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Genomic Regions Associated with Dermal Hyperpigmentation, Polydactyly and Other Morphological Traits in the Silkie Chicken

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Abstract

The Silkie chicken has been a model of melanoctye precursor and neural crest cell migration and proliferation in the developing embryo due to its extensive hyperpigmentation of dermal and connective tissues. Although previous studies have focused on the distribution and structure of the Silkie's pigment or the general mechanisms by which this phenotype presents itself, the causal genetic variants have not been identified. Classical breeding experiments have determined this trait to be controlled by 2 interacting genes, the sex-linked inhibitor of dermal melanin (*Id*) and autosomal fibromelanosis (*Fm*) genes. Genome-wide single nucleotide polymorphism (SNP)-trait association analysis was used to detect genomic regions showing significant association with these pigmentation genes in 2 chicken mapping populations designed to segregate independently for *Id* and *Fm*. The SNP showing the highest association with *Id* was located at 72.3 Mb on chromosome Z and 10.3–13.1 Mb on chromosome 20 showed the highest association with *Fm*. Prior to this study, the linkage group to which *Fm* belonged was unknown. Although the primary focus of this study was to identify loci contributing to dermal pigmentation in the Silkie chicken, loci associated with various other morphological traits segregating in these populations were also detected. A single SNP in a highly conserved cis-regulatory region of Sonic Hedgehog was significantly associated with polydactyly (*Po*). Genomic regions in association with silkie feathering or hookless (*h*), feathered legs (*Pti*), vulture hock (*V*), rose comb (*R*), and duplex comb (*D*) were also identified.

Key words: *fibromelanosis, hyperpigmentation, inhibitor of dermal melanin, polydactyly, Silkie*

The Silkie chicken (*Gallus gallus*) is noteworthy as a source of phenotypic variations not commonly seen in other domestic breeds of chicken, representing a few of the vast array of morphological differences present across various domesticated poultry species. Thought to originate in China and described by Marco Polo in the 13th century during his explorations of Asia, the Silkie breed is believed to have been established well before the 13th century due to references to the unusual fowl in ancient Chinese writings (Haw 2006). Although the Silkie chicken is named for the hair-like appearance of adult feathers, one of the more distinguishing traits of this breed is hyperpigmentation of the dermis (Figure 1) and connective tissue throughout the entire body. This presents an opportunity to utilize the Silkie chicken as a biological model of hyperpigmentation that can be used to identify genes regulating potentially novel mechanisms of melanoctye migration, proliferation, and differentiation. A gene mapping approach was chosen utilizing a synthetically derived segregating population for the hyperpigmentation genes of interest in conjunction with genome-wide SNP (single nucleotide polymorphism) marker-trait association analysis. The combination of historically described chicken mutants with modern day gene mapping technologies is an area that remains understudied and which may have profound contributions to our understanding of basic developmental processes.

In the Silkie, pigmentation is found extensively in the dermal layer of skin, sheaths of muscles and nerves, tendons, gut mesenteries, blood vessel walls, trachea, and air sacs (Kuklenski 1915; Hutt 1949; Faraco et al. 2001). Analyses of the structural properties of melanin and the process of melanogenesis have revealed no qualitative differences between the pigment present in the hyperpigmented Silkie breed as compared with normally pigmented

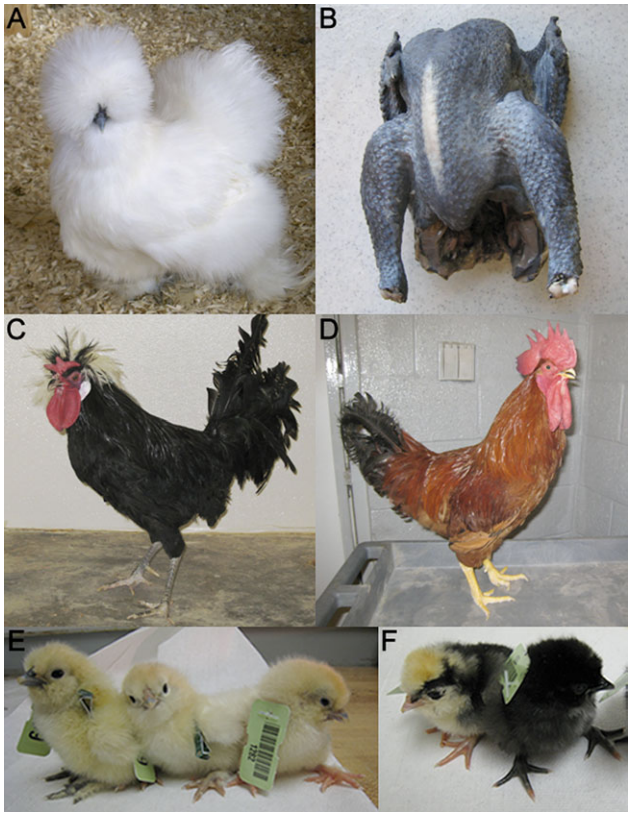

Figure 1. Founders of the mapping populations and chick shank pigmentation phenotypes. Representative individuals of the breeds used to generate the mapping population segregating for pigmentation and morphological traits in this study ((**A**) White Silkie and (**B**) White Silkie as prepared for meat consumption. Note the extensive pigmentation of the dermis across the entire body. (**C**) White Crested Black Polish and (**D**) New Hampshire). Day-old chickens from the mapping populations displaying different shank pigmentation phenotypes ((**E**) Cross I from left to right are putative id^+ (black), Id/id^+ (gray) and Id/Id (white) representative individuals. (**F**) Cross II shows a putative fm^+/fm^+ (white shank) individual on the left and an Fm/fm^+ individual (black shank) on the right).

