Polymorphism of the myostatin gene and its association with growth traits in chicken

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ABSTRACT An experiment was carried out on myostatin gene with the objectives of identification of polymorphism in the myostatin gene and estimation of the effect of polymorphism on growth traits in chickens. Single-stranded conformation polymorphism followed by sequencing was performed to reveal polymorphism of the gene. A total of 13 haplotypes were observed across 3 chicken lines (PB-1 and CB as broiler lines and IWI as the layer line). Myostatin haplogroups had a significant effect on BW at 28, 42, and 49 d of age in the PB-1 line. The significant association of haplogroups was observed with BW at d 14 and 49 in the CB line. In the IWI layer line, the myostatin gene was polymorphic but had no significant association with growth traits. It is concluded that the myostatin gene was polymorphic and had a significant effect on growth traits in broiler chickens.

Key words: association, growth, myostatin, polymorphism

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INTRODUCTION

Several genes are involved in regulating growth in chickens. Some of the genes enhance muscular growth, whereas a gene such as myostatin (***MSTN***) regulates growth negatively by limiting muscular growth during the prehatch and posthatch period (Sato et al., 2006). Myostatin is a member of transforming growth factor-β family expressed primarily in muscle tissues and involved in regulating development at the embryonic stage and tissue homeostasis in the adult stage (Lee and McPherron, 2001; Hickford et al., 2010). Functionally, lack of MSTN function results in the excessive growth of skeletal muscle, which delineates the existence of a powerful mechanism to control muscle size in normal individuals (McPherron et al., 1997). The sequence and function of MSTN appears to be highly conserved among vertebrate species, and its role during myogenesis of chickens was expected to be similar to that observed in mammals (Scheuermann et al., 2004). During the embryonic stage, MSTN downregulates the *Pax-3* gene, which is associated with proliferation of myogenic cells and also prevents expression of the *Myo-D* gene involved in activation of the myogenic program in chickens (Amthor et al., 2002). McPherron et al. (1997) revealed that mice with a deleted *MSTN* gene had an enormous increase in skeletal muscle mass, making it approximately twice that of wild type mice on account of muscle fiber hyperplasia and hypertrophy. Mutated *MSTN* gene showed double muscling in cattle with a drastic increase in skeletal muscle (Grobet et al., 1997; McPherron and Lee, 1997). In humans, the mutation caused gross muscular hypertrophy (Schuelke et al., 2004). Increased expression of MSTN was also observed in humans with chronic illness, HIV infection, and early aging due to muscle atrophy (Gonzalez-Cadavid et al., 1998; Ivey et al., 2000; Reardon et al., 2001). Keeping these facts in view, the objectives of the study were designed to identify genetic polymorphism of the *MSTN* gene and to estimate association of polymorphism with growth traits in chickens.

MATERIALS AND METHODS

Experimental Birds

The study was conducted in 2 broiler chicken lines, PB-1 and CB and a layer chicken line, IWI, maintained at the Institute farm, Rajendranagar, Hyderabad, India. All the lines were pedigreed and closed populations. In the PB-1, CB, and IWI lines, study was undertaken on 422, 214, and 462 birds, respectively. The number of sires used for the study in all the lines was 50 for each line. The PB-1 was a synthetic color broiler line selected initially for 6-wk BW and later on for 5-wk BW for the last 18 generations. The overall BW of PB-1 at 5 wk of age was 924.3 ± 0.12 g (Project Directorate on Poultry, 2009). This line is used as a male line for commercial broiler production. The CB line is a synthetic

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Table 1. Primer sequences used for amplification of fragments of the myostatin gene

Primer name[1]	Sequence (5′–3′)	Fragment	Size (bp)
MYTmF	ATGCAAAAGCTAGCAGTCTATG	Exon 1	373
MYTE1R	ACTCCGTAGGCATTGTGATAAT		
MYTE2F	CTGATTTTCTTGTACAAATGGAG	Exon 2	374
MYTE2R	CAATCCATCTTCACCCGGTC		
MYTE3F	AACCCATTTTTAGAGGTCAGAG	Exon 3	381
MYTmR	TCATGAGCACCCGCAACGAT		

[1]F = forward primer and R = reverse primer used for amplification by PCR.

