One subspecies of the red junglefowl (*Gallus gallus gallus*) suffices as the matriarchic ancestor of all domestic breeds

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ABSTRACT The noncoding control region of the mitochondrial DNA of various gallinaceous birds was studied with regard to its restriction fragment length polymorphism (RFLP) and sequences of the first 400 bases. Tandem duplication of the 60-base unit was established as a trait unique to the genus *Gallus*, which is shared neither by pheasants nor by quails. Unlike its close ally *Gallus varius* (green junglefowl), the red junglefowl *Gallus gallus* is a genetically very diverse species; the 7.0% sequence divergence was seen between those from Thailand (*G. g. gallus* and *G. g. spadiceus*) and the other from the Indonesian island of Java (*G. g. Bankiva*). Furthermore, the divergence increased to 27.83% if each transversion is regarded as an equivalent of 10 transitions. On the other hand, a mere 0.5–3.0% difference (all transitions) separated various domestic breeds of the chicken from two *G. g. gallus* of Thailand, thus indicating a single domestication event in the area inhabited by this subspecies of the red junglefowl as the origin of all domestic breeds. Only transitions separated six diverse domesticated breeds. Nevertheless, a 2.75% difference was seen between RFLP type I breeds (White Leghorn and Nagoya) and a RFLP type VIII breed (Ayam Pelung). The above data suggested that although the mitochondrion of RFLP type V was the main contributor to domestication, hens of other RFLP types also contributed to this event.

There is little doubt that the domestication of various wild animals as the beasts of burden, the source of protein and fat, and the instrument of war and recreation played many pivotal roles in the cultural evolution of mankind. Of special interest has been the various divine rites performed in association with various domesticated animals, particularly the chicken. For documentation of so recent an event as domestication, nuclear genes with their low mutation rate would be of little use. On the contrary, the mitochondrial genome appears particularly suitable. Its high mutation rate is expected to remain constant, being relatively impervious to generation time differences between species. It may be recalled that an organism does not start its life with a single copy but with hundreds of thousands of copies of the mitochondrial genome harbored by the egg cytoplasm. Accordingly, generation changes do not constitute significant epochs in the life history of mitochondrial DNA. Furthermore, the extremely useful landmark was established by recent studies on two hypervariable subregions of the control region of human mitochondria. The average sequence divergence between all races of mankind was established as 2.0% and the rate of evolution was estimated to be 1% sequence divergence per 71,000–167,000 years (1, 2). It follows that any mitochondrial sequence divergence substantially above 2.0% within a given domesticated species creates a peculiar paradox of either

domestication occurring before the emergence of mankind or at least domestication occurring within the African cradle before the exodus of certain bands to the Near East and outward.

Indeed, such a paradox was encountered in a recent study on the mitochondrial control region of various breeds of cattle. Two distinct mitochondrial lineages separated by a 5.01% sequence difference were observed. Furthermore, this dichotomy did not follow the customary *Bos taurus/Bos indicus* split, for the African zebu was more similar to European taurine breeds than to Indian zebu. This paradox was resolved by the assumed presence of two subspecies of the aurochs (*Bos primigenius*) prior to the emergence of humans and the two subsequent independent domestication events (3).

In view of the above data, we have decided to study the control region light chain (L chain) of the avian mitochondria on various gallinaceous birds with regard to its restriction fragment length polymorphism (RFLP) as well as sequences of the first 400 bases of the control region. In human studies, 64% of the total polymorphism in the entire control region was found among the first 400 bases (4).

MATERIALS AND METHODS

Material. Materials used for the present study are summarized in Fig. 1. As to junglefowls, 10 of the red junglefowl (*G. g. gallus* and *G. g. spadiceus*) were gifts from the Department of Forestry of the Thai government. Five specimens of *G. g. bankiva* were obtained from the Indonesian island of Java, and so are all of the green junglefowl (*Gallus varius*). Four additional Thai red junglefowls sampled were from those kept in the Tama Zoological Garden (Tokyo).

Of various domestic breeds, samples from all those classified as "occidental breeds" were collected at the Domestic Fowl Trust of England. As to Asiatic breeds, those starting with "ayam" were all Indonesian breeds and were collected there. Others were either collected in their native habitats or obtained from one of the following three institutions in Japan: Yamashima Institute for Ornithology, The Research Institute of Evolutionary Biology, or Hiroshima Animal Husbandry Experimental Station.

