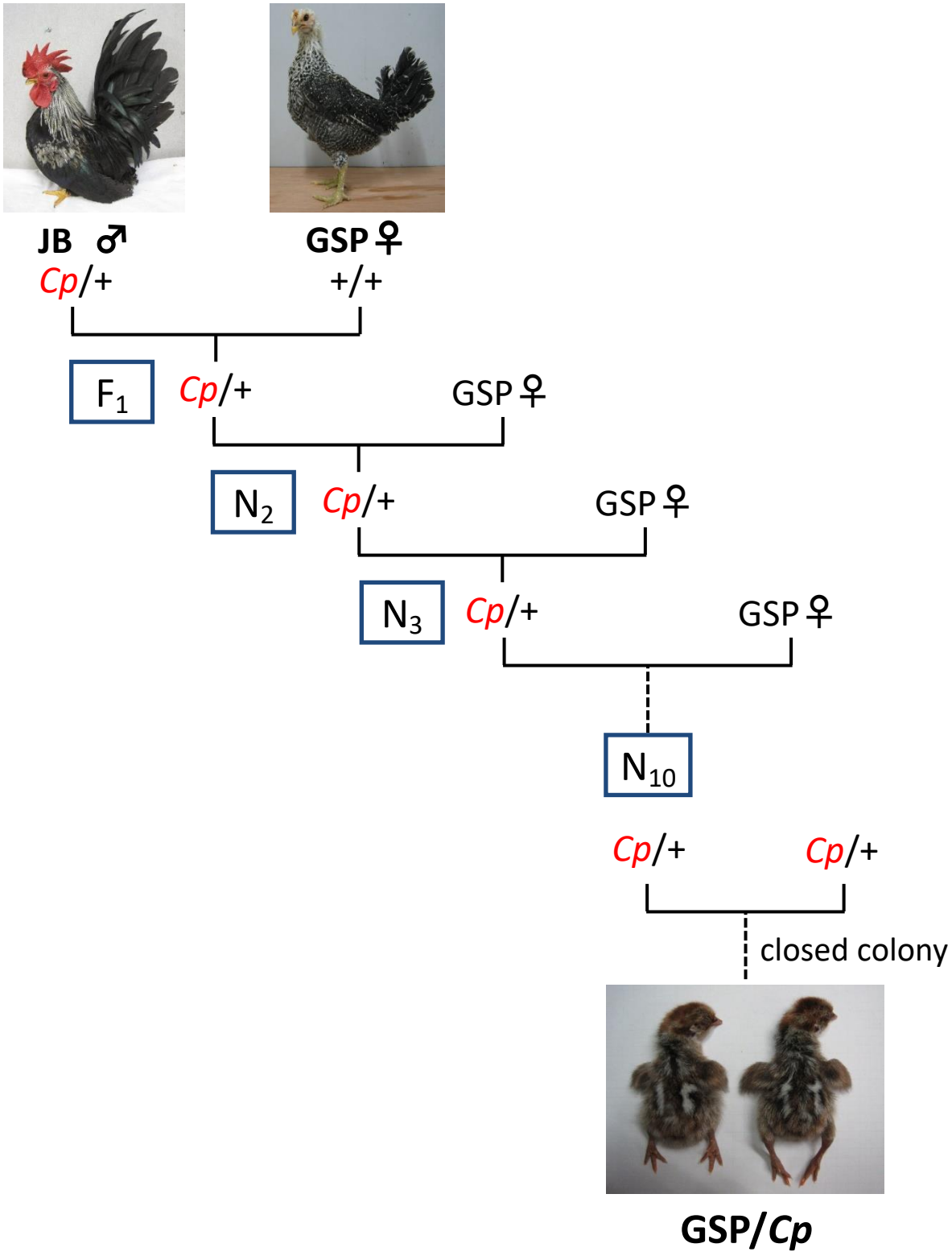
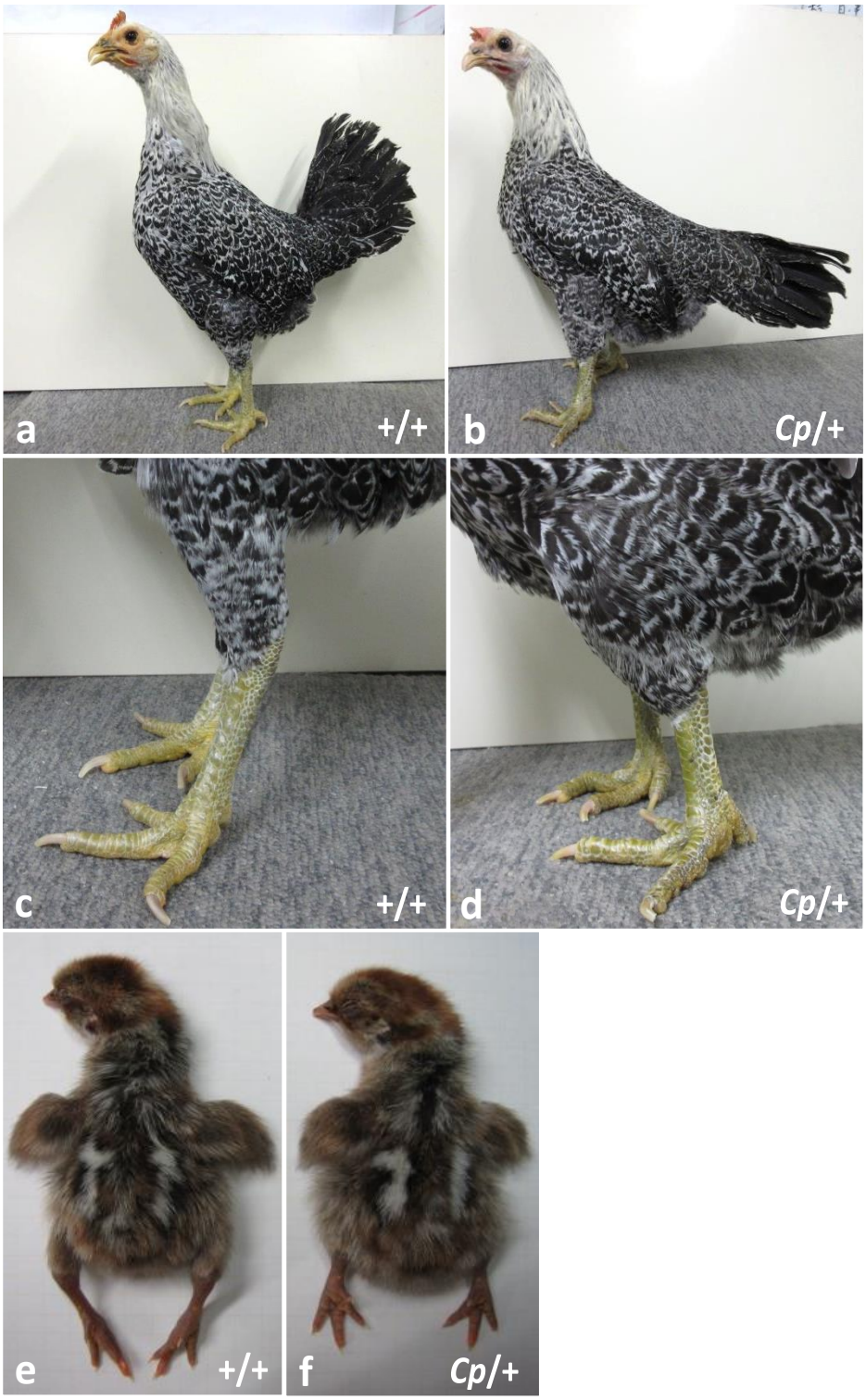
Supplementary Figures