color broiler line that is random bred pedigreed over last 9 generations. The BW of CB line at 5 wk of age was 625.5 ± 0.13 g (PDP Annual Report, 2010). The IWI line was a selected line for egg production and egg weight developed by selecting over 12 generations. The birds were kept in the brooder house till the age of 6 wk and then shifted to the grower house. All the birds were reared on deep litter system in the same shed under intensive management of farming providing same management regimen with ad libitum feeding and watering up to 6 wk. All the birds were hatched at the same time and housed all along in the shed at the age of 16 wk. The feeding schedule for these birds were 22% CP and 2,900 kcal of ME for d 1 to 21 and 20% CP and 3,000 kcal of ME for d 22 to 49 in the PB-1 and CB lines, and 20% CP and 2,600 kcal of ME for d 1 to 49 in IWI line. Cooling facilities were provided during summer season by water sprinkling on the roof, and proper lighting was arranged in the shed so that birds had congenial environment for performing to optimum potential.

Isolation

Blood samples were collected from all the birds at 4 wk of age in a 0.5-mL tube containing 20 μL of 0.05 *M* EDTA anticoagulant. Genomic DNA was isolated from blood cells following the standard protocol (Sambrook and Russell, 2001).

PCR

Myostatin gene consisting of 3 exons was considered to explore polymorphism at the coding region of the gene. Three pairs of primers were designed from the chicken MSTN sequence available at the National Center for Biotechnology Information (accession number AF346599) with DNASTAR software (Lasergene Inc., Madison, WI). The primer sequences are given in Table 1. The PCR reactions included 50 μg of DNA template, 10 p*M* of each primer, 1.5 m*M* of $MgCl_2$, 100 μ*M* of each dNTP, 1× assay buffer, and 0.25 U of *Taq* DNA polymerase (MBI Fermentas, St. Leon-Rot, Germany). The amplification conditions for all exons were initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 45 s, annealing at 55°C, and extension at 72°C for 45 s with final extension at 72°C for 10 min.

Single-Stranded Conformation Polymorphism and Sequencing

Single-stranded conformation polymorphism was carried out on 12% native PAGE (50:1, acrylamide and bisacrylamide) with 5% glycerol. A volume of 3 μL of PCR product mixed with 15 μL of formamide dye (95% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue, 0.5 *M* EDTA) was denatured at 95°C for 5 min followed by snap cooling on ice for 15 min. Then the product was loaded in the gel and electrophoresis was performed at 4°C for 12 h at 200 V. After electrophoresis was over, the gel was stained with silver nitrate to visualize banding patterns of the fragments (Bhattacharya et al., 2011).

Three PCR products amplified from each genotypic group pertaining to each of 3 fragments of the *MSTN* gene, derived with HotStar HiFidelity DNA Polymerase (MBI Farmentas) were sequenced using fragment-specific primers from both ends by the automated dye–terminator cycle sequencing method in ABI PRIZM 377 DNA sequencer (Perkin-Elmer, Waltham, MA).

Preparation of Haplotypes; Traits

Haplotypes were prepared by combining single-stranded conformation polymorphism patterns of all the fragments of the gene in each individual. Haplotypes in diploid state of an individual reveal the haplotype combinations of that animal. Haplotype sequences were analyzed with DNASTAR Software (Lasergene Inc.). Frequencies of haplotype and its combinations were calculated by the gene counting method.

Body weights at d 1, 14, 28, 42, and 49 d of age were measured in all the birds under study.

Statistical Analysis

The association of haplotype combinations and traits were estimated following the least-square maximum likelihood method of the LSML90 package (Harvey, 1991), where haplotype and breed were used as fixed effect and sire as the random effect. Thus, the model used for this analysis was

$$Y_{ijklm} = \mu + S_i + B_j + HPL_k + B_jXHPL_k + e_{ijkl},$$

where μ = overall mean, S_i = ith sire effect, B_j = jth breed effect, HPL_k = kth haplotype effect, B_jXHPL_k = interaction effect, and e_{ijkl} = random error with NID (errors are normally independently distributed having mean as 0 and SD as σ^2_e). The allele/haplotype substitution effect was calculated by the formula of Falconer and Mackay, 1996;

$$\alpha_1 = q[a + d(q - p)];$$

$$\alpha_2 = -p[a + d(q - p)],$$

where p = frequency of A1 allele; q = frequency of A2 allele; a = genotypic value of A1A1 genotype; d = genotypic value of the A1A2 genotype.

