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Geographic and Breed Distribution Patterns of an A/G Polymorphism Present in the Mx Gene Suggests Balanced Selection in Village Chickens

S. Ommeh[1]*, L.N. Jin[2,3]*, H. Eding[14], F.C. Muchadeyi[14], S. Sulandari[4], M.S.A. Zein[4], G. Danbaro[5], C.E. Wani[6], S.G. Zhao[2,3], Q.H. Nie[7], X.Q. Zhang[7], M. Ndila[1], R. Preisinger[8], G.H. Chen[9], I.A. Yousif[10], K.-N. Heo[11], S.J. Oh[1,11], M. Tapio[1], D. Masiga[12], O. Hanotte[1,13], H. Jianlin[1,3] and S. Weigend[14]

[1]International Livestock Research Institute (ILRI), P.O. Box 30709, GPO 00100, Nairobi, Kenya
[2]College of Animal Science and Technology, Gansu Agricultural University, Lanzhou 730070, Gansu, P.R. China
[3]CAAS-ILRI Joint Laboratory on Livestock and Forage Genetic Resources, Institute of Animal Science, Chinese Academy of Agricultural Sciences (CAAS), Beijing 100094, P.R. China
[4]Research Centre for Biology, The Indonesian Institute of Science (LIPI), Cibinong 16911, Indonesia
[5]Department of Agriculture, University of Technology, PMB, Lae, Papua New Guinea
[6]Department of Animal Production, College of Veterinary Sciences, UBG, P.O. Box 10739, Khartoum, Sudan
[7]Department of Animal Genetics, Breeding and Reproduction, College of Animal Science, South China Agricultural University, Guangzhou 510642, Guangdong, P.R. China
[8]Lohmann Tierzucht GmbH, Cuxhaven, Germany
[9]Department of Animal Science, College of Animal Science and Veterinary Medicine, Yangzhou University, Yangzhou 225009, Jiangsu, P.R. China
[10]Department of Animal Genetics and Breeding, University of Khartoum, P.O. Box 13314, Khartoum, Sudan
[11]National Institute of Animal Science, RDA, Suwon, Korea
[12]International Centre for Insect Physiology and Ecology (ICIPE), P.O. Box 30772, GPO 00100, Nairobi, Kenya
[13]University of Nottingham, School of Biology, University Park, NG2 2RD Nottingham, United Kingdom
[14]Institute of Farm Animal Genetics, Friedrich Loeffler Institut (FLI), Hoeltystrasse 10, 31535 Neustadt, Germany

Abstract: An A/G Single Nucleotide Polymorphism (SNP) at position 1,892 of the Mx gene coding sequence has been linked to susceptibility/resistance to avian viral infection *in vitro*. Using PCR-RFLP and sequencing methods, 1,946 samples from 109 populations from Asia, Africa and Europe; grouped as indigenous village, commercial, fancy chicken as well as wild junglefowl were genotyped for the polymorphism. Allele and genotype frequencies were calculated. Only the G allele was present in Ceylon junglefowl *Gallus lafayetti*. Using the wild red junglefowl *G. gallus* population as reference, we assessed if the A/G alleles and genotypes frequencies have been affected by the breeding history and the geographic dispersion of domestic chicken. Within group variation was high but overall there were no significant variation in distribution of alleles and genotypes frequencies between the red junglefowl and indigenous village chickens ($p>0.1946$), with the exception of the East Asian group ($p<0.0001$). However, allele and genotype frequencies were significantly different between the red junglefowl and the commercial or fancy groups ($p<0.0001$). A small but significant negative correlation ($r = - 0.166$, $p<0.0003$) was observed between allelic and geographic distance matrices amongst indigenous village chicken populations. Human selection and genetic drift are likely the main factors having shaped today's observed allele and genotype frequencies in commercial and fancy breeds. In indigenous village chicken and red junglefowl, we propose that both A and G alleles have been maintained by natural selection for disease resistance through a balancing selection mechanism.

Key words: Indigenous village chicken, junglefowl, commercial chicken, viral infection, poultry

INTRODUCTION

Mx proteins which are induced by type I α/β interferons have highly conserved domains in the amino terminus, consisting of a tripartite GTP-binding motif and a dynamin family signature sequence. The less conserved C terminus contains a Central Interactive Domain (CID) and an effector domain which includes leucine zipper motifs (Haller and Kochs, 2002). The chicken Mx protein

Corresponding Author: O. Hanotte, University of Nottingham, School of Biology, University Park, NG2 2RD Nottingham, United Kingdom
*These two authors contributed equally to this article

is predominantly present in the cytoplasm and consists of 705 amino acids encoded by 13 exons. The chicken Mx gene has 14 exons and the translational initiation codon is located in the second exon of the gene (Schumacher *et al*., 1994). An A/G polymorphism at nucleotide position 1,892 in the 13th protein coding exon (Livant *et al*., 2007) leads to an amino acid change at position 631 which was referred to as nucleotide position 2,032 of Mx cDNA sequence by Ko *et al*. (2002). Previous studies have suggested a functional role in viral disease resistance-susceptibility for the studied A/G polymorphism (Ko *et al*., 2002;2004), with the A allele encoding for an asparagine conferring more resistance against recombinant VSV and AIV infection in cell cultures compared to the G allele, encoding for serine (Ko *et al*., 2002; 2004). However, more recently, the relevance of this polymorphism for resistance/susceptibility to viral infection in poultry has been questioned (Benfield *et al*., 2008) and no significant association was found between viral infection (H7N1) and the A/G genotypes in five chicken lines (Sironi *et al*., 2008).