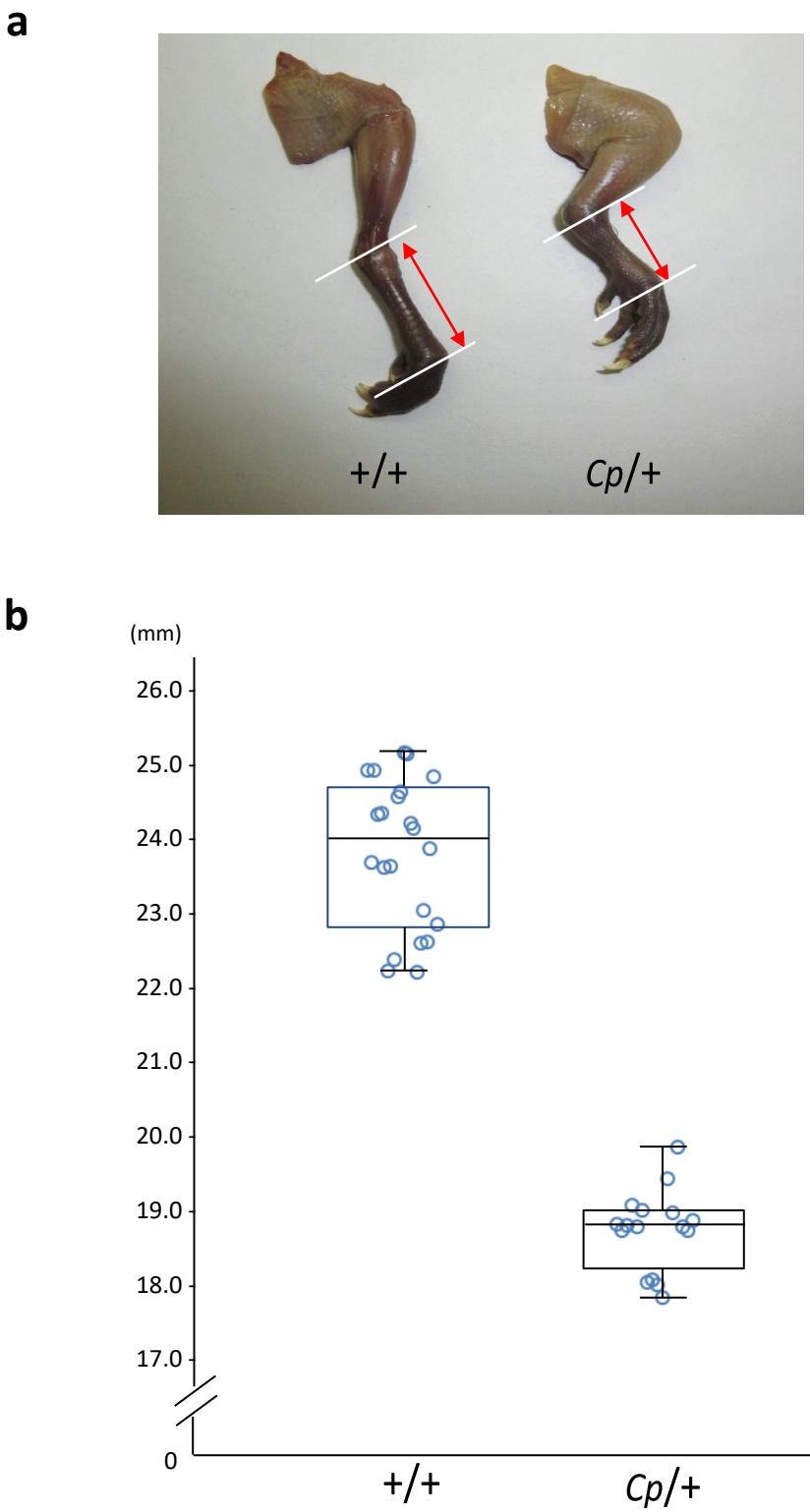
Supplementary Fig. 1 Phenotypes of Creeper chickens. Whole body images of heterozygous (*Cp*/+) male (**a**) and female (**b**) from the Japanese bantam (JB) strain.



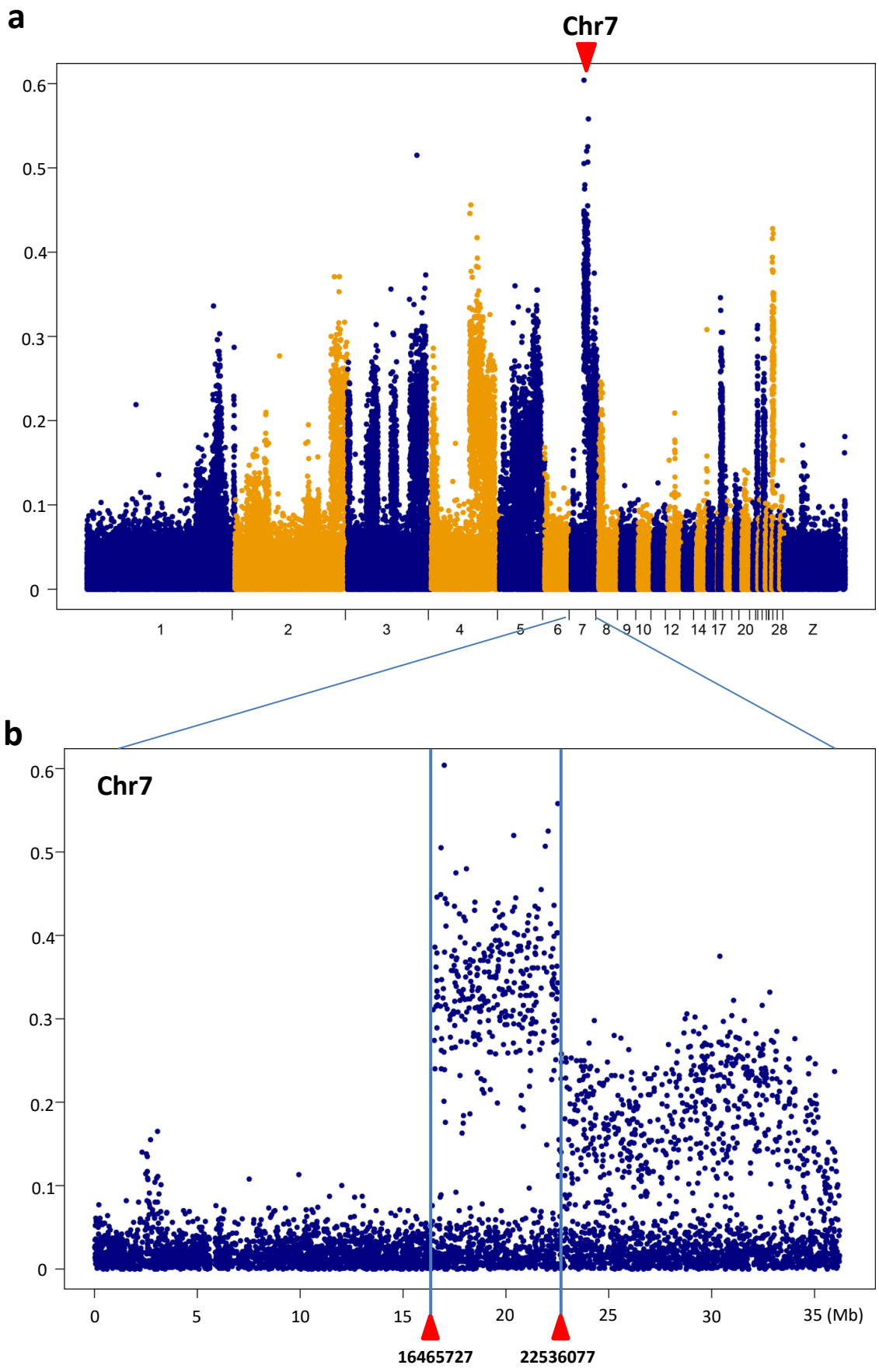
Supplementary Fig. 2 Mating scheme for establishing a congenic strain of the *Cp* gene (GSP/*Cp*). F_1 hybrids were obtained by mating a female of the GSP strain with a male of the JB strain that was heterozygous for the *Cp* allele. The congenic strain of the *Cp* gene in the GSP genetic background was established by backcrossing an F_1 hybrid male to GSP females, followed by backcrossing with GSP females eight times. This strain has been maintained as a closed colony.