Preparation of Cell Lysate and Extraction of DNA. At least 5 μl of peripheral blood was blotted on a small piece of filter paper (approximately 5 × 5 mm) and kept dried during transportation. Blood was eluted from a filter paper in 500 μl of phosphate-buffered saline. After centrifugation at 5000 rpm for 2 min, cell pellets were suspended in 100 μl of 10 mM Tris·HCl (pH 8.3) buffer containing 50 mM KCl, 1.5 mM MgCl$_2$, 0.001% gelatin, 0.45% Nonidet P-40, 0.45% Tween 20, and 200 μg of proteinase K per ml. The suspension was incubated for 30 min at 60°C and was heated at 94°C for 15 min

Abbreviation: RFLP, restriction fragment length polymorphism.

TYP	RFLP						JUNGLEFOWLS	DOMESTIC FOWLS	
	V	A	Ms	Mb	Ms	Mb		ASIATIC	OCCIDENTAL
I	-	-	-	-	+	+		NAGOYA 1/1 GIFU-JIDORI 2/2 BLACK SILKY 1/3 THAI BANTAM 5/8 AYAM KATAI 1/5	WHITE LEGHORN 3/3 INDIAN GAME 2/2
II	-	-	-	-	-	+		BLACK SILKY 1/3	
III	+	-	-	-	+	+	THAI RED 3/14	THAI BANTAM 3/8	
IV	+	-	-	+	+	+		WHITE LEGHORN (HIROSHIMA VAR.)1/2 TOHMARU 1/2 BLACK SILKY 1/3	
V	+	+	-	+	+	+	THAI RED 7/14 INDONESIAN RED 3/5	WHITE LEGHORN (HIROSHIMA VAR.)1/2 TOHMARU 1/2 WHITE SILKY 1/1 DARK BRAHMA 1/1 HOUDAN 2/2 FAYOMI 1/1 MALAY GAME 2/2 SUMATRA GAME 2/2 AYAM BANGKOK 2/2 AYAM BEKISAR 2/4 AYAM CEMANI 1/2 AYAM KEDU 2/9 AYAM PELUNG 1/5	BARRED PLYMOUTH ROCK 3/3 WHITE PLYMOUTH ROCK 1/1 RHODE ISLAND RED 1/1 LIGHT SUSSEX 2/2 BUFF COCHIN 2/2 PARTRIDGE COCHIN 2/2 SILVER GREY DORKING 2/2 JERSEY BLACK GIANT 2/2 BROWN LEGHORN 1/1 LA FRECHE 1/1 ARAUCANA 1/1
VI	+	+	-	+	-	+	THAI RED 1/14 INDONESIAN RED 1/5	AYAM CEMANI 1/2	
VII	+	+	+	+	+	+	THAI RED 3/14		
VIII	+	+	-	-	+	+	INDONESIAN RED 1/5	AYAM BEKISAR 2/4 AYAM KATAI 4/5 AYAM KEDU 7/9 AYAM PELUNG 4/5	
A	-	-	-	-	+	-	GREEN 1/30		
B	-	-	-	-	-	-	GREEN 1/30		
C	-	-	+	-	-	-	GREEN 18/30 GREEN 3/30 (60-BASE-LONG INSERTION)		
D	-	-	+	-	+	-	GREEN 2/30		
E	+	-	+	-	-	-	GREEN 3/30 GREEN 1/30 (120-BASE-LONG INSERTION)		
F	-	+	-	-	-	-	GREEN 1/30		

FIG. 1. Distribution of 14 RFLP types among 121 individuals of *G. gallus* (red junglefowls and domestic breeds) and *G. varius* (green junglefowls) is shown. Mitochondrial control region amplified by PCR contained six polymorphic sites for four restriction enzymes. V, *Vsp* I; A, *Alu* I; Ms, *Mse* I; Mb, *Mbo* II. On the left, each RFLP type is defined as cleavable (+) or not cleavable (−) at each of the six sites. RFLP types of *G. gallus* are numbered in Roman numerals I–VIII, whereas those of *G. varius* are shown as A–F. Nevertheless, types I and II of the former and types A and B of the latter are related (see text). With regard to each wild species and subspecies as well as to each domestic breed, distribution is expressed as number of individuals of a particular RFLP type per total number studied. Aside from 14 Thai red junglefowls (10 *G. g. gallus* and 4 *G. g. spadiceus*) and 5 Indonesian red junglefowls (*G. g. bankiva*) and 30 green junglefowls, 72 individuals representing 26 diverse domestic breeds, 3 of them in 2 varieties each were studied. Although all domestic breeds are ultimately of Asiatic origin, those long established in Europe and the New World were classified as occidental in contrast to those that stayed in Asia.