We examined here a large set of domestic chicken *Gallus domesticus* and wild red junglefowl populations *G. gallus ssp*, from across the Old World geographic distribution of the species (Asia, Africa and Europe) and with different breeding histories (commercial, fancy, indigenous village chicken and wild junglefowl), to address the issue of the origin, geographic distribution and evolutionary history of the A/G genotypes. We hypothesize that in commercial breeds, managed in relatively disease free environments with veterinarian controls and preventive measures, the polymorphism will not be under strong direct selection and alleles and genotypes frequencies will vary through genetic drift or hitchhiking for non-disease selected traits. Conversely in fancy breeds, the population sizes are rather small and breeders select for exterior traits forcing inbreeding. These evolutionary factors may also be of importance in indigenous village chicken and wild junglefowl, but in these populations continuously exposed to pathogen challenges we expect much stronger disease related selection, with either one allele favoured against the other (positive selection) or either both alleles under selection with their frequencies shaped through a balancing selection mechanism.

MATERIALS AND METHODS

Chicken populations: A total of 1,946 samples in 109 populations were used in this study (Table 1). These samples included indigenous village and commercial chicken populations, red junglefowl and Ceylon junglefowl *G. lafayetti* populations. Commercial populations encompassed commercial white layers, brown layers and broilers. The white layers were all from the White Leghorn breed and included three commercial strains and one experimental strain. The brown layers consisted of eight strains, while the broilers were made up of 11 strains. The six fancy breeds used in this study were obtained from Germany and they belong to the Northwest European chicken type. Asian indigenous populations from Bangladesh, China, Indonesia, Korea, Pakistan, Papua New Guinea and Vietnam were included in the dataset, as were indigenous populations from 14 African countries. Additionally, four populations of red junglefowl and one population of the Ceylon junglefowl were included in the study (Table 1).

The 109 populations were divided into 10 groups: 1) commercial white layers; 2) commercial brown layers; 3) commercial broilers; 4) German fancy breeds; 5) indigenous chickens from East Asia (China and Korea); 6) indigenous chickens from Southeast Asia (Vietnam, Indonesia and Papua New Guinea); 7) indigenous chickens from South Asia (Bangladesh, Pakistan and Sri Lanka); 8) indigenous chickens from Africa including Botswana, Burkina Faso, Chad, Egypt, Ethiopia, Gambia, Kenya, Madagascar, Malawi, Mali, Senegal, Sudan, Uganda and Zimbabwe; 9) various subspecies of wild red junglefowl which included *G. g. gallus*, *G. g. spadiceus*, *G. g. jabouillei* and *G. g. murghi* and 10) Ceylon junglefowl *G. lafayetti* (Table 1 and 2).

DNA extraction: Venous blood from chickens and junglefowls was collected in EDTA buffer or on Whatman FTA® filter paper (Whatman BioScience, Maidstone, UK). DNA from blood collected in EDTA buffer was extracted using the phenol-chloroform method (Sambrook and Russell, 2001). DNA from the FTA filter paper was extracted using the method described by Smith and Burgoyne (2004). Part of the DNA samples were taken from the DNA bank established during the AVIANDIV (http://aviandiv.tzv.fal.de/) project.

Genotyping: Three methods were employed to genotype the A/G SNP at nucleotide position 1,892 in the 13th protein coding exon of the Mx gene: i) PCR-RFLPs using both regular (Institute of Farm Animal Genetics, Neustadt, Germany) and mismatched primers (CAAS-ILRI joint lab, Beijing, China) and ii) direct sequencing of the PCR fragment (ILRI, Nairobi, Kenya and CAAS-ILRI joint laboratory Beijing, China). The datasets were standardized and merged between laboratories using a common set of samples.

PCR-RFLPs using regular primers. A restriction enzyme was identified (Hpy8I; Fermentas, St. Leon-Rot, Germany) to cut the PCR fragment of allele G (5' GTN | NAC 3' or 3' CAN | NTG 5') at two base pairs (bp) downstream from the A/G SNP. PCR-RFLP primers were constructed to amplify a fragment of 323-327 bp in length. Size variation was due to the presence of 7-11 poly Ts in the intron 15 bp before the 13th protein coding exon and 74 bp downstream from the forward primer.