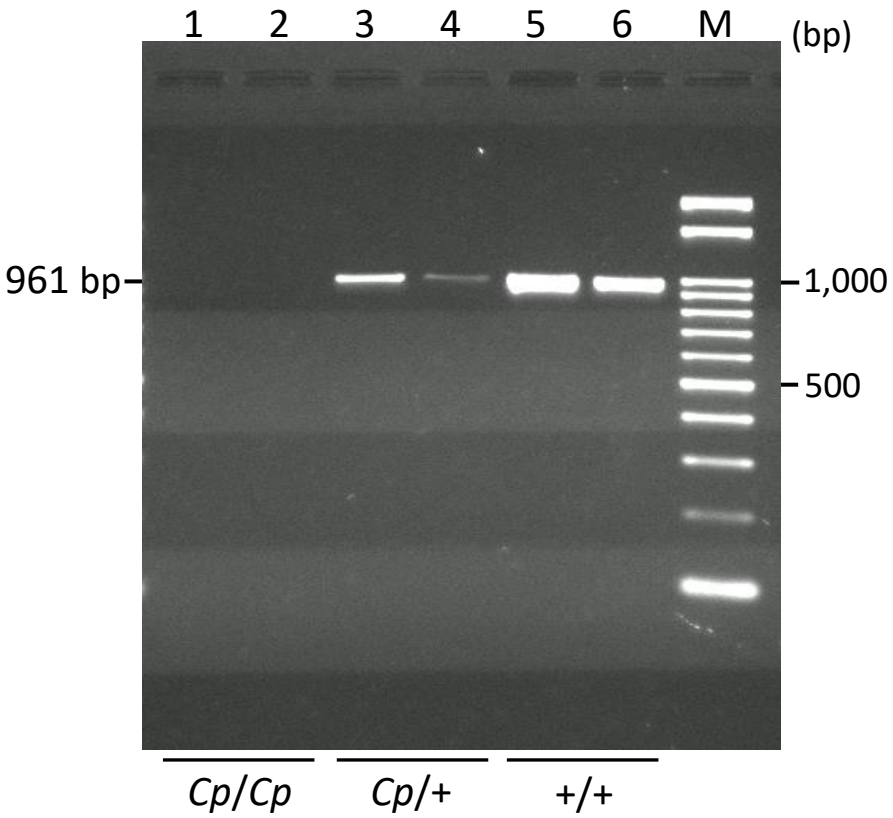
Supplementary Fig. 3 Phenotypes of the wild-type and Creeper chickens in the congenic GSP/*Cp* strain. **a–d** Pictures of whole bodies and legs of a wild-type chicken (+/+) (**a**, **c**) and a Creeper chicken (*Cp*/+) (**b**, **d**). The legs of the Creeper chicken (**d**) are shorter than those of the wild-type chicken (**c**). **e, f** A wild-type chick (+/+) (**e**) and a Creeper chick (*Cp*/+) (**f**) 1 day after hatching.



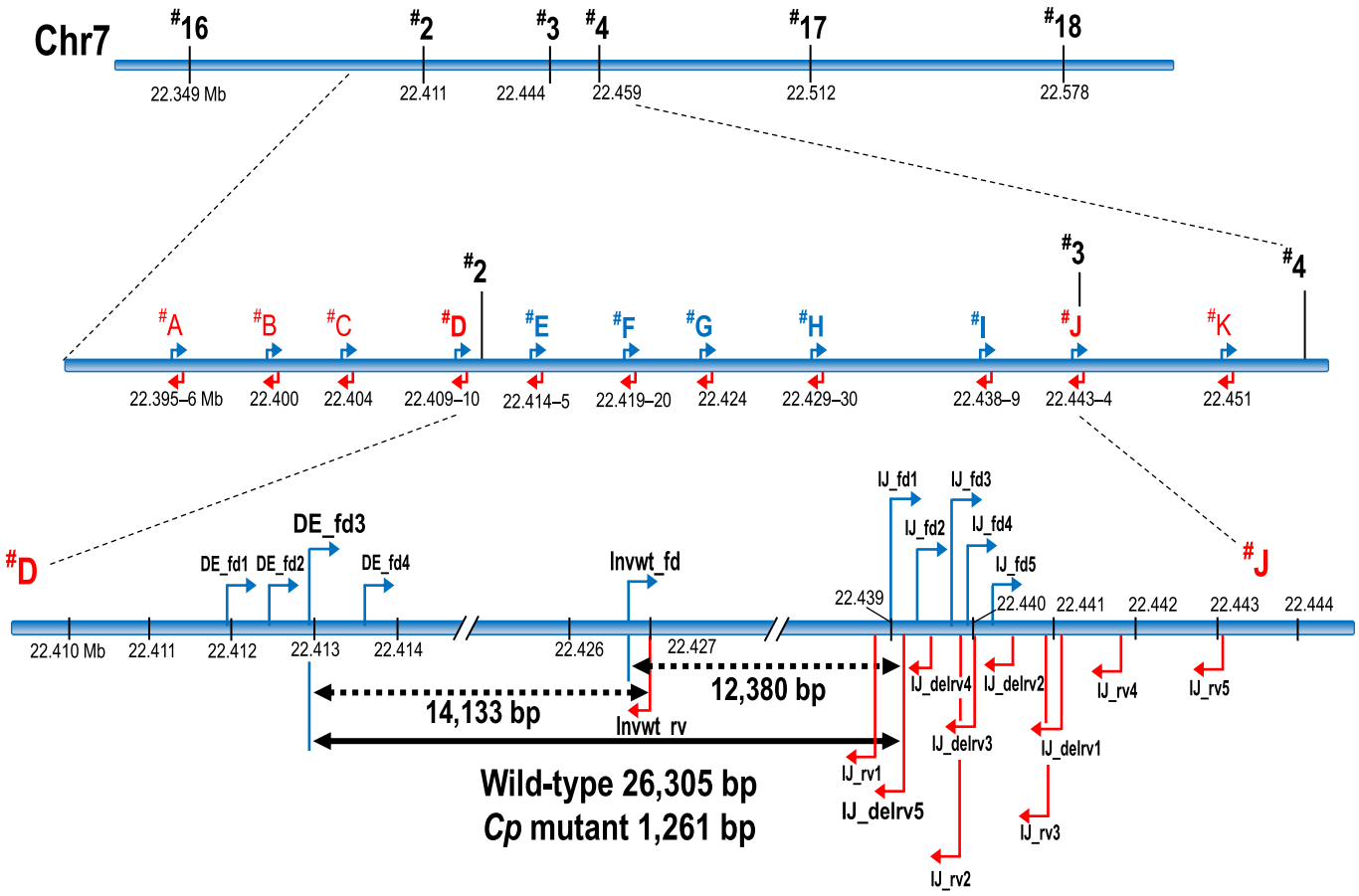
Supplementary Fig. 4 Shank lengths of the wild-type and Creeper chicks in the GSP/*Cp* strain. **a** The right legs of a wild-type chick (+/+) and a Creeper chick (*Cp*/+) 1 day after hatching. The lengths of metatarsal bones (double-headed arrows) were measured using an electronic caliper. **b** Boxes and whisker plots show the lengths of metatarsal bones of wild-type chicks (+/+, n = 22) and Creeper chicks (*Cp*/+, n = 16). Error bars extend to the maximum and minimum values of each group. The shank length of Creeper chicks was approximately 20% shorter (average = 18.8 mm) than that of the wild-type chicks (average = 23.9 mm) (Welch's t-test, $P = 1.55 \times 10^{-20}$).