breeds (Muroya et al. 2000; Ortolani-Machado et al. 2009). Melanocytes differentiate from a multipotent migratory population of cells termed neural crest cells (NCCs) that also give rise to neurons and glial cell lineages (Le Douarin and Dupin 2003). Many studies have used the Silkie chicken as a model of NCC specification, migration, differentiation, and function (Hallet and Ferrand 1984; Erickson 1993; Lecoin et al. 1995; Jacobs-Cohen et al. 2002) and have found that clear differences exist in the choice of migratory path traveled by melanoctye precursors (melanoblasts). In chicken embryos of the Leghorn breed, which lacks dermal pigmentation, melanoblasts only invade the dorsolateral pathway. In the Silkie embryo, this same population of cells is also able to migrate in the ventral pathway normally reserved for cells of neuronal and glial lineages (Reedy et al. 1998; Faraco et al. 2001). Additionally, Silkie embryo extracts have been shown to have a proliferative effect on NCCs in vitro (Lecoin et al. 1994) as well as increasing proliferation of quail melanoblasts when grafted into Silkie embryos (Ferrand and L'Hermite 1985). These results suggest that the hyperpigmentation phenotype of the Silkie may be mediated by an abnormal choice of migratory path by melanoblasts as well as increased proliferation of this cell lineage.

Through experimental crosses it was shown that the hyperpigmentation phenotype of the Silkie chicken is controlled by 2 genes, autosomal dominant fibromelanosis (Fm/fm^+) and the sex-linked incompletely dominant inhibitor of dermal melanin (Id/id^+) (Bateson and Punnett 1911; Dunn and Jull 1927). It was found id^+ allows pigmentation of the shank in the dermal layer of skin and in conjunction with *Fm* causes the hyperpigmented state in the Silkie chicken (Hutt 1949; Smyth 1990). In combination with fm^+, id^+ results in pigmentation of only the shank and is present in many other breeds of fowl, including the Polish breed, which was utilized to develop the mapping populations in this study. The incompletely dominant nature of *Id* in heterozygous males in the presence of *Fm* is manifested by small isolated spots of dermal pigmentation on an otherwise starkly unpigmented background. *Id* is known to be 13.7 map units proximal to the centromere from the sex-linked barring gene (*B*) (Bitgood 1988) and located between 185 cM and 201 cM on the genetic map of chromosome Z (Levin et al. 1993; Groenen et al. 2000; Wright et al. 2006). The location of *Id* on the distal end of the long arm of chromosome Z has remained questionable due to our inability to replicate the randomly amplified polymorphic DNA markers developed by Levin et al. (1993) and the greater than 50 cM map distance between *Id* and the closest marker as shown by Wright et al. (2006). However, *B* has recently been mapped to a 355 Kb region at 71.8 Mb on chromosome Z (Dorshorst and Ashwell 2009) suggesting that *Id* is indeed located on the distal end of the long arm of chromosome Z. To the knowledge of these authors, no published studies have been conducted to determine the linkage group or genomic location to which *Fm* belongs.

This work utilizes the Silkie chicken, a naturally occurring pigmentation mutant, to further the understanding of NCC migration and differentiation. Previous studies have identified 2 primary differences in melanocyte precursor migration and proliferation in the Silkie chicken that may directly correspond to the 2-gene model of inheritance described from classical mating experiments. Analysis at the cellular level of all possible mechanisms by which this abnormal process of NCC migration and proliferation takes place would prove a monumental task. In lieu of that approach, we have chosen to generate 2 populations segregating independently for the primary genes of interest, *Fm* and *Id*, and use a gene mapping methodology to determine their genomic location allowing

for more focused candidate gene analysis. Although the primary goal of this research was to identify genomic regions in association with *Fm* and *Id*, the use of 3 morphologically diverse chicken breeds at the parental generation (Silkie, Polish, and New Hampshire) presented the opportunity to map numerous unrelated segregating traits in these 2 populations. Herein, we report the identification of 9 genomic regions associated with various traits in these populations including the 2 primary traits of interest, 2 traits for which causal mutations have been previously characterized, and 5 traits for which limited to no genomic location information was previously available.

Materials and Methods

Due to the incomplete dominance and sex-linked nature of *Id* and the interaction between *Fm* and *Id*, 2 separate mapping populations were developed to allow for the individual segregation of each gene and unambiguous classification of skin pigmentation phenotypes. Cross I was developed to map *Id* and was generated by mating a New Hampshire male (fm^+/fm^+, *Id/Id*) to a Silkie female with the genotype *Fm/Fm*, id^+/w. A single resulting male (*Fm*/fm^+, *Id*/id^+) was crossed to 10 Silkie females in order to generate more than 250 chicks segregating for *Id* in the presence of *Fm*. Cross II was developed to map *Fm* and was generated by mating a Polish male (fm^+/fm^+, id^+/id^+) to a Silkie female (*Fm/Fm*, id^+/w). The resulting females (*Fm*/fm^+, id^+/w) were mated to the same Polish male, which allowed for the segregation of *Fm* on an id^+ background in more than 350 individuals. Trait phenotypes were recorded at hatch and at 12 weeks of age in all populations. Founding individuals and representative photographs of day-old chicks segregating for *Fm* and *Id* are shown in Figure 1.

Founder individuals from Cross I and II were genotyped for 3072 SNPs on a custom Illumina GoldenGate BeadArray SNP panel in order to identify informative SNPs. Subsequently, an Illumina GoldenGate BeadXpress 384 SNP panel was designed to maximize the number of fully informative shared SNPs between Cross I and II and to cover all chromosomes with known DNA sequence in the chicken genome. DNA sequence analysis of the zone of polarizing activity (ZPA) regulatory sequence (ZRS) region of Sonic hedgehog (*SHH*), a candidate region for association with polydactyly, was performed using standard polymerase chain reaction conditions and dye terminator sequencing with the primers ZRS_F (5′-CACCTGCCTTAAGCATACGA-3′), ZRS_R (5′-TTTACCATCGTGAGAAACTGG-3′), ZRS_F_nested (5′-GCAGTACGCGATTTTCCTCTC-3′), and ZRS_R_nested (5′-AAAAATTTGAGGTAACTTCCTTGC-3′). A single SNP was detected between the founders of Cross II, which was then included in the 384 SNP panel and submitted to dbSNP (ss161109890).