Table 1: Number of observed and expected (in brackets) genotypes under Hardy-Weinberg Equilibrium (HWE), frequency of allele A (P(A)) and F_{is} estimates of the G/A SNP at nucleotide position 1,892 of coding sequence of the Mx gene in a wide range of chicken and junglefowl populations

			Genotype			HWE			
Population	Category	n[1]	AA	AG	GG	x^2	P(x^2)	P(A)	F_{is}
Commercial chicken									
LSS (White Leghorn experimental line)	Experimental white layer	21	20 (19.02)	0 (1.95)	1 (0.02)	41.03	0***	0.95	1.000[*a]
WLA_1 (White Leghorn)	Commercial white layer	40	31 (31.46)	9 (8.09)	0 (0.46)	0.57	0.452	0.89	-0.114
WLB (White Leghorn)	Commercial white layer	40	40 (40)	0 (0)	0 (0)	0	1.000	1.00	1.000
WLF (White Leghorn F line, Korea)	Commercial white layer	10	2 (3.47)	8 (5.05)	0 (1.47)	3.82	0.051	0.60	-0.636
BLA_1 (Rhode Island Red)	Commercial brown layer	40	0 (0)	0 (0)	40 (40)	0	1.000	0	1.000
BLB (Rhode Island Red cross)	Commercial brown layer	21	13 (12.88)	7 (7.24)	1 (0.88)	0.03	0.871	0.79	0.034
BLC (Rhode Island Red)	Commercial brown layer	26	0 (0)	0 (0)	26 (26)	0	1.000	0	1.000
BLD (Rhode Island Red)	Commercial brown layer	20	0 (0)	0 (0)	20 (20)	0	1.000	0	1.000
BLE (Australorps)	Commercial brown layer	21	0 (0)	0 (0)	21 (21)	0	1.000	0	1.000
BLF (White Rock)	Commercial brown layer	40	20 (20.92)	18 (16.15)	2 (2.92)	0.54	0.461	0.73	-0.116
CBL_A (Hy-Line variety brown, China)	Commercial brown layer	90	2 (15.92)	72 (44.16)	16 (29.92)	36.2	0***	0.42	0.636[***b]
RIR_D (Rhode Island Red, dam line, Korea)	Commercial brown layer	10	8 (8.05)	2 (1.90)	0 (0.05)	0.06	0.808	0.90	-0.059
RIR_S (Rhode Island Red, sire line, Korea)	Commercial broiler	28	0 (0.11)	4 (3.78)	24 (24.11)	0.12	0.727	0.07	-0.059
BRD_A (unknown)	Commercial broiler	21	2 (0.51)	3 (5.98)	16 (14.51)	5.96	0.015*	0.17	0.504
BRD_B (unknown)	Commercial broiler	20	10 (9.69)	8 (8.62)	2 (1.69)	0.11	0.741	0.70	0.073
BRD_C (unknown)	Commercial broiler	21	0 (0)	0 (0)	21 (21)	0.00	1.000	0	1.000
BRD_D (unknown)	Commercial broiler	21	1 (0.15)	2 (3.71)	18 (17.15)	5.81	0.016*	0.10	0.467
BRD_DD (ANAK40, sire line, China)	Commercial broiler	10	0 (0.79)	6 (4.42)	4 (4.79)	1.48	0.223	0.30	-0.385
BRS_A (unknown)	Commercial broiler	20	0 (0.15)	4 (3.69)	16 (16.15)	0.18	0.671	0.10	-0.086
BRS_B (unknown)	Commercial broiler	21	3 (1.10)	4 (7.81)	14 (12.10)	5.45	0.020*	0.24	0.494[*a]
BRS_C (unknown)	Commercial broiler	20	0 (0)	1 (1)	19 (19)	0	1.000	0.03	0
BRS_D (unknown)	Commercial broiler	21	5 (3.73)	8 (10.54)	8 (6.73)	1.28	0.258	0.43	0.245
BRS_E (unknown)	Commercial broiler	10	0 (0.05)	2 (1.89)	8 (8.05)	0.06	0.808	0.10	-0.059
Fancy breeds									
Bergische Schlotterkaemmer, Germany	German fancy	20	12 (9)	3 (9)	5 (2)	9.50	0.002**	0.68	0.672[**a]