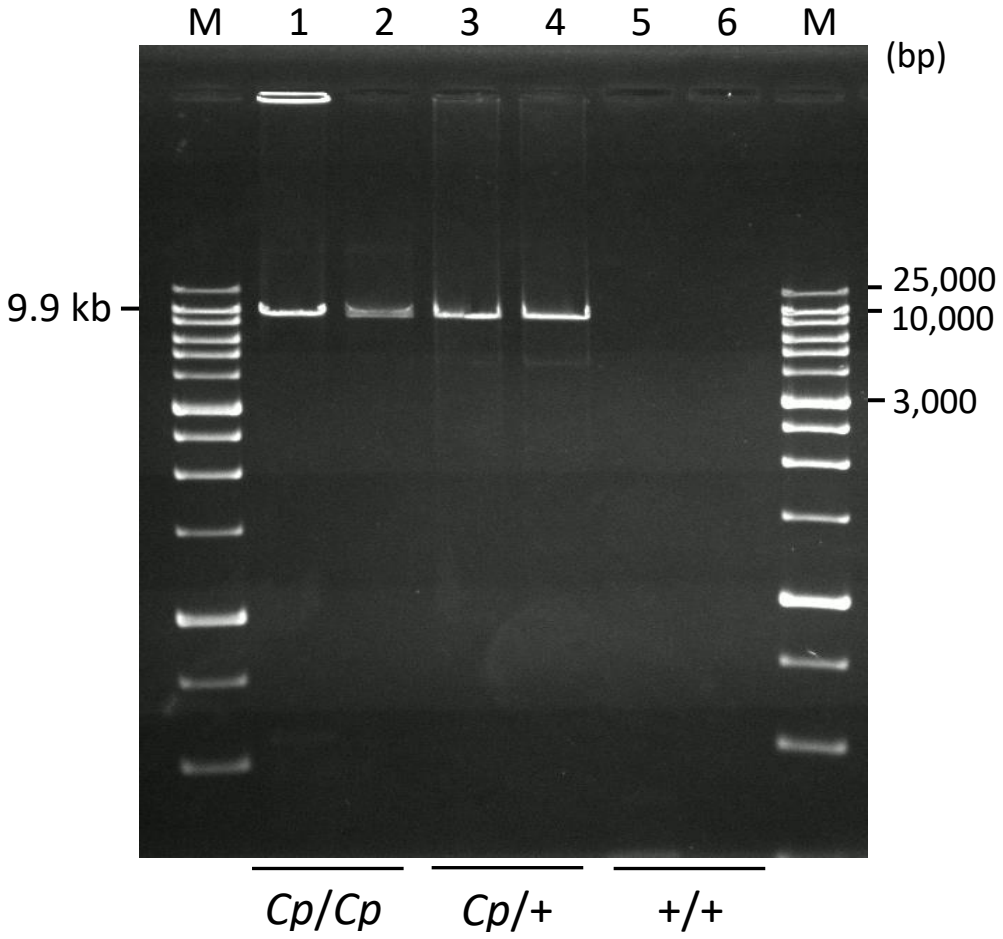
Supplementary Fig. 5 Distribution of genome-wide absRAFdif values obtained using a 600K genome-wide SNP array. **a** The highest peak of absRAFdif values between the wild-type (+/+) and heterozygous (*Cp*/+) chickens located on chromosome 7. **b** The peak of absRAFdif values was localized within 6.1 Mb at nucleotide position 16465727 – 22536077 in the chicken reference genome Gallus_gallus-4.0 (17032895 – 23162069 in Gallus_gallus-5.0).



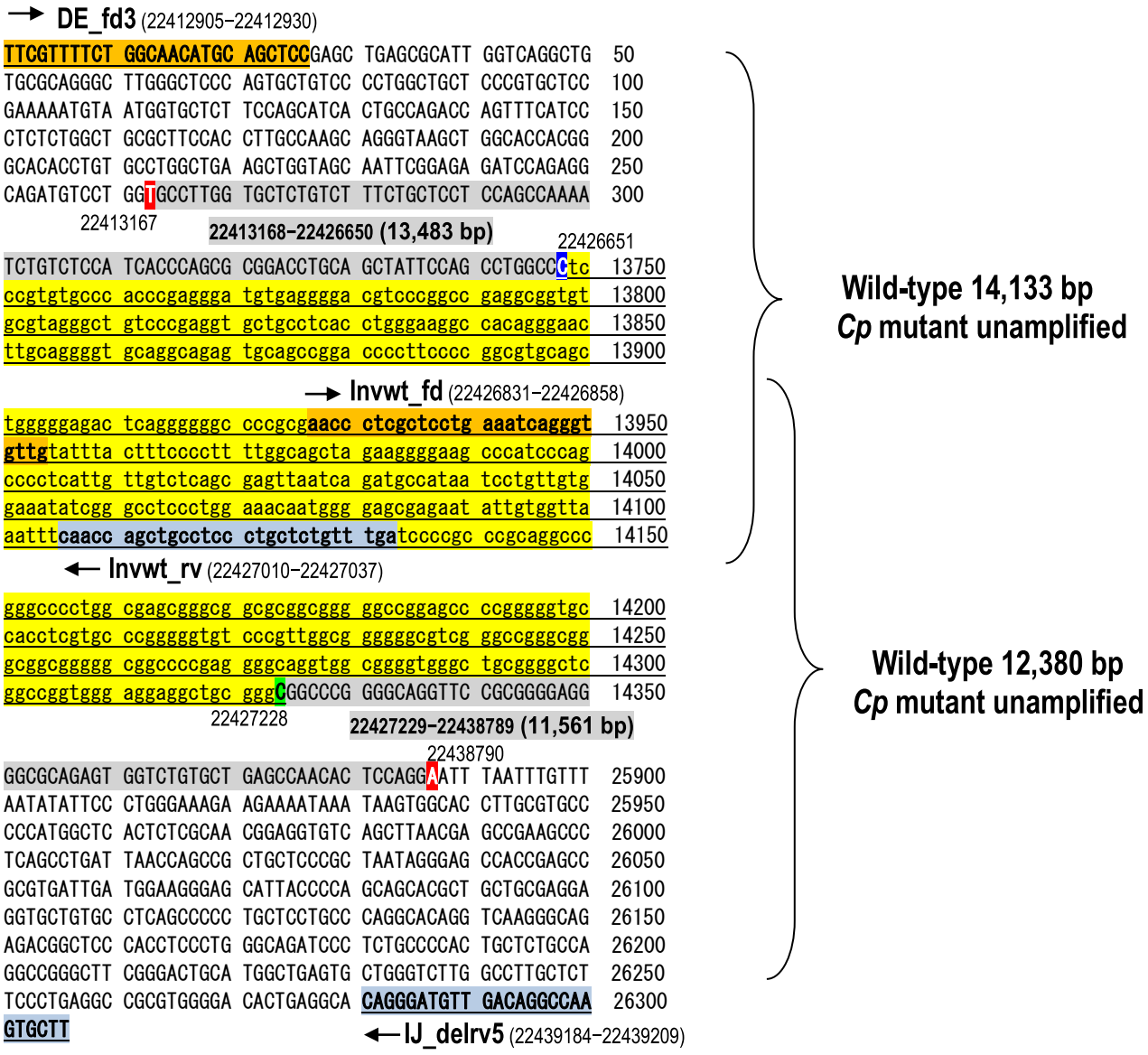
Supplementary Fig. 6 Detection of the genomic deletion of the *IHH* gene at the *Cp* locus. The 961 bp fragment was amplified from the wild-type (+/+) and heterozygous (*Cp*/+) chickens, but not amplified in the *Cp*/*Cp* homozygous embryos using a pair of primers designed in the fifth intron and sixth exon of the *IHH* gene. M, molecular size marker: Gene Ladder 100 (0.1 – 2 kbp) (NIPPON GENE, Tokyo, Japan).