All founder and F_1 level individuals were genotyped using the 384 SNP panel as well as 180 segregating individuals from Cross I and 272 segregating individuals from Cross II. A larger number of individuals from Cross II were genotyped due to the unknown chromosomal location of the segregating gene (*Fm*) within this population as compared with Cross I in which the segregating gene (*Id*) was known to be on chromosome Z. In each cross, markers were excluded that were not polymorphic (217 and 133) or displayed Mendelian inheritance errors (7 and 4) resulting in a total of 160 and 247 markers in cross I and II, respectively. A single marker association analysis was performed using PLINK (Purcell et al. 2007) and *P* values were adjusted for multiple testing using false discovery rate (FDR) (Benjamini and Hochberg 1995). The significance threshold of the corrected *P* values was set at 0.001.

Results and Discussion

The FDR corrected *P* value plots (Figure 2) for the genome-wide single SNP-trait association tests identified 9 genomic regions that show significant association with pigmentation and morphological traits in these mapping populations (Table 1). The association of chick down and adult feather color to the 180–195 Mb region of chromosome 1 spans the location of recessive white (*C*), mapped to a retroviral insertion in the gene encoding the enzyme tyrosinase (*TYR*) at 192 Mb on chromosome 1 (Chang et al. 2006). The association of chick beak color, representative of epidermal pigmentation, to the 20.7 Mb region of chromosome 11 correlates with the location of the extended black (*E*) locus encoding the melanocortin 1-receptor (*MC1R*) located at 20.8 Mb. *MC1R* is recognized as the major locus contributing to epidermal pigmentation status across a wide variety of animal species with the most dominant allele conferring black pigmentation in the chicken (Kerje et al. 2003; Ling et al. 2003). The identification of the previously mapped *C* and *E* loci confirms the role these genes play in the overall process of feather and other epidermal structure pigmentation as well as validates our experimental approach.

Skin Pigment

Highly significant *P* values were obtained for the primary traits of interest, shank and skin pigmentation, on chromosome Z and chromosome 20, respectively. In cross I, 2 different traits directed at measuring dermal pigmentation showed significant association to 2 SNPs located at 67.1 Mb and 72.3 Mb on the 74.6 Mb chromosome Z (Table 1). In cross II, 3 different dermal pigmentation phenotypes representing *Fm* were found to be significantly associated with 3 SNPs located within 10.3–13.1 Mb on chromosome 20. A single SNP in cross I at 7.2 Mb on chromosome 20 showed significant association with pigmentation of the beak in a day-old chicks suggesting that *Fm*, although completely dominant in the adult chicken, has an additive effect on chick pigmentation traits. This cross was designed to segregate *Id*/__ or id^+/__ on an *Fm/Fm* or *Fm*/fm^+ background. Under the assumption that *Fm* is completely dominant it was not expected to detect an