Deutsche Sperber, Germany	German fancy	18	1 (0.60)	5 (5.80)	12 (11.60)	0.39	0.532	0.19	0.141
Friesenhuhn, Germany	German fancy	20	5 (3.49)	7 (10.03)	8 (6.49)	1.92	0.166	0.43	0.307
Ostfriesische Moewen, Germany	German fancy	14	6 (5.67)	6 (6.67)	2 (1.67)	0.15	0.696	0.64	0.103
Vorwerkhuehner, Germany	German fancy	20	0 (0.03)	2 (1.95)	18 (18.03)	0.03	0.869	0.05	-0.027
Westfaelische Totleger, Germany	German fancy	22	0 (0.23)	5 (4.53)	17 (17.23)	0.28	0.595	0.11	-0.105
Indigenous village chicken									
Beijing fatty, China	East Asia, indigenous	21	2 (0.15)	0 (3.71)	19 (17.15)	27.39	0***	0.10	1.000[**a]
Dagu, China	East Asia, indigenous	21	0 (0.07)	3 (2.85)	18 (18.07)	0.08	0.776	0.07	-0.053
Langshan, China	East Asia, indigenous	20	0 (0)	0 (0)	20 (20)	0.00	1.000	0	1.000
Tibetan, China	East Asia, indigenous	20	9 (9.69)	10 (8.62)	1 (1.69)	0.56	0.456	0.70	-0.166
Xiaoshan, China	East Asia, indigenous	21	2 (1.10)	6 (7.80)	13 (12.10)	1.23	0.268	0.24	0.236
Minqin, China	East Asia, indigenous	26	4 (1.08)	3 (8.84)	19 (16.08)	12.3	0.001***	0.21	0.665[**a]
Jingning, China	East Asia, indigenous	10	1 (0.32)	2 (3.37)	7 (6.32)	2.11	0.146	0.20	0.419
Huining, China	East Asia, indigenous	34	0 (0.42)	8 (7.16)	26 (26.42)	0.52	0.470	0.12	-0.119
Wuding, China	East Asia, indigenous	40	2 (1.15)	10 (11.70)	28 (27.15)	0.90	0.343	0.18	0.147
Gushi_1, China (provided by G. H. Chen)	East Asia, indigenous	25	1 (1.86)	12 (10.29)	12 (12.86)	0.74	0.390	0.28	-0.171
Gushi_2, China (provided by Q. H. Nie)	East Asia, indigenous	21	5 (6.73)	14 (10.54)	2 (3.73)	2.39	0.122	0.57	-0.340
Henan Fight, China	East Asia, indigenous	21	2 (1.10)	6 (7.81)	13 (12.10)	1.23	0.268	0.24	0.236
Korean Black chicken, Korea	East Asia, indigenous	39	2 (1.36)	11 (12.27)	26 (25.36)	0.45	0.505	0.19	0.105
Korean Yellow chicken, Korea	East Asia, indigenous	9	0 (0.18)	3 (2.65)	6 (6.18)	0.23	0.633	0.17	-0.143
Korean Red chicken, Korea	East Asia, indigenous	10	0 (0.16)	3 (2.68)	7 (7.16)	0.20	0.656	0.15	-0.125
Ac, Vietnam	Southeast Asia, indigenous	10	0 (0)	1 (1)	9 (9)	0	1.000	0.05	0
Tre, Vietnam	Southeast Asia, indigenous	10	0 (0.79)	6 (4.42)	4 (4.79)	1.48	0.223	0.30	-0.385
Te, Vietnam	Southeast Asia, indigenous	10	2 (1.47)	4 (5.05)	4 (3.47)	0.49	0.485	0.40	0.217
Dia phuong, Vietnam	Southeast Asia, indigenous	15	1 (2.69)	11 (7.62)	3 (4.69)	3.17	0.075	0.43	-0.467
H'mong_1, Vietman (provided by L.T. Thuy)	Southeast Asia, indigenous	10	0 (1.11)	7 (4.79)	3 (4.11)	2.42	0.120	0.35	-0.500
H'mong_2, Vietnam (provided by N.T.K. Cuc)	Southeast Asia, indigenous	20	12 (11.15)	6 (7.69)	2 (1.15)	1.06	0.304	0.75	0.224