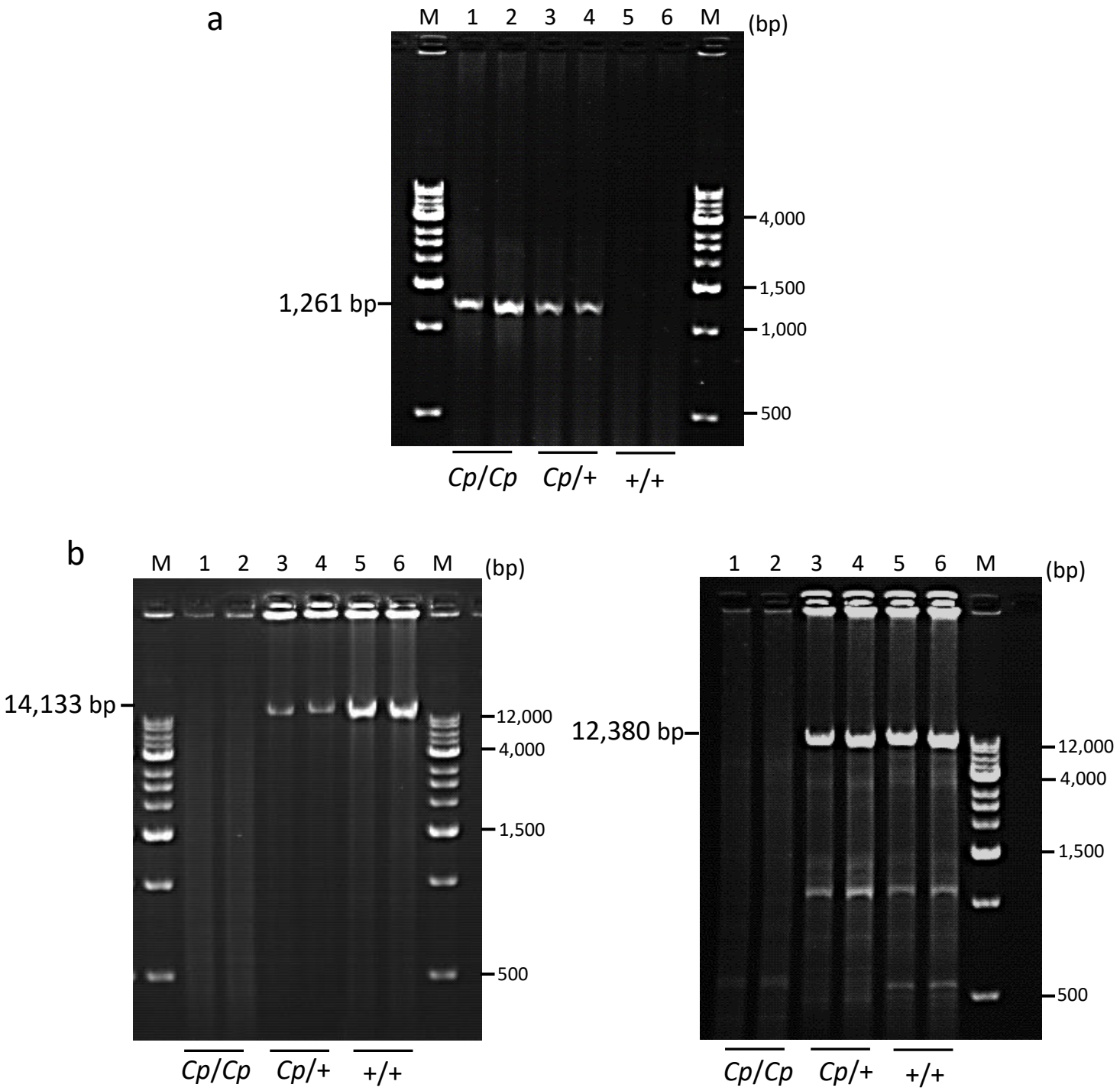
Supplementary Fig. 7 The locations of PCR primers used for detecting deletions at the *Cp* locus. Six SNPs ($^{\#}$2, $^{\#}$3, $^{\#}$4, $^{\#}$16, $^{\#}$17, and $^{\#}$18) in the vicinity of the *Cp* locus were used as anchors for detecting deletions. The key-shaped arrows indicate the positions of PCR primers.