The predominant haplotypes with frequency 0.01 or more were included in the haplotype substitution study. The haplotype substitution effects were estimated with respect to only the most predominant haplotype.

RESULTS AND DISCUSSION

Polymorphism

Myostatin gene consisting of 3 exons was amplified for detection of polymorphism, and consequently haplotypes were screened for all the birds. In total, 13 haplotypes were found across the lines. In the PB-1 line, only 11 haplotypes were observed of which h1 haplotype was the predominant one (0.49) and 2 haplotypes, h7 and h8, were the least frequent haplotypes (0.01). Accordingly, 11 haplotype combinations were found of which h1h4 and h1h7 were the most (0.20) and least (0.03) frequent haplotype combinations, respectively. However, h9 and h10 haplotypes were absent in this line, which may be the line characteristic and be used as a molecular marker for differentiating the population from other chicken lines.

In the CB line, 7 haplotypes were observed of which h1 showed the highest (0.48) frequency and h3 revealed the lowest (0.01) frequency (Table 2). A total of 6 haplotype combinations were found of which h1h5 and h1h3 revealed the highest (0.39) and lowest (0.03) frequency, respectively. In this line, the h8, h9, h10, h11, h12, and h13 haplotypes were absent. In the IWI line, 10 haplotypes and 9 haplotype combinations were observed in which the h1 haplotype had the highest frequency (0.41) and 3 haplotypes; namely, h9, h10, and h13 possessed the lowest frequency of 0.01. The haplotype combinations h1h5 and h1h13 had the highest (0.27) and lowest (0.02) frequency, respectively. However, h8, h11, and h12 haplotypes were absent in this line, though they were present in PB-1 line. The absence of haplotypes in the population may help in differentiating the line from other lines where they are present. The sequences of different fragments were used to prepare haplotypes, and the sequences were submitted to the National Center for Biotechnology Information GenBank with the accession numbers GU181321, GU181322, GU181323, GU181324, GU181325, GU181326, GU181327, and GU181328. Our results were in agreement with the findings of Ye et al. (2007), which revealed 8 haplotypes in the *MSTN* gene of commercial broiler chickens. Kumar et al. (2007) reported SNP in promoter (5 SNP), intron 1 (1 SNP), intron 2 (1 SNP), and exon 1 (3 SNP) of *MSTN* gene in indigenous poultry and Red Junglefowl. The polymorphism in exon 1 of MSTN was determined by Zhu et al. (2007) in the Wenling grass-chicken. Gu et al. (2002) depicted 3 SNP in the 5′-regulatory region and 2 SNP in the 3′-regulatory region of the *MSTN* gene in Beijing Youji, Balerji, Shiqiza, Dwarf yellow chicken, mini yellow chicken, Huiyang Huxuji, Recessive white chicken, and Hyline layer chickens.

Table 2. Frequency of myostatin haplotypes in different lines of chicken

Haplotype[1]	PB-1[2]	CB[3]	IWI[4]
h1	0.49	0.48	0.41
h2	0.04	0.02	0.04
h3	0.05	0.01	0.02
h4	0.10	0.08	0.05
h5	0.07	0.19	0.13
h6	0.04	0.14	0.12
h7	0.01	0.02	0.02
h8	0.01	—	—
h9	—	—	0.01
h10	—	—	0.01
h11	0.03	—	—
h12	0.02	—	—
h13	0.11	—	0.01

[1]Frequency of each haplotype denotes the occurrence of each haplotype among all the haplotypes in the population within a specific line.

[2]PB-1 is a color broiler line under selection for BW at 5 wk of age practiced for last 18 generations.

[3]CB is a color broiler line bred randomly without practicing any selection.

[4]IWI is a layer line selected for egg production and egg weight for the last 12 generations.