Ayam Pelung Cianjur, Java, Indonesia	Southeast Asia, indigenous	10	5 (5.53)	5 (3. 95)	0 (0.53)	0.86	0.355	0.75	-0.286
Ayam Sentul, Java, Indonesia	Southeast Asia, indigenous	10	3 (2.90)	5 (5.21)	2 (1.90)	0.02	0.893	0.55	0.043
Ayam Sentul Jatiwangi, Java, Indonesia	Southeast Asia, indigenous	10	4 (3.47)	4 (5.05)	2 (1.47)	0.49	0.485	0.60	0.217
Ayam Kedu, Java, Indonesia	Southeast Asia, indigenous	10	4 (2.37)	2 (5.26)	4 (2.37)	4.27	0.039*	0.50	0.633
Ayam Kedu Hitam, Java, Indonesia	Southeast Asia, indigenous	10	4 (4.11)	5 (4.79)	1 (1.11)	0.02	0.882	0.65	-0.047
Ayam Gaok, Java, Indonesia	Southeast Asia, indigenous	10	4 (4.79)	6 (4.42)	0 (0.79)	1.48	0.223	0.70	-0.385
Ayam Kedu Putih, Java, Indonesia	Southeast Asia, indigenous	10	2 (2.90)	7 (5.21)	1 (1.90)	1.31	0.252	0.55	-0.370
Ayam Kate, Java, Indonesia	Southeast Asia, indigenous	10	3 (4.11)	7 (4.79)	0 (1.11)	2.42	0.120	0.65	-0.500
Ayam Cemani, Java, Indonesia	Southeast Asia, indigenous	9	7 (7.06)	2 (1.88)	0 (0.06)	0.07	0.796	0.89	-0.067
Ayam Kedu Putih Jatiwangi, Java, Indonesia	Southeast Asia, indigenous	9	5 (5.35)	4 (3.29)	0 (0.35)	0.53	0.468	0.78	-0.231
Ayam Wareng, Java, Indonesia	Southeast Asia, indigenous	11	4 (3.14)	4 (5.71)	3 (2.14)	1.09	0.296	0.55	0.310
Ayam Pelung, Sumatra, Indonesia	Southeast Asia, indigenous	10	3 (2.90)	5 (5.21)	2 (1.90)	0.02	0.893	0.55	0.043
Ayam Arab Silver, Sumatra, Indonesia	Southeast Asia, indigenous	10	2 (1.47)	4 (5.05)	4 (3.47)	0.49	0.485	0.40	0.217
Ayam Arab Gold, Sumatra, Indonesia	Southeast Asia, indigenous	10	4 (3.47)	4 (5.05)	2 (1.47)	0.49	0.485	0.60	0.217
Ayam Kapas, Sumatra, Indonesia	Southeast Asia, indigenous	10	1 (1.11)	5 (4.79)	4 (4.11)	0.02	0.882	0.35	-0.047
Ayam Merawang, Sumatra, Indonesia	Southeast Asia, indigenous	10	5 (5.53)	5 (3.95)	0 (0.53)	0.86	0.345	0.75	-0.286
Alotau, Papua New Guinea	Southeast Asia, indigenous	37	12 (9.63)	14 (18.74)	11 (8.63)	2.43	0.119	0.51	0.256
Madang, Papua New Guinea	Southeast Asia, indigenous	35	10 (11.30)	20 (17.39)	5 (6.30)	0.81	0.367	0.57	-0.153
Port Moresby, Papua New Guinea	Southeast Asia, indigenous	25	4 (2.78)	9 (11.45)	12 (10.78)	1.20	0.273	0.34	0.217
Sri Lanka	South Asia, indigenous	25	4 (4.29)	13 (12.43)	8 (8.29)	0.06	0.814	0.42	-0.047
Aseel, Pakistan	South Asia, indigenous	10	5 (3.47)	2 (5.05)	3 (1.47)	4.10	0.043*	0.60	0.617
Desi, Pakistan	South Asia, indigenous	30	17 (13.22)	6 (13.56)	7 (3.22)	9.73	0.002**	0.67	0.562[**a]
Naked neck, Pakistan	South Asia, indigenous	10	1 (1.90)	7 (5.21)	2 (2.90)	1.31	0.252	0.45	-0.370
Mymensigh, Bangladesh	South Asia, indigenous	30	20 (17.54)	6 (10.92)	4 (1.54)	6.47	0.011*	0.77	0.455[*a]
Chittagong, Bangladesh	South Asia, indigenous	10	5 (4.79)	4 (4.42)	1 (0.79)	0.11	0.745	0.70	0.100