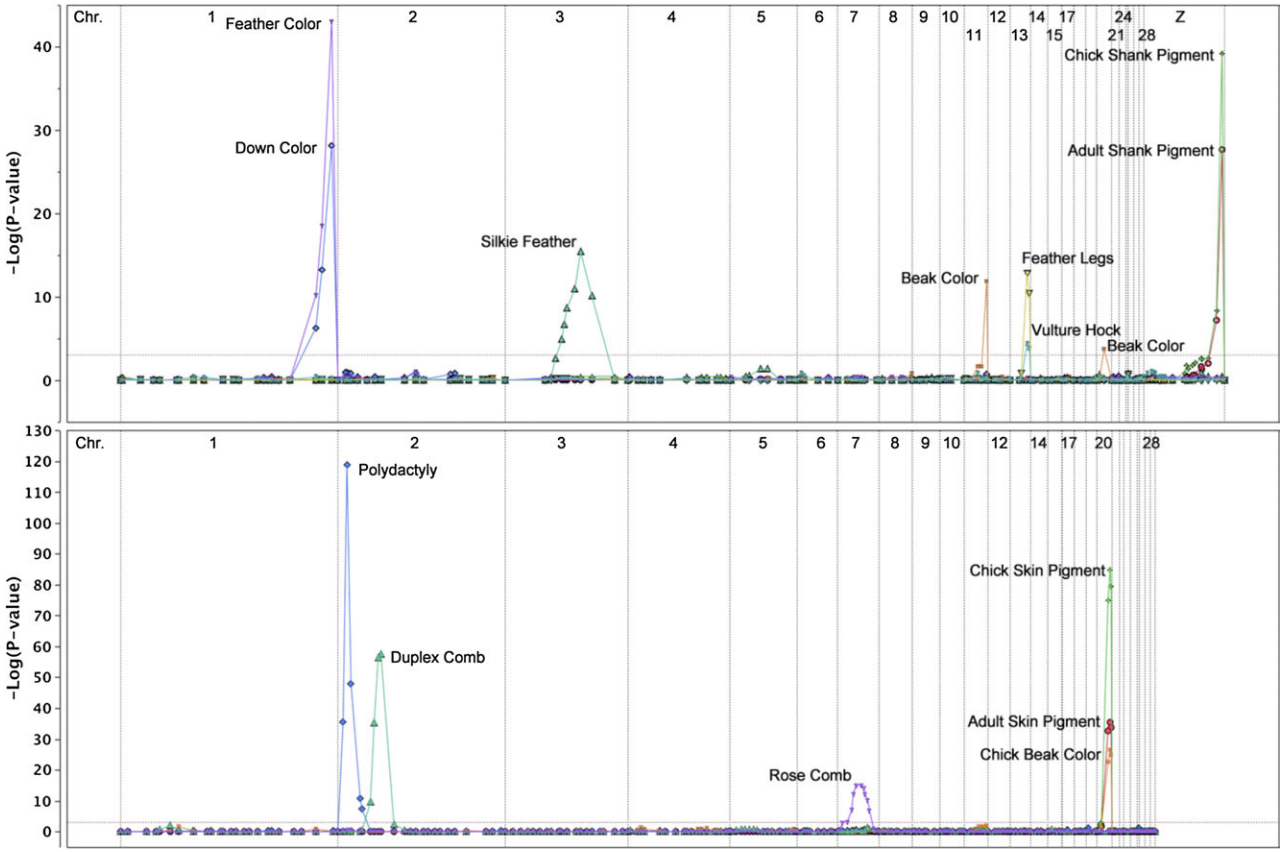

Figure 2. Genome-wide SNP-trait association test results. Genome-wide plot of SNP-trait association test in cross I (top) and cross II (bottom) for all phenotypic traits. Chromosome boundaries are demarcated by vertical dashed lines with chromosome number displayed along the top of each plot. Chromosome Z is absent in cross II due to the backcross design of this population.

association between the *Fm* locus and pigmentation status in this cross in which all birds possessed at least one dominant allele at *Fm*. However, this is the same chromosome and approximate location to the SNPs identified as being associated with *Fm* in cross II suggesting that *Fm* has an additive effect on chick pigmentation status but overall adheres to a dominant mode of inheritance.

The hyperpigmentation phenotype of the Silkie chicken is believed to be caused by an abnormal migration and proliferation of NCCs, the multipotent precursors of the melanocyte, in the developing embryo (Lecoin et al. 1994; Reedy et al. 1998; Faraco et al. 2001). The genomic regions identified as being associated with *Id* and *Fm* are on the order of several Mb making the analysis of candidate genes tedious due to the large number of genes they contain. However, due to previous studies of the mechanisms impacting the process of cellular migration and melanoctye proliferation in the Silkie and other species, we suggest Endothelin 3 (*EDN3*) as a candidate gene for *Fm* and beta 1,4-Galactosyltransferase, polypeptide 1 (*B4GALT1*) and versican (*VCAN*) as candidate genes for *Id*.

Labeling with the lectin peanut agglutinin (PNA) has been shown to define the migratory pathways taken by NCCs in the developing chicken embryo (Oakley and Tosney 1991; Oakley et al. 1994; Krull et al. 1995; de Freitas et al. 2003). The lack of PNA labeling in the Silkie embryo corresponds in a temporal and spatial manner to the invasion of melanoblasts into the ventral pathway normally reserved for neuronal and glial cell lineages. The *Id* candidate gene *B4GALT1* functions in migratory cell lamellipodia formation by localizing to the leading edge of the cell (Hathaway and Shur 1992; Appeddu and Shur 1994). Injection of anti-GalTase IgG has been shown to inhibit ventrolateral migration of NCCs in the cranial region confirming the role of *B4GALT1* in NCC migration (Hathaway and Shur 1992). The Gal-β(1-4)-GlcNAc glycosidic linkage that *B4GALT1* catalyzes is perhaps coincidentally similar to the Gal-β(1-3)-GalNAc linkage bound by PNA lectin in a highly specific manner. However, the non–cell-autonomous explanation of abnormal NCC migration in the Silkie embryo (Faraco et al. 2001) does not fit with the known role of *B4GALT1* in cell migration. This suggests that either a difference in spatial expression pattern in the embryonic region in which melanoblasts migrate or an altered acceptor/donor specificity of *B4GALT1* may be mediating the abnormal

Table 1 Genomic regions showing significant association with pigmentation and morphological variant phenotypes

Region	Phenotype	Classical gene symbol	Cross	Age	SNP	Chr	Mb[a]	−Log (*P* value)	Previously reported gene
1	Down color	*C*	I	Chick	rs14924023	1	180.6	6.30	TYR[b]
					rs14929246		186.3	13.24	
					rs13994687		195.3	28.10	
	Feather color		I	Adult	rs14924023		180.6	10.17	
					rs14929246		186.3	18.56	
					rs13994687		195.3	43.04	
2	Polydactyly	*Po*	II	Chick	rs15056847	2	4.8	35.57	
					ss161109890		8.4	118.67	
					rs15892452		12.2	47.74	
3	Duplex comb	*D*	II	Adult	rs15082359	2	33.6	35.11	
					rs15951989		37.3	56.19	
					rs15958477		39.8	57.56	
4	Silkie feather	*H*	I	Adult	rs16287115	3	64.5	10.93	
					rs14371625		69.7	15.41	
					rs14381094		80.2	10.07	
5	Rose comb	*R*	II	Adult	rs15846462	7	14.5	12.15	
					rs16589956		16.9	14.91	
					rs16594915		22.4	14.91	
					rs15861828		24.2	14.11	
6	Beak color	*E*	I	Chick	rs14968348	11	20.7	11.88	MC1R[c]
7	Feather legs	*Pti*	I	Adult	rs14999343	13	15.6	12.83	
					rs13505642		17.8	10.40	
	Vulture hock	*V*	I	Adult	rs14999343		15.6	4.42	
					rs13505642		17.8	3.88	
8	Beak color	*Fm*	I	Chick	rs13633288	20	7.2	3.72	
	Skin color		II	Adult	rs16172148		10.3	32.61	
					rs14280427		12.2	35.41	
					rs14280857		13.1	33.72	
	Skin color		II	Chick	rs16172148		10.3	74.78	
					rs14280427		12.2	84.79	
					rs14280857		13.1	79.46	
	Beak color		II	Chick	rs16172148		10.3	22.66	
					rs14280427		12.2	26.58	
					rs14280857		13.1	24.94	
9	Shank color	*Id*	I	Adult	rs14779590	Z	67.1	7.19	
					rs14686603		72.3	27.65	
	Shank color		I	Chick	rs14779590		67.1	8.38	
					rs14686603		72.3	39.22	

[a] SNP location in megabase pairs in WASHUC2 chicken genome assembly.

[b] Chang et al. (2006).