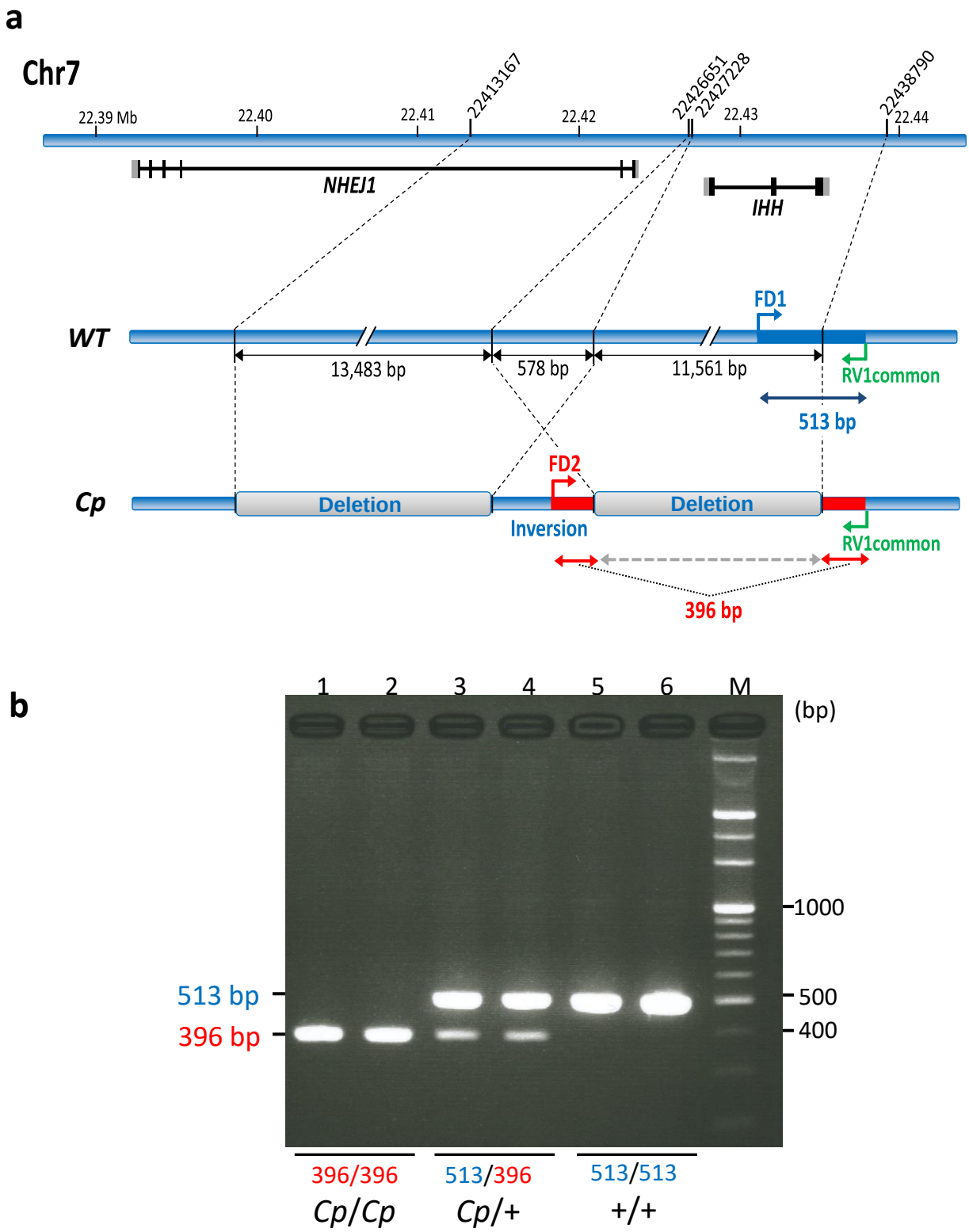
Supplementary Fig. 8 PCR amplification for detecting the deletion at the *Cp* locus. A 9.9 kb PCR product was only amplified from the *Cp*/+ chickens and the *Cp*/*Cp* embryos but not in the wild-type (+/+) chickens by LA-PCR using primers #D_fd and #J_rv in the 35 kb region at 22.409 – 22.444 Mb in Gallus_gallus-5.0. M, molecular size marker: ExcelBand XL 25 kb DNA Ladder, Broad Range (up to 25 kb) (SMOBIO Technology, Hsinchu, Taiwan).



Supplementary Fig. 9 The nucleotide sequence of the 1,261 bp fragment amplified from the *Cp*/*Cp* embryos with primers DE_fd3 and IJ_delrv5. The inverted 578 bp sequence remains in the 25 kb deleted region in the *Cp* allele, which is located between nucleotide positions 22413167 and 22438790.



Supplementary Fig. 10 PCR amplification for detecting the deletion at the *Cp* locus. **a** A 1,261 bp PCR product was only amplified from the *Cp*/+ chickens and the *Cp*/*Cp* embryos but not in the wild-type (+/+) chickens by LA-PCR using primers DE_fd3 and IJ_delrv5 in the 26 kb region at 22.413 – 22.439 Mb in Gallus_gallus-5.0. **b** 14,133 bp (left) and 12,380 bp (right) PCR products were only amplified from the wild-type (+/+) and *Cp*/+ chickens but not in the *Cp*/*Cp* embryos by LA-PCR using DE_fd3 and Invwt_rv primer pair and Invwt_fd and IJ_delrv5 primer pair, respectively (see Supplementary Fig. S8 and Supplementary Table 9). M, molecular size marker: DL10001 DNA ladder (500 – 12,000 bp) (Generay Biotech, Shanghai, China).



Supplementary Fig. 11 Genotyping of the wild-type and Creeper alleles using a PCR assay. **a** A physical map of two deletions and the remaining inverted fragment in the deleted region at the *Cp* locus. The breakpoints located in the fourth intron of *NHEJ1* and behind the 3' UTR of *IHH*. The key-shaped arrows indicate the positions of PCR primers, FD1, FD2, and RV1common, used for genotyping. **b** Electrophoretic pattern of the DNA fragments amplified from the three genotypes. A 513 bp fragment was amplified from the wild-type allele by primers FD1 and RV1common, and a 396 bp fragment from the *Cp* allele by primers FD2 and RV1common. M, molecular size marker: Gene Ladder 100 (0.1 – 2 kbp) (NIPPON GENE, Tokyo, Japan).

Supplementary Tables

Supplementary Table 1 Segregation of Creeper and wild-type phenotypes in F_1, F_2, and backcross progeny that were obtained from mating between the JB line and the two wild-type strains, PNP/DO and GSP

Mating		No. of progeny	Phenotype of progeny		Expected ratio	χ^2	*P*
Male (Expected genotype) (No. of individuals)	Female (Expected genotype) (No. of individuals)		Creeper	Wild-type			
JB (*Cp* /+) (1)	PNP/DO (+/+) (1)	2	1	1	–	–	–
F_1 (*Cp* /+) (1)	F_1 (+/+) (1)	113	56	57	1 : 1	0.009	*P* <0.05
JB (*Cp* /+) (1)	GSP (+/+) (1)	24	10	14	1 : 1	0.667	*P* <0.05
F_1 (*Cp* /+) (2)	F_1 (*Cp* /+) (4)	175	118	57	2 : 1	0.043	*P* <0.05
F_1 (*Cp* /+) (2)	F_1 (+/+) (2)	120	62	58	1 : 1	0.133	*P* <0.05
F_1 (*Cp* /+) (1)	GSP (+/+) (1)	15	7	8	1 : 1	0.067	*P* <0.05

Supplementary Table 2 Genotyping of the 25 kb deletion (del) at the *Cp* locus in the E2.5 – 3.0 embryos from mating between *Cp* /+ heterozygotes from the GSP/*Cp* congenic strain, and morphological characteristics of the embryos

Stage of developemt	No. of embryos examined	No. of dead embryos	No. of live embryos	Morphology of live embryos					
				Normal			Abnormal		
				+/+	del/+	del/del	+/+	del/+	del/del
E2.5 – 3.0[a]	110	4	106	27	43	3	3	3	27

[a] Collected 3 days after the start of incubation

Supplementary Table 3 Classification of morphological abnormalities in the E2.5 – 3.0 embryos obtained from a mating between *Cp* /+ heterozygotes from the GSP/*Cp* congenic strain

Genotype	No. of abnormal embryos[a]	Morphological abnormality		
		Hypoplasia of brain	Abnormal blood island formation	Heart hypoplasia
+/+	3	3	0	0
del/+	3	1	2	0
del/del	27	26	19	23

[a] Collected 3 days after the start of incubation in the experiment shown in Supplementary Table 2

Supplementary Table 4 Eighteen haplotype regions that showed high absRAFdif values between the wild-type (+/+) chickens from the GSP strain and the heterozygoous (*Cp* /+) chickens from the GSP/*Cp* strain by SNP genotyping assay