Nucleotide Variability

In MSTN haplotypes, nucleotide substitution was found in 11 positions of which substitutions at first 5 positions had the silent mutations (Table 3). The remaining 6 positions at 478, 908, 998, 1006, 1069, and 1120 revealed nonsynonymous types of mutation, which showed changes of amino acids in the protein composition of different haplotypes. Our results suggest that the haplotypes of the *MSTN* gene that varied both in nucleotide as well as amino acid composition may be used as different MSTN variants in layer and broiler chicken lines.

Association

The haplogroups were significantly ($P < 0.05$) associated with BW at different ages. Chicken lines showed a significant effect ($P < 0.05$) on BW in the model, whereas the interaction effects between lines and haplogroups were found to be nonsignificant for the traits. The BW at 28, 42, and 49 d of age were significantly

Table 3. Nucleotide and amino acid changes among myostatin haplotypes in chickens[1]

	Position in the gene										
Haplotype	154	195	234	324	477	478	908	998	1006	1069	1120
h1	c	c	g	c	t	a	g	g	g	g	g
						M	R	R	A	E	G
h2	a	c	a	t	t	a	a	a	g	g	g
						M	K	K	A	E	G
h3	a	c	a	t	t	a	g	g	c	a	t
						M	R	R	P	K	C
h4	a	c	a	t	c	t	g	g	g	g	g
						L	R	R	A	E	G
h5	a	c	a	t	c	t	a	a	g	g	g
						L	K	K	A	E	G
h6	a	c	a	t	c	t	g	g	c	a	t
						L	R	R	P	K	C
h7	a	g	a	t	c	t	a	a	g	g	g
						L	K	K	A	E	G
h8	c	c	g	c	t	a	a	a	g	g	g
						M	K	K	A	E	G
h9	a	g	a	t	t	a	a	a	g	g	g
						M	K	K	A	E	G
h10	a	g	a	t	c	t	g	g	c	a	t
						L	R	R	P	K	C
h11	a	g	a	t	t	a	g	g	g	g	g
						M	R	R	A	E	G
h12	a	g	a	t	c	t	g	g	g	g	g
						L	R	R	A	E	G
h13	a	c	a	t	t	a	g	g	g	g	g
						M	R	R	A	E	G

[1]Lowercase letters are nucleotides, and uppercase letters are amino acids. Nonsynonymous type of mutation in the gene showed a change of amino acids among haplotypes, whereas synonymous types of mutation did not cause any change in amino acid structure.

($P < 0.05$) associated with haplogroups in PB-1 line. The birds having h1h7 haplogroup showed highest BW, whereas h1h13, h1h11, and h1h2 haplogroups showed the lowest BW at 28, 42, and 49 d of age, respectively (Table 4). The birds of h1h7 had 12% higher BW than h1h13 group at 28 d, whereas h1h7 revealed 18% higher weight than h1h11 haplogroup at 42 d of age. At 49 d of age, the birds with the h1h7 haplogroup had 17% higher weight than the h1h2 group. However, overall haplotype performance trend for BW at 28 d of age was found to be higher in haplogroup cluster h1h7/h1h6/h1h4 followed by h1h5/h1h11/h1h13. At 42 d of age, the h1h7/h1h5/h1h6 haplogroup cluster had the highest BW followed by h1h2/h1h3/h1h4/h1h13 and h1h11 haplogroups. At 49 d of age, the h1h7 haplogroup showed highest BW followed by h1h3/h1h4/h1h5/h1h6/h1h11/h1h13 and h1h2/h1h12 cluster.

In the CB line, BW at 14 and 49 d were significantly ($P < 0.05$) associated with haplotype combinations (Table 5). At 14 d age, the h1h3 haplogroup showed 106% higher BW than the h1h2 haplogroup. But, at 49 d of age, birds of the h1h5 group revealed 34% higher BW than the h1h2 haplogroup. The trend of haplotypes for 14-d BW was found to be highest in the h1h3 group followed by the h1h5/h1h6/h1h7 and h1h2 haplogroups. At 49 d of age, the highest BW was observed in the h1h5/h1h6 groups followed by h1h3/h1h7 and h1h2, respectively. Body weights at different ages were not found to be significantly associated with haplotype combinations in IWI line of chicken (Table 6). There

Table 4. Myostatin haplotype BW in PB-1 chicken[1]