[c] Kerje et al. (2003), Ling et al. (2003).

migration of melanocyte precursor cells in the Silkie embryo if *B4GALT1* is involved.

In the current assembly of the chicken genome (WASHUC2) *B4GALT1* is located at 68.7 Mb and the presence of a *B4GALT1* pseudogene at 72.0 Mb is suggested. Sex-linked barring (B) has been located within 71.79–72.15 Mb (Dorshorst and Ashwell 2009) and is known to be 13.7 cM more distal from the centromere than *Id* (Bitgood 1988). Our results show almost complete linkage of shank pigmentation reflective of *Id* to a SNP at 72.31 Mb and highly significant but not nearly as complete association to a SNP at 67.1 Mb. These data suggest that the *B4GALT1* pseudogene is more likely to correspond to *Id*; however, this would invalidate the known linkage order of *Id* more centromeric than *B* suggesting that the assembly of the genome in this region may be inaccurate. An alternative candidate gene for *Id* is the chondroitin sulfate proteoglycan versican (*VCAN*) which acts as an inhibitory molecule to NCC migration in both the dorsolateral and ventral migratory pathways in the trunk region of avian embryos (Landolt et al. 1995) and is known to bind to PNA (Apostolski et al. 1994; Dutt et al. 2006). However, *VCAN* (61.3 Mb) is currently located outside of the region showing significant association to *Id* (67.1–72.3 Mb) on chromosome Z. An updated assembly of the most distal portion of the Z chromosome in the chicken will be necessary to distinguish between these 2 candidate genes.

In the mouse ectopic expression of *EDN3* by a keratin promoter results in extensive pigmentation of the skin tissue (Garcia et al. 2008; Aoki et al. 2009) in a manner that is

strikingly similar to the Silkie chicken's hyperpigmentation phenotype. In this system, *EDN3* is required both at an early embryonic stage and postnataly for the hyperpigmentation phenotype to occur and persist, demonstrating a role for *EDN3* in melanoblast proliferation and maintenance. In the chicken, *EDN3* has been shown to be expressed in the subectodermal mesenchyme during embryonic stages when melanoblasts are migrating (Nataf et al. 1998; Nagy and Goldstein 2006) and to have a potent mitogenic effect on melanoblasts in vitro (Lahav et al. 1996; Lahav et al. 1998). However, no published studies have documented the pattern or magnitude of *EDN3* expression in the Silkie chicken. The Polish breed of chicken has pigment present in the dermis of the shank but lacks pigment in the rest of the dermal and connective tissue throughout the body. This breed has the same genotype at the *Id* locus as the Silkie but lacks the dominant allele of *Fm*. This suggests that if id^{+} reflects the abnormal migratory properties of melanocyte precursors in the Silkie embryo, which are then maintained by the action of *Fm* (*EDN3*) throughout the entire body, maintenance of melanocytes in the shank of the Polish breed in the absence of *Fm* is overcome by some other factor endogenously expressed in this region. Although *EDN3* has been shown to be expressed by the mesenchymal tissue in the chicken trunk it remains to be seen if *EDN3* is constitutively expressed in the hindlimb environment of the chicken or if perhaps another endothelin is supporting melanocyte survival in this region in the case of the Polish breed shank pigmentation phenotype.

Polydactyly

Commonly referred to as preaxial polydactyly or PPD in other species, polydactyly (*Po*) in the chicken (Figure 3) is thought to be an incompletely dominant autosomal gene (Somes 1990a). In these backcross populations, birds with the wild-type number of 8 toes and those with the polydactylous combinations of 9, 10, 11, and 12 toes were observed. The single SNP (ss161109890) identified in the *SHH* regulatory region was detected as being highly associated with the polydactyly condition (Table 1). Of 272 individuals, all those (144) which inherited both alleles from the Polish nonpolydactyly founder possessed 8 toes. Of those individuals which were heterozygous, 124 individuals had 9–12 toes and 4 individuals had only 8 toes. Due to the backcross design of the population no individuals were homozygous for the Silkie allele. These results support the incompletely dominant nature of *Po* in the chicken, with a heterozygote penetrance of 96.9%, and highlight the variable expressivity of the polydactyly condition.

Previous work mapped the location of *Po* in the chicken to chromosome 2 (Pitel et al. 2000) and focused on *LMBR1* as the gene involved in the development of extra digits (Huang et al. 2006). Recent work in the mouse has identified several different causal mutations of preaxial polydactyly (PPD) within the cis-regulatory region of Sonic hedgehog (*SHH*), located within intron 5 of *LMBR1* and 1 Mb upstream of the *SHH* transcription initiation site (Maas and Fallon 2005). PPD in Hemingway cats of Key West, FL as well as other populations of PPD cats in United Kingdom was recently shown to be caused by point mutations in the 778 bp conserved noncoding element of the limb-specific cis-regulator of *SHH* termed the ZRS (Lettice et al. 2008). Analysis of the corresponding 794 bp ZRS region, that is 328 Kb upstream of *SHH* in the chicken, between the founder individuals of cross II in this study revealed a single SNP not previously observed in the mouse, cat, or human. Inclusion of this polymorphic marker (ss161109890) in the SNP genotyping panel revealed nearly complete association with the polydactyly phenotype in a manner supportive of the incomplete dominance of *Po*. This SNP does not overlap with any of the 14 known mutations associated with PPD in human, mouse, and cat as shown in Figure 4 (Lettice et al. 2003, 2008; Gurnett et al. 2007; Masuya et al. 2007). Analysis of transcription factor binding sites involving this SNP using TFSEARCH (Heinemeyer et al. 1998) and rVISTA (Loots and Ovcharenko 2004) indicates that the allele present in the Silkie generates binding sites for OCT-1, NKX2-5, PRRX2, MSX1, and RORA. Based on gene ontology PRRX2 and MSX1 have known roles in limb morphogenesis and may be directing ectopic expression of *SHH* in the Silkie limb bud resulting in the formation of a fifth more anterior digit.