Chromosome	Nucleotide position[a]		Median of absRAFdif values	Haplotype block size (Mb)
	5' region	3' region		
7	16465727	22536077	0.335	6.1
26	394415	2705165	0.24	2.3
4	50446006	69851627	0.221	19.4
7	22677582	35976759	0.191	13.3
3	81609740	104480421	0.187	22.9
2	126787922	148326478	0.181	21.5
17	3395054	6096006	0.178	2.7
4	2426384	3874078	0.172	1.4
3	53045	3228287	0.171	3.2
4	70002537	83960114	0.171	14
5	15847877	55356475	0.167	39.5
3	30919389	40051721	0.164	9.1
21	5795548	6763857	0.159	1
1	170335509	177682674	0.157	7.3
3	57002224	64541911	0.149	7.5
23	2762047	5694912	0.147	2.9
8	2310802	5061393	0.141	2.8
1	193871308	194405013	0.128	0.5

[a] Nucleotide position in the reference genome Gallus_gallus-4.0

Supplementary Table 5 Primer sets of 16 SNP markers used for mapping the *Cp* locus on chromosome 7

Marker		Nucleotide sequence of primer (5' to 3')	SNP position (Gallus_gallus-4.0)	SNP position (Gallus_gallus-5.0)	SNP[a] (JB/GSP)	Size of PCR product (bp)	Annealing temparature (°C)	Restriction enzyme (No. of restriction sites)
CHR7_#5	Forward	AAACAAGACAGAGCCAGCTTCC	2523701	2555424	C/T	458	59.5	*Pst* I (1)
	Reverse	TTGGGTCGTGTAGATAGGCAAG						
CHR7_#6	Forward	AGAAAGACATGCAGCCACAGAG	7468194	7977228	C/A	424	59.5	*Eco* RI (1)
	Reverse	TTGGGCTTAGCAAAACAGCACC						
CHR7_#19	Forward	ACCATATTTCTTCAATAAG	15711523	16274591	C/T	317	47.5	*Hin* dIII (1)
	Reverse	AGCTTGTAGACTACGTGAGC						
CHR7_#8	Forward	AGGTAGAGTCTGTAGGTCTCAG	17462758	18049452	C/T	438	59.5	*Pst* I (1)
	Reverse	TGGTCATAATGAGTGGCAGCAG						
CHR7_#9	Forward	AAGTGCTCCCAAGTAGGATCAG	19709516	20305921	A/G	459	59.5	*Pst* I (1)
	Reverse	ACTTACCCTTCCACTCTCCTAC						
CHR7_#14	Forward	TTCAGTGGGATTTCTCAGGAGC	21435690	22049676	T/G	758	59.5	*Pst* I (1)
	Reverse	TCTCTTCAGGAGATTGAGACGG						
CHR7_#15	Forward	TGTCCTCGTAAACGAAGCTCAG	21647789	22268282	A/G	732	58.5	*Nme* AIII (1)
	Reverse	ATGCCCCATGATCATGTGCATG						
CHR7_#16	Forward	TAAAAGCTGGACTGCTGTAGCC	21726140	22348862	T/G	662	59.5	*Nde* I (1)
	Reverse	TTCCTTCAGGAAGCCGATGAAG						
CHR7_#2	Forward	AACTGTGAGCCGATGAAGCAAG	21783101	22411354	A/G[b]	654	59.5	*Bci* VI (1)
	Reverse	AGCACAACCGGCTTCCAATTAG						
CHR7_#3	Forward	AGATTTTCTCCAGCTCTCGTGG	21815955	22444190	C/T[b]	656	59.5	*Pml* I (1)
	Reverse	AAACCTTCTTGCTCCTGCATGC						
CHR7_#4	Forward	ATTTCTCCAGGAGCTGCATGGG	21830848	22458582	C/G	449	59.5	*Pvu* II (2 sites including 1 common site)
	Reverse	TCACAGGAAACTTCGGAGGCAG						
CHR7_#17	Forward	ATGTGGGAGTACCATGTCATGC	21884855	22512418	T/C	782	59.5	*Tsp* 45I (1)
	Reverse	AGCCCCATAGGGAAACTCTTTC						
CHR7_#18	Forward	ACTCAGCACCTTTAGGACTTGC	21951698	22578237	T/C	922	59.5	*Hph* I (2 sites including 1 common site)
	Reverse	AGCCAGCAAGTATGGGATTTCC						
CHR7_#11	Forward	TTCCAAGTGACTCCACCATCTC	23830947	24456802	T/C	486	59.5	*Pst* I (1)
	Reverse	TCCTCTCTTTCAGGGTAGAAGG						
CHR7_#12	Forward	ATCACTGAGAGGCTTCTCGTTG	30424765	31088900	C/G	456	59.5	*Hin* dIII (2 sites including 1 common sit
	Reverse	ATTGACCACTCAGAAGGCTCTG						
CHR7_#13	Forward	AGCTGTCCAGGAAAGCCTTATC	35185180	35875738	A/G	424	59.5	*Pst* I (1)
	Reverse	AAGCAGGTTTGCACTTGGTAGG						

[a] SNPs detected between the JB strain and the GSP strain

[b] SNPs detected between the JB strain and the GSP strain in the deleted region

Supplementary Table 6 Linkage analysis of the *Cp* locus using 16 SNP markers and three microsatellite markers on chromosome 7

Marker	Position of marker (Gallus_gallus-4.0)	Position of marker (Gallus_gallus-5.0)	Rough mapping using 95 F_2 progeny		Marker	Position of marker (Gallus_gallus-5.0)	Fine mapping using 175 F_2 progeny	
			Map distance (cM)	LOD score			Map distance (cM)	LOD score
CHR7_#5	2523701	2555424						
			21.6	8.7			–	–
CHR7_#6	7468194	7977228						
			29.6	3.0			–	–
CHR7_#19	15711523	16274591			CHR7_#19	16274591		
			3.8	19.5				
CHR7_#8	17462758	18049452						
			2.1	19.0			8.6	33.8
CHR7_#9	19709516	20305921						
			1.1	21.9				
MCW0133	20859924 – 20860054	21473824–21473954			*MCW0133*	21473824–21473954		
			1.1	22.8			1.2	61.6
CHR7_#14	21435690	22049676			CHR7_#14	22049676		
			0.0	26.6			0.0	70.7
CHR7_#15	21647789	22268282			CHR7_#15	22268282		
			0.5	24.4			0.6	65.4
CHR7_#16	**21726140**	**22348862**			**CHR7_#16**	**22348862**		
			0.5	24.1			**0.3**	69.8
CHR7_#2	21783101	22411354			**CHR7_#2**	22411354		
			0.0	26.1			**0.0**	69.8
***Cp* phenotype**	–	–			***Cp* phenotype**	–		
			0.0	26.1			**0.3**	66.9
CHR7_#3	**21815955**	**22444190**			**CHR7_#3**	**22444190**		
			0.0	26.1			0.0	69.5
CHR7_#4	21830848	22458582			CHR7_#4	22458582		
			0.0	26.1			0.0	69.5
CHR7_#17	21884855	22512418			CHR7_#17	22512418		
			0.5	23.8			0.3	66.6
CHR7_#18	21951698	22578237			CHR7_#18	22578237		
			1.1	21.8			1.4	58.8
MCW183	22208672–22208965	22834668–22834961			*MCW183*	22834668–22834961		
			7.3	16.3				
CHR7_#11	23830947	24456802					13.1	24.4
			6.6	20.7				
ADL180	26581206–26581339	27230408–27230541			*ADL180*	27230408–27230541		
			8.4	20.2			–	–
CHR7_#12	30424765	31088900						
			15.4	15.0			–	–
CHR7_#13	35185180	35875738						

Supplementary Table 7 Primer pairs used for identifying the deletion in a candidate region of the *Cp* locus