(Table 1 Continued)

Population	Category	n[1]	Genotype			HWE			
			AA	AG	GG	x^2	P(x^2)	P(A)	F_{is}
Rangpur, Bangladesh	South Asia, indigenous	10	5 (4.79)	4 (4.42)	1 (0.79)	0.11	0.745	0.70	0.100
Khulna, Bangladesh	South Asia, indigenous	10	6 (6.32)	4 (3.37)	0 (0.32)	0.45	0.502	0.80	-0.200
ECO I, Zimbabwe	Africa, indigenous	21	6 (3.32)	5 (10.37)	10 (7.32)	5.93	0.015*	0.41	0.524**
ECO II, Zimbabwe	Africa, indigenous	18	3 (1.89)	6 (8.23)	9 (7.89)	1.42	0.233	0.33	0.277
ECO III, Zimbabwe	Africa, indigenous	20	4 (2.00)	5 (9.00)	11 (9.00)	4.22	0.040*	0.33	0.451
ECO IV, Zimbabwe	Africa, indigenous	20	6 (3.92)	6 (10.15)	8 (5.92)	3.53	0.060	0.45	0.415
ECO V, Zimbabwe	Africa, indigenous	19	4 (1.49)	3 (8.03)	12 (9.49)	8.06	0.006**	0.29	0.633**
Malawi	Africa, indigenous	20	6 (3.08)	4 (9.85)	10 (7.08)	7.46	0.006**	0.40	0.600**
Nekemte, Ethiopia	Africa, indigenous	10	3 (2.90)	5 (5.21)	2 (1.90)	0.02	0.893	0.55	0.043
Jimna, Ethiopia	Africa, indigenous	10	1 (1.47)	6 (5.05)	3 (3.47)	0.40	0.530	0.40	-0.200
Debre, Ethiopia	Africa, indigenous	10	3 (3.47)	6 (5.05)	1 (1.47)	0.40	0.530	0.60	-0.200
Northern Kenya	Africa, indigenous	11	0 (0.14)	3 (2.71)	8 (8.14)	0.18	0.675	0.14	-0.111
KARI, Kenya	Africa, indigenous	10	3 (1.11)	1 (4.79)	6 (4.11)	7.12	0.008**	0.35	0.800**
Kianjasoa, Madagascar	Africa, indigenous	10	0 (0.79)	6 (4.42)	4 (4.79)	1.48	0.223	0.30	-0.385
Mahasolo, Madagascar	Africa, indigenous	10	1 (1.11)	5 (4.79)	4 (4.11)	0.02	0.882	0.35	-0.047
Botswana	Africa, indigenous	10	3 (2.37)	4 (5.26)	3 (2.37)	0.64	0.423	0.50	0.250
Burkina Faso	Africa, indigenous	10	5 (4.11)	3 (4.79)	2 (1.11)	1.59	0.208	0.65	0.386
Chad	Africa, indigenous	10	4 (3.47)	4 (5.05)	2 (1.47)	0.49	0.485	0.60	0.217
Fayoumi, Egypt	Africa, indigenous	10	1 (1.47)	6 (5.05)	3 (3.47)	0.40	0.530	0.40	-0.200
Ai cap, introduced from Egypt to Vietnam	Africa, indigenous	10	1 (0.79)	4 (4.42)	5 (4.79)	0.11	0.745	0.30	0.100
Gambia	Africa, indigenous	10	0 (0)	0 (0)	10 (10)	0	1.000	0	1.000
Mali	Africa, indigenous	10	6 (5.53)	3 (3.95)	1 (0.53)	0.69	0.405	0.75	0.250
Senegal	Africa, indigenous	10	0 (0.05)	2 (1.90)	8 (8.05)	0.06	0.808	0.10	-0.059
Southern Sudan	Africa, indigenous	10	6 (4.11)	1 (4.79)	3 (1.11)	7.12	0.008**	0.65	0.800**
Baladi, Sudan	Africa, indigenous	18	9 (6.60)	4 (8.80)	5 (2.60)	5.71	0.017*	0.61	0.553**
Large Baladi, Sudan	Africa, indigenous	11	5 (5.71)	6 (4.57)	0 (0.71)	1.25	0.264	0.73	-0.333
Betwil, Sudan	Africa, indigenous	12	6 (5.91)	5 (5.17)	1 (0.91)	0.02	0.901	0.71	0.035
Bareneck, Sudan	Africa, indigenous	10	4 (4.11)	5 (4.79)	1 (1.11)	0.02	0.882	0.65	-0.047
Uganda	Africa, indigenous	10	1 (1.47)	6 (5.05)	3 (3.47)	0.39	0.530	0.40	-0.200
Red jungle fowl (most likely *G. g. jabouillei*)[2]	Red junglefowl	32	13 (12.38)	14 (15.24)	5 (4.38)	0.22	0.640	0.63	0.082
G. g. murghi	Red junglefowl	10	8 (8.05)	2 (1.90)	0 (0.05)	0.06	0.808	0.90	-0.059
G. g. gallus	Red junglefowl	20	2 (2.69)	11 (9.62)	7 (7.69)	0.44	0.507	0.38	-0.148
G. g. spadicus	Red junglefowl	22	2 (3.16)	13 (10.67)	7 (8.16)	1.10	0.294	0.39	-0.224
G. lafayetti	Ceylon junglefowl	12	0 (0)	0 (0)	12 (12)	0	1.000	0	1.000

*,p<0.05; **, p<0.01; ***, p<0.001; [1]Number of samples; [2]These red junglefowl samples were collected from Guangxi Province, China by X.Q. Zhang. They were also included in the study by Li *et al.* (2006)

The forward primer sequence was 5' AGC AAC TCC ATA CCG TGT TTT 3', while the reverse primer sequence was 5' TTG GTA GGC TTT GTT GAG GTG 3'. Based on a repeat size of nine Ts of the poly T stretch (fragment size of 325 bp), the polymorphic G/A site was located at 126 bp, resulting in a recognition site of the Hyp8I at 126 of the amplified fragment of allele G. A second recognition site of the Hpy8I is located at position 26 bp of this fragment. Both primers were tailed with M13 universal forward (M13/pUC(-20), 17-mer 5' GTA AAA CGA CGG CCA GT 3') and reverse (M13/pUC(-26), 17-mer 5'-CAG GAA ACA GCT ATG AC-3) sequences so that the same primers could be used for sequencing the PCR products (not used in the current study). Consequently, the total length of the fragment was increased by 34 bp with the size of the M13 tails. A cut at position 28 bp (two nucleotides downstream from the A/G SNP at position 26 bp) resulted in a fragment of 45 bp (28 bp + 17 bp) that was hardly visible on an agarose gel. The A/G SNP at position 126 bp of the fragment yielded either one visible fragment of 314 bp [(325 bp-28 bp) + 17 bp] for allele A without the recognition site at the A/G SNP position or two visible fragments for allele G of 100 bp (128 bp-28 bp) and 214 bp [(325 bp-128 bp) + 17bp]. PCR was performed using HotStarTaq Master Mix Kit (Qiagen, Hilden, Germany) on an Eppendorf Mastercycler. An initial denaturation was at 95°C for 15 min, followed by 35 cycles at 94°C for 1 min, 66°C for 1 min and 72°C for 1 min and finished with a final extension at 72°C for 10 min. A volume of 2.5 μl of the PCR product was digested with 10 units of the Hpy8I for 3 h at 37°C. The digested DNA fragments were analyzed by electrophoresis on a 2% agarose gel for 2 h at 5V/cm and visualized with ethidium bromide staining on UV light.