To confirm the role that this point mutation plays in the polydactyly phenotype the entire 794 bp ZRS was sequenced in representative samples of 4 other polydactylous breeds (Salmon Faverolle, Mottled Houdan, Silver Gray Dorking, and White Sultan) and 8 nonpolydactylous breeds (Table 2). No other mutations were found across all breeds except for the same single-point mutation identified in our Silkie population. This mutation was only present in the White Sultan breed, whereas the 3 other polydactylous breeds did not posses this or any other mutation within the ZRS. The White Sultan breed displays a high degree of phenotypic similarity to the Silkie, which suggests that the 2 breeds may be closely related. The White Sultan was originally developed in Istanbul, Turkey as a favorite bird of Turkish rulers (The American Poultry Association 1998). The Faverolle and Houdan breeds are believed to be derived from the Dorking breed of chicken, which originated in Italy (The American Poultry Association 1998). The presence of the same SNP in a highly conserved regulatory region of *SHH* in 2 polydactylous breeds believed to have a common genetic origin suggests that this mutation is a candidate for one of at least 2 causal mutations of polydactyly in the chicken. The absence of this SNP in 3 polydactylous breeds believed to have a different genetic origin as the Silkie and White Sultan breeds suggests the existence of an allele outside the 794 bp ZRS or that of a second locus contributing to *Po*.

Duplex Comb

Silkie chickens display the walnut comb phenotype, which is the result of the combination of loci that separately

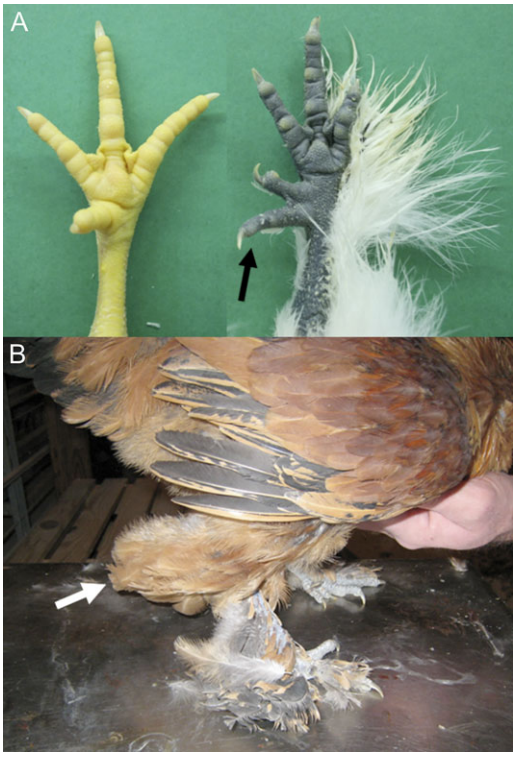

Figure 3. Polydactyly and vulture hock phenotypes in the chicken. (**A**) Shank of the New Hampshire breed (left) and the White Silkie (right) demonstrating the extra fifth toe present in the Silkie breed (black arrow). Differences in skin pigmentation, feathered shank, and the silkie feathering phenotypes can also be seen. (**B**) An individual from cross II of the mapping population showing the presence of both the vulture hock and feathered leg conditions. Vulture hock is seen as a group of elongated and rigid feathers (white arrow) extending from the base of the tibia, whereas the feathered leg phenotype can be seen as feathers on the shank and foot.

represent the pea (*P*) and rose (*R*) comb types. The Polish breed has the duplex v-shaped (D^v) comb phenotype, whereas the New Hampshire breed has the wild-type single comb (Somes 1990b). The combination of multiple loci affecting comb and crest type contributed to a wide diversity of comb phenotypes displayed in our backcross populations. The Silkie chicken is also known to carry an abnormal type of trifid comb modifier, which is further truncated by the crested nature of the skull (Punnett 1923). In cross I single, rose and walnut comb phenotypes were observed. In cross II, rose, pea, walnut, and duplex comb types were segregating. Although no significant association with comb type was observed in cross I, the 33.6–39.8 Mb region of chromosome 2 showed a significant association with comb type in cross II (Table 1). Previous studies have shown that *D* is within the same linkage group as *Po* (Hutt and Mueller 1943) and is comprised of several different alleles, D^v being the most dominant (Somes 1991). These results match our findings of both polydactyly and duplex comb being most highly associated with SNPs on chromosome 2.

Rose Comb

No significant regions were identified for Rose (*R*) or Pea (*P*) combs in either cross when comb type was considered a single trait with up to 6 categories (Single, Rose, Pea, Walnut, Duplex, and Mixed). Due to the number of comb modifying genes segregating in this population, phenotying became difficult and there is no doubt that some birds were misclassified. In order to combat these difficulties and accommodate for the known epistasis of *R* and *P* resulting in the Walnut comb phenotype birds were classified as putative carriers of at least one dominant allele or the recessive allele at *R* and *P*. This resulted in a region of chromosome 7 detected as having significant association with *R*, whereas no significant regions were detected for *P*.

The Pea comb locus has recently been mapped to a tandem duplication in *SOX5* on chromosome 1 (Wright et al. 2009) and is described as a reduced version of the single comb with 3 rows of points, the central being the largest (Somes 1990b). We were unable to detect an association with *P* and this region in our population most likely due to the difficulty in phenotyping the large variety of comb types segregating. Rose comb is of similar height as many single combs but much broader at the top with numerous small papillae and most often a single spike at the rear (Somes 1990b). Rose comb is an autosomal dominant trait (Bateson and Saunders 1902) and has been well documented as having detrimental effects on fertility in matings involving homozygous dominant males (Crawford 1965; Wehrhahn and Crawford 1965). The poor fertility of homozygous *R* males is associated with reduced spermatozoa metabolic rate, motility, and filling of sperm storage tubules in vivo (Kirby et al. 1994; McLean and Froman 1996b). Rose comb has been mapped to 32.5 cM from the Lavender (*LAV*) locus (Brumbaugh et al. 1972) which has been mapped to the melanophilin gene (*MLPH*) located at ~4.7 Mb on chromosome 7 (Vaez et al. 2008). These results corroborate our finding of R being significantly associated with several SNPs on chromosome 7 with the highest degree of association from 16.9–22.4 Mb. No obvious candidate genes are identifiable in this region given the limited knowledge of the molecular mechanisms of the rose comb phenotype.