Haplotype combination	*f*	BW1	BW14	BW28	BW42	BW49
h1h2	0.09	48.4 ± 1.0	289 ± 8	721 ± 21^{ab}	1,234 ± 36^{b}	1,354 ± 38^{a}
h1h3	0.11	46.3 ± 0.9	278 ± 8	725 ± 20^{ab}	1,221 ± 32^{b}	1,462 ± 46^{b}
h1h4	0.20	46.7 ± 0.7	285 ± 6	736 ± 16^{b}	1,228 ± 21^{b}	1,423 ± 40^{b}
h1h5	0.15	46.9 ± 0.8	285 ± 7	699 ± 15^{a}	1,261 ± 25^{c}	1,483 ± 34^{b}
h1h6	0.09	48.2 ± 1.0	289 ± 8	769 ± 20^{b}	1,294 ± 39^{c}	1,422 ± 39^{b}
h1h7	0.03	49.6 ± 1.8	305 ± 14	771 ± 15^{b}	1,331 ± 24^{c}	1,581 ± 40^{c}
h1h11	0.06	45.8 ± 0.7	282 ± 5	691 ± 18^{a}	1,132 ± 32^{a}	1,433 ± 42^{b}
h1h12	0.04	46.5 ± 1.3	297 ± 12	718 ± 22^{ab}	1,274 ± 37^{bc}	1,364 ± 41^{a}
h1h13	0.22	48.2 ± 1.6	290 ± 12	687 ± 17^{a}	1,197 ± 18^{b}	1,448 ± 38^{b}

[a–c]Within a column, different superscripts indicate significance at $P < 0.05$.

[1]BW1, BW14, BW28, BW42, and BW49 are the BW (g) at 1, 14, 28, 42, and 49 d of age, respectively, and *f* is the frequency. Body weights on d 28 (BW28), 42 (BW42), and 49 (BW49) had a significant association with haplogroups. PB-1 is a color broiler line under selection for BW at 5 wk of age practiced for last 18 generations.

Table 5. Myostatin haplotype BW in CB chicken[1]

Haplotype combination	*f*	BW1	BW14	BW28	BW42	BW49
h1h2	0.05	34.9 ± 1.2	81 ± 6[a]	337 ± 23	675 ± 29	746 ± 41[a]
h1h3	0.03	32.9 ± 1.3	167 ± 7[c]	377 ± 23	674 ± 29	879 ± 35[b]
h1h4	0.17	37.5 ± 1.3	98 ± 6[ab]	410 ± 21	713 ± 29	931 ± 30[bc]
h1h5	0.39	34.2 ± 0.6	104 ± 3[b]	373 ± 14	707 ± 27	998 ± 18[c]
h1h6	0.29	34.3 ± 0.4	114 ± 5[b]	352 ± 9	675 ± 18	950 ± 24[c]
h1h7	0.04	33.1 ± 0.4	107 ± 11[b]	362 ± 11	720 ± 20	901 ± 33[b]

[a–c]Within a column, different superscripts indicate significance at $P < 0.05$.

[1]BW1, BW14, BW28, BW42 and BW49 are the BW (g) at 1, 14, 28, 42, and 49 d of age, respectively, and *f* is the frequency. Body weights on d 14 (BW14) and 49 (BW49) had a significant association with haplogroups. CB is a color broiler line bred randomly without practicing any selection.

was no clear trend of haplogroup performance in BW of IWI birds.

The substitution effects of haplotypes have been analyzed for growth traits where significant associations of haplotype with the traits have been obtained through haplotype-trait association analysis. Accordingly, haplotype substitution effect was analyzed for BW at 28, 42, and 49 d in the PB-1 line and BW at 14 and 49 d in the CB line (Table 7). For BW at 28 d of age, in the PB-1 line substitution effect was higher in h7 than h1 and the lower in h13 than h1. However, variable magnitudes of haplotype substitution effects were observed for different traits indicating the exact impact of substitution of one haplotype over others. The substitution effects delineated the impact of individual haplotype on the trait while substituting the predominant haplotype at the multi-allelic system in the population. Ye et al. (2007) delineated the significant association of haplotypes with growth and antibody titer to infectious bursal disease virus vaccine. They suggested that the *MSTN* gene had pleiotropic effects on broiler performance.