to stop the reaction. The cell lysate was then extracted twice with 400 μl of phenol/chloroform/isoamyl alcohol (25:24:1) and total DNA was recovered from ethanol precipitation. The DNA pellet was dissolved in 200 μl of 10 mM Tris·HCl (pH 7.5) buffer containing 1 mM EDTA.

PCR. Conserved primer pair H1255 (5′-CATCTTG-GCATCTTCAGTGCC-3′) and L16750 (5′-AGGACTACG-GCTTGAAAAGC-3′) was used to amplify the control region for RFLP analysis. L and H refer to the light and heavy chains and the number designates the position of the 3′ end of the primer in the reference sequence (5).

Two microliters of the total DNA or cell lysates was subjected to 35 amplification cycles by using *Taq (Thermus aquaticus)* DNA polymerase (Takara Shuzo, Kyoto) according to the manufacturer's instructions, with denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min.

Detection of RFLP. After testing 36 restriction enzymes, the following four were chosen as suitable: *Alu* I (recognition sequence, AGCT), *Mse* I (TTAA), *Mbo II* (TCTTC), and *Vsp* I (ATTAAT). RFLPs were detected on either 1.5% or 4.0% agarose gels after 30 min to 1 hr of electrophoresis at 80 V.

Nucleotide Sequencing. Because of the presence of an *Eco*RI site within, the primer H1254 (5′-ATGAATTCTTGGCATCT-TCAGTGCCA-3′) was used instead of H1255 to obtain PCR products for cloning. The base sequence of another primer already given, L16775, did contain a *Hin*dIII site. When the above H1254/L16775 pair was used for PCR amplification, 3.0 mM MgCl₂ replaced the 1.5 mM concentration recommended. PCR products were digested with *Eco*RI and *Hin*dIII and purified by agarose gel electrophoresis. Ligation of the DNA segments into the *Eco*RI/*Hin*dIII site of the cloning vector pUC118, transformation of *Escherichia coli* JM109, and sin-

gle-strand DNA preparation by using helper phage M13K07 were performed as described (6). To minimize errors introduced by *Taq* DNA polymerase during PCR, two or three clones obtained from each sample were used for sequencing. Sequencing was carried out with the BcaBEST dideoxynucleotide sequencing kit (Takara Shuzo) using fluorescein isothiocyanate labeled M13 forward primer (Shimazu, Kyoto) and DNA sequencer DSQ1 (Shimazu).

OBSERVATIONS

RFLP Within the 1200- to 1300-Base Control Region. As noted in *Materials and Methods*, four restriction enzymes recognized specific polymorphic cleavage sites within the control region, thus yielding different sized fragments readily distinguishable by gel electrophoresis. These four restriction enzymes were *Vsp* I, *Alu* I, *Mse* I, and *Mbo* II. Inasmuch as the last two enzymes recognized two polymorphic sites each, a total of six sites were involved in RFLP (Fig. 1). The first four polymorphic sites are identified in Fig. 2. Of the potential 64 (2^6) types involving six sites, eight were found among domestic chickens and their wild ancestor, red junglefowls. Six additional types were seen among more distantly related green junglefowls. Thus, 14 of the 64 potential types are in existence.