PCR-RFLPs using mismatch primers. For the PCR-RFLP method using mismatched primers, the *Hpa* I enzyme (Beijing SBS Genetech Co., Ltd, Beijing, China) was used with a recognition sequence of 5' GTT | AAC 3' or 3' CAA | TTG 5' to cut the fragment of allele G. The mismatch primer sequences which amplify approximately a 101 bp long fragment (due to the poly Ts) were: the forward primer 5' GAG TAC CTT CAG CCT GTT TT 3' and the reverse primer 5' ATC TGA TTG CTC AGG CGT TAA 3'. The reverse primer produced a mismatch at the 3' end by introducing an A nucleotide at its second last position. As a consequence, a recognition site for the *Hpa* I is resulted in the presence of a nucleotide G. The G/A SNP is located at position 80 bp (based on nine poly Ts) of the amplified fragment. The enzyme cuts at position 82 bp when an allele G is present. The mismatch RFLPs yielded one visible fragment of either 101 bp for allele A without a recognition site or 82 bp for allele G. PCR was done using an initial denaturation at 92°C for 5 min, followed by 30 cycles at 92°C for 30 s, 56°C for 30 s and

Table 2: Observed genotype and A allele frequencies P(A) in different groups of domestic chickens and wild junglefowl

			Genotype frequency ± SD			
Population group[1]	Category	N*	AA	AG	GG	P(A)
White layers	Commercial	4	0.73±0.37	0.26±0.38	0.01±0.02	0.86±0.18
Brown layers	Commercial	8	0.24±0.34	0.22±0.29	0.53±0.50	0.36±0.40
Broilers	Commercial	11	0.09±0.16	0.22±0.18	0.69±0.28	0.20±0.21
	Average		0.26±0.35	0.23±0.25	0.52±0.42	0.37±0.36
German fancy breeds	Fancy	6	0.22±0.25	0.26±0.12	0.52±0.30	0.35±0.27
East Asia	Village	21	0.11±0.16	0.33±0.22	0.56±0.28	0.27±0.20
Southeast Asia	Village	19	0.36±0.16	0.46±0.13	0.18±0.16	0.59±0.14
South Asia	Village	8	0.45±0.21	0.38±0.18	0.17±0.11	0.64±0.14
Africa	Village	27	0.26±0.19	0.36±0.18	0.38±0.24	0.44±0.20
	Average		0.26±0.20	0.38±0.18	0.36±0.27	0.45±0.22
Red junglefowl	Wild	4	0.35±0.33	0.44±0.18	0.21±0.16	0.58±0.21
Ceylon junglefowl	Wild	1	0	0	1.00	0

*Number of populations included; [1]For grouping details see Table 1

72°C for 30 s and completed by a final extension at 72°C for 1 min. PCR products were digested using *Hpa* I (1 U/μg) for 6-8 h at 37°C following the manufacturer's instructions. The digested fragments were analyzed on a 12% polyacrylamide gel for 3 h at 150 V. The gel was stained with silver nitrate and scanned for an image using Adobe Photoshop.

Direct sequencing of the PCR fragment. The primer sequences were: Mx forward primer 5' GGT TAG CAG AGA GAG GGA GA 3' and Mx reverse primer 5' AGG TTG CTG CTA ATG GAG GA 3'. PCR was carried out with an initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing 55°C for 30 s and primer extension of 72°C for 1 min and a final extension of 72°C for 10 min. The PCR product varied from 609-612 bp long due to the poly Ts. The purification was done using Qiaquick purification kit (Qiagen, AMBION Inc, Austin, Texas, USA) following the manufacturer's instructions. The PCR products were directly sequenced using the PCR primers and BigDye Terminators v 3.0 kit on an automated DNA sequencer (ABI 3730 DNA Analyzer, Applied Biosystems, USA). The ABI trace files of chromatograms were aligned using the software NovoSNP version 2.0.3 (Weckx *et al*., 2005) to detect SNPs.

Statistical analysis: Allele frequencies were calculated in each population from allele counts using FSTAT version 2.9.3 (Goudet, 2001). Arlequin version 3.11 (Excoffier *et al*., 2005) was used to compute the Analysis for Molecular Variance (AMOVA) among the indigenous populations according to the country and geographic distribution. Zt version 1.1 (Bonnet and Van de Peer, 2002) was used to perform the simple Mantel test between allelic and geographic distance matrices. GenAlEx version 6.1 (Peakall and Smouse, 2006) as an add-on in Microsoft Excel was used to plot the regression graph between the allelic and the geographic distances. The Fisher's exact test was used for comparison of allele and genotype frequencies between the groups (R stats package version 2.8.0. http://www.r-project.org/).