Silkie Feathering

The silkie feathering trait also referred to as hookless (Figure 3) is known to be directed by a lack of barbicel formation on the highly branched structure of the feather and is inherited as an autosomal recessive trait (Somes 1990b). Initial linkage mapping studies suggested the Silkie gene (*h*) was 43 map units from the naked neck gene (*Na*) in linkage group III,

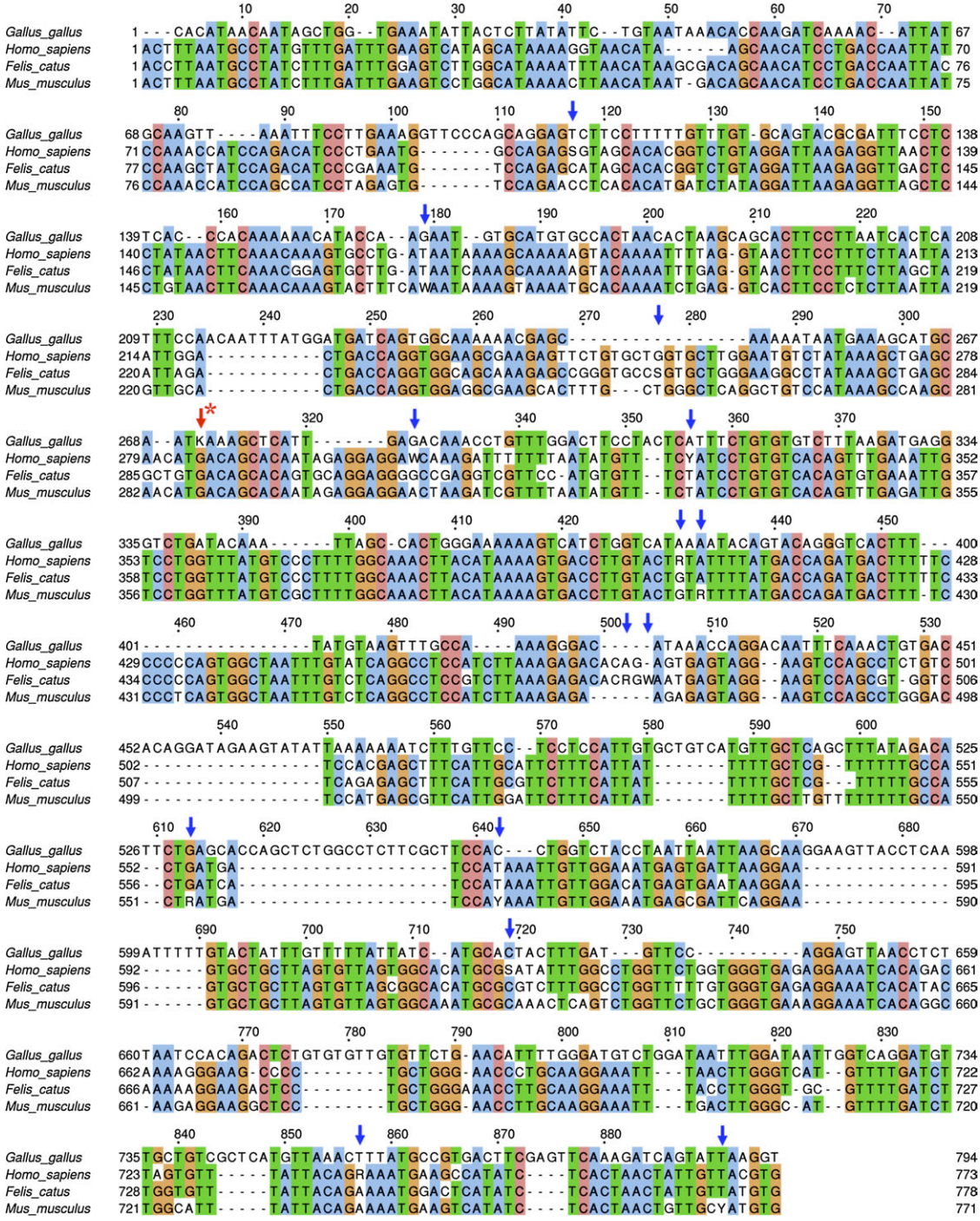

Figure 4. *SHH* ZRS multiple sequence alignment. Comparison of chicken, human, cat, and mouse *SHH* ZRS DNA sequence. The SNP ss161109890 is associated with polydactyly in the chicken is marked with a red arrow with a star. Known mutations associated with PPD are marked with a blue arrow (Lettice et al. 2003, 2008; Gurnett et al. 2007; Masuya et al. 2007). R = A or G, W = A or T, S = C or G, Y = C or T, K = G or T.

now known as chromosome 1 (Warren 1938; Hutt 1949). However, it was subsequently shown that *Na* was located on chromosome 3 suggesting that *h* was also on this autosome (Pitel et al. 2000). In agreement with these prior findings a 15.7 Mb region on chromosome 3 is significantly associated with silkie feathering (Table 1) with rs14371625 located at 69.7 Mb showing the highest degree of association.

Table 2 *SHH* ZRS sequencing results

Breed	No. of birds	No. of toes	ss161109890
White Silkie	10	5	T
White Sultan	2	5	T
Salmon Faverolle	6	5	G
Mottled Houdan	6	5	G
Silver Gray Dorking	6	5	G
Various breeds[a]	12	4	G

Table of breeds in which the highly conserved zone of polarizing activity (ZPA) regulatory sequence (ZRS) of Sonic Hedgehog was sequenced for detection of SNPs associated with the polydactyly phenotype. The White Silkie and White Sultan breeds were both found to have the *T* allele at the SNP ss161109890, whereas all other breeds, both 4 and 5 toed, had the *G* allele. No other mutations were detected within the 794 bp ZRS in the chicken breeds shown below.

