where no change in antibody levels was observed.

Breed significantly affected immune response. Quemoy had high and rapid responses to the three vaccines and to H6N1 LPAVI challenge test, in contrast to Ju-Chi which had low immune response to vaccines and challenge test. Breed's effect on the antibody titers at day 0 revealed differences in the duration of immunity to previous vaccines. Quemoy appeared to have a better immunity than other breeds, Shek-Ki had a slow response to ND and IB, and Ju-Chi presented lowest response to IBD. The better immune response of Quemoy is consistent with previous results [2].

H6N1 LPAIV challenge effect on IB immune response

H6N1 LPAIV challenge had a negative effect on antibody levels to IB even before the vaccination at 11 weeks of age. Immune response to IB vaccine took place in H6N1 challenge groups with some delay (after 14 days). The negative relationship of H6N1 LPAIV challenge and IB vaccine response could be related to the fact that both viruses target the lung tissues. Recently, Haghighat-Jahromi et al. [4] showed that coinfection of H9N2 AI virus with IB live virus enhanced the virulence of H9N2 and increased the rate of mortality. In addition, Karimi-Madab et al. [5] showed that IB live vaccine

could be an important risk factor resulting in enhanced virulence of H9N2 LPAIV in field conditions. Although these studies were focusing on broilers and H9N2 LPAIV, the present study shows also an interaction between IB and H6N1 LPAIV infection in some local chicken breeds. One could speculate that the type of local immune response induced by H6N1 LPAIV infection was not favourable for birds to make optimum IB-specific antibody response.

In conclusion, local breeds have different immune response to H6N1 LPAIV challenge and subsequent vaccines. The H6N1 LPAIV challenge influenced the response to subsequent vaccination against IB, but had no effect on ND and IBD subsequent vaccines.

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Authors' contributions
MTB and BB conceived the main idea of the study and helped to draft the manuscript. CSC carried out animal experiments, performed the statistical analysis and drafted the manuscript. YPL and CFC participated in the design of the study and provided experimental animals. PCC participated in the design of the study, and provided H6N1 virus. JWC participated in the design of the study, the analysis and the interpretation of the data. OC participated in the design of the study and helped to draft the manuscript. All authors contributed to the manuscript and all authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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