Primer name	Nucleotide sequence (5' to 3')	Nucleotide position (Gallus_gallus-4.0)	Nucleotide position (Gallus_gallus-5.0)	Size of PCR product (bp)
#A_fd	TTCTACAGAGCTTAGAGCAGCTGG	21766687–21766710	22394939–22394962	646
#A_rv	ATGTTCTGCAGAAACGTCTCCTCC	21767332–21767309	22395584–22395561	
#B_fd	TTAATGAGCGCAAGGTAGCTGTGG	21771370–21771393	22399622–22399645	686
#B_rv	AGTCTGGTTTTAGCACTGTGCAGC	21772055–21772032	22400307–22400284	
#C_fd	TCTTCGCTGTGGACGTTTCCTTTC	21775297–21775320	22403549–22403572	709
#C_rv	TTTAGCCGTGTGTACCGTACAGTC	21776005–21775982	22404257–22404234	
#D_fd	AAACCCAGAAGGGTTTGCAGTGTG	21780960–21780983	22409213–22409236	685
#D_rv	AAACATGCACCAGAAAGAGGCAGG	21781644–21781621	22409897–22409874	
#E_fd	ATTCAGCAACCTGCTCCTGATTGG	21785847–21785870	22414100–22414123	632
#E_rv	ACATGTCTCTTCAGTGGATGCCAG	21786478–21786455	22414731–22414708	
#F_fd	ATCACCTCTATCACCTCTCTCAGC	21790980–21791003	22419233–22419256	756
#F_rv	TAACTGGAAAACAGAGCGAACGGG	21791735–21791712	22419988–22419965	
#G_fd	AAGGGCTCTTCAGCTGAAAGGTTG	21795295–21795318	22423548–22423571	779
#G_rv	TTGAGAGAAAGCAGTGAGCCTGTG	21796073–21796050	22424326–22424303	
#H_fd	AAGCCATTGACCCAACTCCTCATC	21801452–21801475	22429440–22429463	633
#H_rv	AGTCAGACGTTTTGGGAAGGACTG	21802084–21802061	22430072–22430049	
#I_fd	TGAGGAAGGCTTTAATCAGGACCC	21809669–21809692	22437793–22437816	797
#I_rv	TCTTCAAAACCCAATGCCCTGGAG	21810465–21810442	22438589–22438566	
#J_fd	ATTGTTGGGTGAAGCACCTTCACC	21815259–21815282	22443494–22443517	674
#J_rv	TCATGGTTCAGTCCTGGAATCTCC	21815932–21815909	22444167–22444144	
#K_fd	TTAATGCAAGGCAACAGCTGTGGG	21822337–21822360	22450571–22450594	935
#K_rv	AAACAGTGATGAAGAACCGCGTGG	21823271–21823248	22451505–22451482	

Supplementary Table 8 Primers used for amplifying nucleotide sequences in the deletion region of the *Cp* locus

Primer name	Nucleotide sequence (5' to 3')	Nucleotide position (Gallus_gallus-4.0)	Nucleotide position (Gallus_gallus-5.0)
5' Breakpoint			
DE_fd1	TTTGCACAGTACACATCTCTGGGC	21783611–21783634	22411864–22411887
DE_fd2	ATATATCGGCAGGGTTTGTGTCCC	21784167–21784190	22412420–22412443
DE_fd3	TTCGTTTTCTGGCAACATGCAGCTCC	21784652–21784677	22412905–22412930
DE_fd4	TGTGGGCAATGAGGTTTGGTTTCC	21785304–21785327	22413557–22413580
578 bp region that remains in the *Cp* allele			
Invwt_fd	AACCCTCGCTCCTGAAATCAGGGTGTTG	21798578-21798605	22426831–22426858
Invwt_rv	TCAAACAGAGCAGGGAGGCAGCTGGTTG	Not applicable	22427037–22427010
3' Breakpoint			
IJ_fd1	ATTGATGGAAGGGAGCATTACCCC	21810835–21810858	22438959–22438982
IJ_fd2	TCGTTGCAGAGACATCAGCAGTC	21811217–21811239	22439341–22439363
IJ_fd3	TTCCTGAATGATGCCAGGCTGTTG	21811582–21811605	22439706–22439729
IJ_fd4	TTGTTTACCTTCCCCATTGGCCTC	21811806–21811829	22439930–22439953
IJ_fd5	TGAACTGCACTCCTAGATGCTACC	21812167–21812190	22440291–22440314
IJ_rv1	AAATTCCTGGAGTGTTGGCTCAGC	21810670–21810647	22438794–22438771
IJ_delrv5	AAGCACTTGGCCTGTCAACATCCCTG	21811085–21811060	22439209–22439184
IJ_delrv4	ACTGGATAAATCAGCTGCTCCTCC	21811397–21811374	22439521–22439498
IJ_rv2	ATAGTGCTTAATCAGCCCTGAGGC	21811720–21811697	22439844–22439821
IJ_delrv3	TTTGCACCCAACCTACCGTTTTGG	21811973–21811950	22440097–22440074
IJ_delrv2	AAGAGCAAACATCCACGTGGACAC	21812334–21812311	22440458–22440435
IJ_rv3	ATCAGAGCTGGATTAAGGTGTCCG	21812731–21812708	22440855–22440832
IJ_delrv1	ATGGGACAACTTCCTCCTTCTG	21812939–21812918	22441063–22441042
IJ_rv4	TTCCTCTTTGCACCTCTTTGGCAC	21813656–21813633	22441780–22441757
IJ_rv5	AACGGCTCCCACGTTTGTAAGAAC	21814776–21814753	22443127–22443104

Supplementary Table 9 Genotype-phenotype association between the 25 kb deletion (del) and the Creeper phenotype in F_2 progeny of a cross between the JB and GSP strains and 22 chicken strains and/or populations from 17 breeds

Breed (line/population)	Phenotype	No. of samples	Genotype		
			del/del	del/+	+/+
Japanese bantam (JB) (embryo)[a]	embryonic lethal[b]	7	7		
Japanese bantam (JB) (chick)	Creeper	20		20	
	wild-type	18			18
F_2 (*Cp*/+ × *Cp*/+)[c]	Creeper	118		118	
	wild-type	57			57
F_2 (*Cp*/+ × +/+)[c]	Creeper	62		62	
	wild-type	58			58
Miyaji-dori	Creeper	2		2	
	wild-type	1			1
Jitokko	Creeper	6		6	
	wild-type	10			10
Ehime-jidori (EJ)	wild-type	8			8
Cochin bantam (CB)	wild-type	2			2
Shokoku	wild-type	3			3
Polish bantam	wild-type	4			4
Fayoumi (GSP)	wild-type	15			15
Fayoumi (PNP/DO)	wild-type	15			15
Fayoumi (GSN/1)	wild-type	8			8
Fayoumi (YL)	wild-type	8			8
Black Minorka (BM-C)	wild-type	4			4
Brown Leghorn (BL-E)	wild-type	4			4
Rhode Island Red (RIR-Y8/NU)	wild-type	4			4
New Hampshire (413)	wild-type	4			4
Dandarawi (DD)	wild-type	4			4
Japanese Silkie (SIL)	wild-type	8			8
Albino (CAL)	wild-type	4			4
White Leghorn (WL-G)	wild-type	4			4
White Leghorn (M/O)	wild-type	4			4
White Leghorn (OS)	wild-type	4			4
Red junglefowl (RJF/NU)	wild-type	3			3

[a] Embryos were collected 12 – 15 days after the start of incubation

[b] All embryos were dead in the shell

[c] Genotyping was conducted for only hatched F_2 progeny obtained from mating between JB males and GSP fema