[a] Breeds include White Crested Black Polish, Barred Plymouth Rock, Jungle Fowl, German Spitzhauben, Blue Sumatra, Black Australorp, Black Orpington, and Silver Phoenix.

Feathered Leg and Vulture Hock

The Silkie breed displays ptilopody (*Pti*), a phenotype characterized by feathers present on the shank, feet, and toes. The Silkie also possesses the related trait known as vulture hock (*V*) in which feathers in the crural feather tract of the tibia are increased in length and rigidity, taking on the appearance of a flight feather (Figure 3). The presence of several *Pti* loci, with varying modes of inheritance, has been suggested from previous studies although the Silkie's feathered leg condition is thought to be caused by 1 or 2 incompletely dominant loci (Somes 1990b). Review of the literature on the vulture hock condition is also inconclusive with a strong indication of primarily recessive but perhaps incompletely dominant modes of inheritance conditional on the presence of *Pti* (Danforth 1929; Jull and Quinn 1931; Somes 1990b). A single genomic region on chromosome 13 is significantly associated with both of these traits in cross I; the SNP rs14999343 at 15.6 Mb having the highest association (Table 1). Although *Pti* shows a higher degree of association with rs14999343 as compared with *V* and has a stimulatory effect on the vulture hock phenotype, it is unclear which trait is truly associated with this region. The possibility remains of both loci being present within this region or that of *V* being located on a chromosome not represented in this SNP panel.

The feathered leg phenotype (*Pti*) is observed in may different poultry breeds (Breda, Langshan, Faverolle, Brahma, Silkie, Belgian d'Uccle Bantam, Booted Bantam, Cochin, and Sultan), whereas vulture hock (*V*) is found only in the Breda, Silkie, Belgian d'Uccle Bantam, Booted Bantam, and Sultan (Somes 1990b). These traits are not found in any of the wild jungle fowl species, the accepted ancestors of the domestic chicken (Fumihito et al. 1994; Eriksson et al. 2008). Functional characteristics of feathering on the hindlimb of the chicken have never been speculated on during the course of classical breeding experiments and therefore have been presumed to be an example of cultivation of phenotypic mutants by humans. Recently a 124–128 million-year-old dromaeosaur fossil (*Microraptor gui*) discovered in China has been described as having wing-like feathers on the legs (Xu et al. 2003). This extraordinary fossil is heralded as supporting the arboreal-gliding hypothesis as opposed to the powered running theory on the origin of flight in the ancestors of modern avian species (Prum 2003). The elongation of feathers on the tibia to the size of primary wing feathers characteristic of *V* in conjunction with feathers along the entirety of the leg representative of *Pti* (Figure 3) is as similar to the *M. gui* 4-winged phenotype as we have seen in any domesticated poultry species. Suggesting that ancient atavisms are not to be unexpected, the extant talpid[2] chicken developmental mutant was shown to initiate tooth formation after 70–80 million years of divergence from the most recent toothed avian ancestor (Harris et al. 2006). It is unlikely that *Pti* and *V* represent homologous structures controlled by identical genetic elements as the 4-winged *M. gui*, but the use of these developmental mutants may prove beneficial in examining the contribution hindlimb feathering may have had on the ability to sustain flight in ancient taxa.

Summary

The Silkie chicken has a long history in Chinese culture as a natural treatment for various ailments (Li and Luo 2003); however, no recent studies have shown a specific benefit from ingesting the heavily pigmented skin and meat from the Silkie chicken. The Silkie chicken has seen the most interest from those researchers interested in pigment cell biology given the clearly defined roles of 2 interacting genes in directing the extensive hyperpigmentation of dermal tissue. In this report, we describe the generation of 2 mapping populations designed for the purpose of identifying these 2 specific genes, inhibitor of dermal melanin (*Id*) and fibromelanosis (*Fm*), that effect dermal pigmentation in the chicken. Loci significantly associated with both of these primary target genes as well as loci associated with the classically described monogenic traits silkie feathering or hookless (*h*), polydactyly (*Po*), feathered legs (*Pti*), vulture hocks (*V*), rose comb (*R*), and duplex comb (*D*) have been identified using these 2 mapping populations. A single SNP within the highly conserved ZRS of *SHH* that likely corresponds to the causal mutation of polydactyly in the Silkie and White Sultan breeds has been described. The location of *Id* on the distal end of the q arm of chromosome Z is in concordance with prior linkage mapping results. The identification of chromosome 20 as the location of *Fm* is a novel finding. Candidate genes for *Id* (*VCAN* and *B4GALT1*) and *Fm* (*EDN3*) have been suggested based on known functional characteristics of these genes and the molecular mechanisms of the Silkie chicken's hyperpigmentation phenotype. Candidate genes for *h*, *V/Pti*, *R*, and *D* remain elusive due to the size of the genomic regions identified and the lack of prior research on the molecular mechanisms behind these traits.

We have shown that the study of the genetics of morphological variation in the chicken is facilitated by gene

mapping in properly designed segregating populations. Future work using these populations will be directed at more narrowly defining the genomic regions associated with each trait in order to facilitate candidate gene analysis. Identification of potential causal mutations for each trait described in this study will further expand our knowledge of important biological methods of body patterning and development. The number of morphological traits that have been extensively categorized in the chicken and other domesticated poultry species is vast (Hutt 1949; Crawford 1990); yet, only a small proportion of these have been investigated using modern genomic techniques. The coupling of these extremely rich classical descriptions of morphological variation to recently available highly dense sets of SNP markers and the rapidly improving genome sequence will allow the continued use of the chicken in probing fundamental principles of developmental biology.

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