Fig. 1 shows that regardless of whether they belong to the breeds long established in the West (Europe and North America) or to the breeds that remained in Asia, the predominant RFLP type among domesticated chickens was type V, closely followed by type I. While type V was also found in more than half of the red junglefowls of three subspecies sampled, types I, II, and IV have not thus far been found among red junglefowls. Conversely, type VII has been confined to the Thai red junglefowl (*G. g. spadiceus*) in spite of

RFLP

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1)        AYAM PEEUNG #76 (VIII) AATTTTATTTTTTAACCTAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTTATAT 94
2)              NAGOYA # 1   (I)  AATTTTATTTTTTAACCTAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTTATAT 94
3) BARRED PLYMOUTH ROCK # 1   (V)  AATTTTATTTTTTAACCTAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTTATAT 94
4)   THAI RED JUNGLEFOWL #11   (V)  AATTTTATTTTTTAACCTAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTTATAT 94
5)   THAI RED JUNGLEFOWL # 3 (VII)  AATTTTATTTTTTAACCTAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTTATAT 94
6) INDN. RED JUNGLEFOWL #15 (VIII) AATTTTATTTTTTAACCTAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTTATAT 94
7)      GREEN JUNGLEFOWL #32   (C)  AATTTTATTTTTTAACCCAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTTATAT 94
8)      GREEN JUNGLEFOWL # 2   (D)  AATTTTATTTTTTAACCCAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTTATAT 94
9)      GREEN JUNGLEFOWL #50   (E)  AATTTTATTTTTTAACCCAACTCCCCTACTAAGTGTACCCCCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTTATAT 94
10)     JAPANESE QUAIL REF.        AACACT-TTTTTTAACCTAACTCCCCTACTTAGTGTACCCCCCCTTTCCCCCCCAGGGGGGGTATACTATGCATAATCGTGCATACATTTATAT 93
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VspI

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1) DMB (VIII) ACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAAACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAATGTCCATTCTATGCATG 206
2) DMB   (I)  ACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAAACCCATTATATGTATACGGGCATTAACCTATATTCCACATTTCTCCCAATGTCCATTCTATGCATG 206
3) DMB   (V)  ACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAAACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAATGTCCATTCTATGCATG 206
4) T RJF  (V) ACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAAACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAATGTCCATTCTATGCATG 206
5) T RJF(VII) ACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAAACCCATTATATGTATACGGGCATTAATCTATATTCCACATTTCTCCCAATGTCCATTCTATGCATG 206
6) I RJF(VIII) ACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAAACCCATTATATGTATACGGGCATTAATCTACATTCCTCATTTCTCCCAATGTCCATTCCATGCATG 206
7) GJF   (C)  ACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAAACCCATTATATGTATACGGACATTAACCTACATTCCCCATTTCTCCCCATGTACATTCCATGAATG 206
8) GJF   (D)  ACCACATATATTATGGTACCGGTAATATATACTATATATGTACTAAACCCATTATATGTATACGGACATTAACCTACATTCCCCATTTCTCCCCATGTACATTCCATGAATG 206
9) GJF   (E)  ACCACATATACTATGGTACCGGTAATATATACTATATATGTACTAAACCCATTATATGTATACGGACATTAATCTACATTCCCCATTTCTCCCCATGTACATTCCATGAATG 206
10) J Qu     TCCACATATACTATGGTACCGGTAATATATATTATATACGTACTAAACCCATTATATGTATACGGGCATTACATATTGTCCCCATTTCTCCCCATGTACATTAGTGCATG 203
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AluI   MseI   1ST COPY