RESULTS

The Ceylon junglefowl *G. lafayetti* was fixed for the 'susceptible' G allele, while both alleles were present in red junglefowl and domestic chicken (Table 1 and 2). As shown in Table 2, alleles and genotypes frequencies, although showing a lot of variation within chicken groups, varied amongst the groups. In red junglefowl, genotype proportions are close to what will be expected for the two alleles being present roughly at equal frequency (Table 2). Using the red junglefowl as reference group, these frequencies are not different for the ones observed in indigenous village chicken ($p<0.1946$), with the exception of the East Asia chicken group ($p<0.0001$), where the "resistant" A allele is present at low frequency. On the contrary, allele and genotype frequencies differ significantly between the red junglefowl and the commercial or fancy breeds ($p<0.0001$), which are characterized by a significantly higher (white layers) or lower (brown layers, broilers, fancy) A allele frequencies (Table 1 and 2). AMOVA results (Table 3) indicate that most of the genetic variation in indigenous village chicken is found within populations, with only 8.25% of the variation found between geographic regions. Also, we observed only a slight but significant negative correlation ($r = - 0.166$, $p<0.0003$) between allele frequencies and the geographic location in indigenous village populations (Fig. 1).

DISCUSSION

The presence of the 'susceptible' G allele in Ceylon junglefowl (this study) as well as in the grey junglefowl *G. sonnerati* and the green junglefowl *G. varius* (Seyama *et al*., 2006) support that allele A is specific to the red junglefowl lineage and of relatively recent origin, with the G allele likely being the ancestral state of the polymorphism. Moreover, the presence of both A and G

Table 3: AMOVA results for indigenous populations in groups by geographical origins i.e. East Asia, Southeast Asia, South Asia and Africa

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among geographic regions	3	95.331	0.02081 Va*	8.25
Among populations within geographic regions	18	55.649	0.01900 Vb**	7.53
Within populations	4554	967.459	0.21244 Vc**	84.22
Total	4575	1118.440	0.25226	

*p<0.05, **p<0.0001

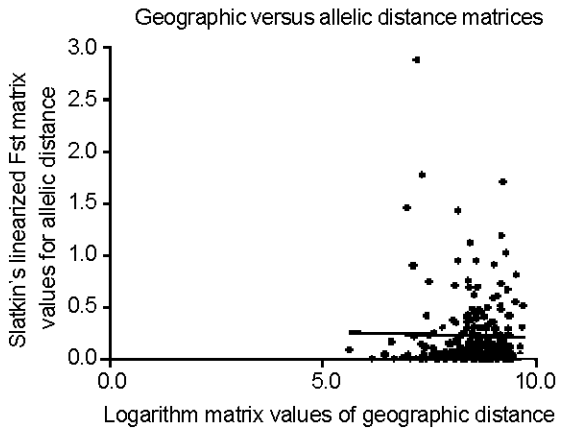

Fig. 1: A regression graph showing the relationship between geographic and allelic distance matrices of indigenous village chickens

alleles in all red junglefowl populations as well as in all indigenous chicken populations from different geographic areas (Table 1 and 2) indicates that the A/G polymorphism was already present in the wild ancestors of domestic chicken and it is today present across the geographic range of the different wild red junglefowl subspecies (Table 1).

If an allele or the A/G polymorphism is under strong natural selection for diseases resistance / susceptibility against pathogens, we would expect to observe comparable genotypes and allele frequencies among groups of indigenous village chicken and wild junglefowl from different geographic regions under natural disease challenges (assuming identical or very similar pathogens circulating in all areas including some sharing epitopes). It should not be the case among populations which are under directed strong human selection for non disease resistance traits and of limited population size, such as for the commercial and fancy breeds. At the contrary if an allele or the polymorphism is not under selection, we would expect to observe 'random' variations in allele and genotype frequencies among all the chicken groups examined. Our results (Table 2) support the former hypothesis rather than the later with similar allele and genotype frequencies amongst indigenous village chicken from different geographic regions and between indigenous village chicken and wild red junglefowl, with the exception of the East Asia indigenous chicken (but see below). Moreover, while both A and G alleles are present among the chicken groups, they are more or less at equal frequencies in the wild and indigenous village chicken groups supporting a balancing rather than a positive ongoing selection mechanism. In the commercial and fancy breeds no similar pattern of allelic variation is observed between the different groups suggesting a random process of allelic frequency variation under the influence of genetic drift and strong human selection for unrelated traits, as illustrated by the difference observed between broiler and egg-layer chicken (Table 2). The results obtained in East Asia include populations from China and Korea. For both countries although local indigenous village chicken were studied, it is likely that recent heavy introgression with commercial stocks has occurred in some population included in this study as supported by mitochondrial DNA results (Han Jianlin personal communication).

Conclusion: Our large scale breed types and geographic analysis support natural selection and more particularly a balancing selection process as an evolutionary force for the maintenance of the A/G Single Nucleotide Polymorphism (SNP) at nucleotide position 1,892 of the Mx gene coding sequence suggesting that both alleles might be of importance for innate immunity in chicken.

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