The significant association of exon 1 SNP with different carcass traits including abdominal fat weight and carcass percent was observed by Zhu et al. (2007) in the Wenling grass-chicken. Likewise, Zhiliang et al. (2004) also reported a strong association between MSTN SNP and traits such as abdominal fat weight, abdominal fat percentage, birth weight, and breast muscle weight and percentage in broiler × Silky F_2 cross chickens in which the AA genotype showed higher breast muscle percentage than the AB genotype. The SNP in exon1 of the *MSTN* gene showed a significant effect on BW at different ages in other species such as goat in which the AB genotype had significantly higher BW at birth and 90 and 300 d of age (Zhang et al., 2012a). Further, Zhang et al. (2012b) revealed supremacy of AA and GA genotypes over GG genotype for BW from 6 to 18 wk of age in Bian chicken. In our study too, certain haplotypes of the *MSTN* gene possess favorable associations with growth traits. The study suggest that the priority of favorable haplotypes may be given over other haplotypes while selecting elite birds for regenerating the next generation to achieve better BW during juvenile age. This event will not only enhance the frequency of superior haplotypes in the farm but will also be able to augment the performance in the desired direction. In conclusion, the *MSTN* gene was highly polymorphic and had significant association with growth traits in chickens.

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Table 6. Myostatin haplotype BW in IWI chicken line[1,2]

Haplotype combination	*f*	BW1	BW14	BW28	BW42	BW49
h1h2	0.07	34.0 ± 0.5	848 ± 3.0	182 ± 9	310 ± 20	369 ± 24
h1h3	0.05	33.7 ± 0.6	850 ± 3.5	194 ± 12	328 ± 24	433 ± 27
h1h4	0.11	34.5 ± 1.1	890 ± 6.1	165 ± 19	288 ± 40	357 ± 45
h1h5	0.27	33.6 ± 0.4	860 ± 2.4	183 ± 8	313 ± 17	391 ± 20
h1h6	0.21	34.5 ± 0.2	876 ± 1.5	187 ± 5	312 ± 10	383 ± 12
h1h7	0.04	34.0 ± 0.3	899 ± 1.7	179 ± 5	299 ± 12	360 ± 14
h1h9	0.03	33.5 ± 0.7	840 ± 3.7	204 ± 12	337 ± 25	371 ± 32
h1h10	0.03	33.8 ± 0.9	878 ± 5.0	157 ± 15	276 ± 35	333 ± 39
h1h13	0.02	31.5 ± 1.1	786 ± 6.1	192 ± 19	337 ± 40	452 ± 50
h2h8	0.02	33.7 ± 0.5	860 ± 2.7	180 ± 9	288 ± 19	344 ± 23
h6h8	0.04	34.2 ± 0.7	852 ± 3.7	180 ± 13	286 ± 25	353 ± 29

[1]Haplogroups had a nonsignificant effect ($P > 0.05$) on BW in the IWI line. IWI is a layer line selected for egg production and egg weight for the last 12 generations.

[2]BW1, BW14, BW28, BW42, and BW49 are the BW (g) at 1, 14, 28, 42, and 49 d of age, respectively, and *f* is the frequency.

Table 7. Estimates of haplotype effect for different growth traits in different chicken lines[1,2]

	PB-1			CB	
Haplotype	BW28	BW42	BW49	BW14	BW49
h2	+324.45	+555.30	+609.30	+37.26	+343.16
h3	+319.00	+537.24	+643.28	+78.49	+413.13
h4	+287.04	+478.92	+554.97	+39.20	+372.40
h5	+293.58	+529.62	+622.86	+30.16	+289.42
h6	+346.05	+528.30	+639.90	+38.76	+323.00
h7	+370.08	+638.88	+758.88	+49.22	+414.46
h11	+317.86	+520.72	+659.18		
h12	+337.46	+598.78	+641.08		
h13	+261.08	+454.86	+550.24		

[1]Haplotype effect is the contribution of each haplotype while substituting the most frequent haplotype in the population.

[2]BW1, BW14, BW28, BW42, and BW49 are the BW (g) at 1, 14, 28, 42, and 49 d of age, respectively. PB-1 is a color broiler line under selection for BW at 5 wk of age practiced for last 18 generations. CB is a color broiler line bred randomly without practicing any selection.

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