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1) DMB (VIII) ATCCAGGACAT-AC-TTATTCACCCTCCCCATAGACAGCT 244  CCAAACCACTACCAAGTCACCTAACTATGAATGGTTGCAGGACATAAATCTCACTCTCATGCT 307
2) DMB   (I)  ATCCAGGACAT-AC-CCATTTACCCTCCCCATAGACAGTT 244  CCAAACCACTATCAAGCCACCTAACTATGAATGGTTACAGGACATAAATCTCACTCTCATGTT 307
3) DMB   (V)  ATCCAGGACAC-AC-TCATTCACCCTCCCCATAGACAGCT 244  CCAAACCACTACCAAGTCACCTAACTATGAATGGTTACAGGACATAAATCTCACTCTCATGTI 307
4) T RJF  (V) ATCCAGGACAT-AC-TCATTCACCCTCCCCATAGACAGCT 244  CCAAACCACTACCAAGTCACCTAACTATGAATGGTTGCAGGACATAAATCTCACTCTCATGCI 307
5) T RJF(VII) ATCCAGGACAT-AC-TCATTCACCCTCCCTACAGACAGCT 244  CCAAACCACCACCAAGTCACCTAACTATGAATGGTTACAGGACATAAATCTCACTCTCATGTL 307
6) I RJF(VIII) ATCCAAGTCAT-TC-TTAGTCATATTCCCCATAAGCAGCT 244 CTCAACCACTACCAAGACACCTAACTATGAATGGTTACAGGACATAACTCTTACTCTCATGCT 306
7) GJF   (C)  ATCCAAGTCATTACATC-GTC--CTACCCTATATCCAACT 243 CTATACCACTAACAGGTCACCTAACTATGAATGGTTACAGGACATACTTCTAATACCAGTGCT 306
8) GJF   (D)  ATCCAAGTCATTACATC-GTC--CTACCCCATATCTAACT 243 CTATACCACTAACAGGTCACCTAACTATGAATGGTTACAGGACATACCTCTAATTCTCATGCT 306
9) GJF   (E)  ATCCAAGTCATTACGTC-GTC--CTACCCTATATCCAACT 243 CTTTACCACTAACAGGTCACCTAACTATGAATGGTTACAGGACATACCTCTAATACTAGTGCT 306
10) J Qu     CTCCAAGACAT-AAACCATAC-GTTCACCTAGTAATAGA- 240 -----------------------------------------------------------
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2ND EXTRA COPY

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7) GJF   (C)  CTACCCCTAACAGGTCACCTAACTATGAATGGTTACAGGACATACTTCTAATACCAGTGCT 367
9) GJF   (E)  CTACCCCCAACAGGTCACCTAACTATGAATGGTTACAGGACATACCTCTAATGTTAGTGCT 367
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3RD EXTRA COPY

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7) GJF   (C)  -----------------------------------------------------
9) GJF   (E)  CTACCCCCAACAGGTCACCTAACTATGAATGGTTACAGGACATACCTCTAATATTAGTGC 428
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MboII   ORIGINAL

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1) DMB (VIII) CCTCCCCC-AACAAGTCACC-TAACTATGAATGGTTACAGGACATACATTTAACTACCATGTT 368 -CTAACCCATTTGGTTATGCTCG-CCGTATCAG 399
2) DMB   (I)  CTCCCCCC-AACAAGTCACC-TAACTATGAATGGTTACAGGACATACATTTAACTACCATGTT 368 -CTAACCCATTTGGTTATGCTCG-CCGTATCAG 399
3) DMB   (V)  CTTCCCCC-AACAAGTCACC-TAACTATGAATGGTTACAGGACATACATTTAACTACCATGTT 368 -CTAACCCATTTGGTTATGCTCG-CCGTATCAG 399
4) T RJF(V)   CTTCCCCC-AACAAGTCACC-CAACTATGAATGGTTACAGGACATACATTTAACTACCATGTT 368 -CTAACCCATTTGGTTATGCTCGTCCGTATCAG 400
5) T RJF(VII) CTTCCCCT-ACCAAGTCACC-TAACTATGAATGGTTGCAGGACATATATTTAACTACCATGTT 368 -CTAACCCATTTGGTTATGCTCG-CCGTATCAG 399
6) I RJF(VIII) CCTCCCCC-AACAAGTCACC-TAACTATGAATGGTTACAGGACATACATTTAACTACCATGAT 367 TCTAACCCATTTGGTTATGCTCGT-GTATCAG 398
7) GJF   (C)  CTACCCCT-AACAGGTCACC-TAACTATGAATGGTTACAGGACATACATCTAACTACCATGAT 430 -CTAACC-ATTTGGTTATGCTCGT-GTACCAG 456
8) GJF   (D)  CTACCCCT-AACAGGTCACC-TAACTATGAATGGTTACAGGACATACATTTAACTACCATGAT 367 -CTAACCCATTTGGTTATGCTCGT-CGTACCAG 398
9) GJF   (E)  CTACCCCC-AACAGGTCACC-TAACTATGAATGGTTACAGGACATACATCTAACTACCATGAT 489 -CTAACC-ATTTGGTTATG-TCGT--GTA-CAG 516
10) J Qu      CTTTCCACTAACAGGACACCATAACTATGAATGGTTGCAGGACATAAGCTTA-CTA-AATACT 302 TAGCTCCCATTTGGTTATGCTAG-ACGTACCAG 334
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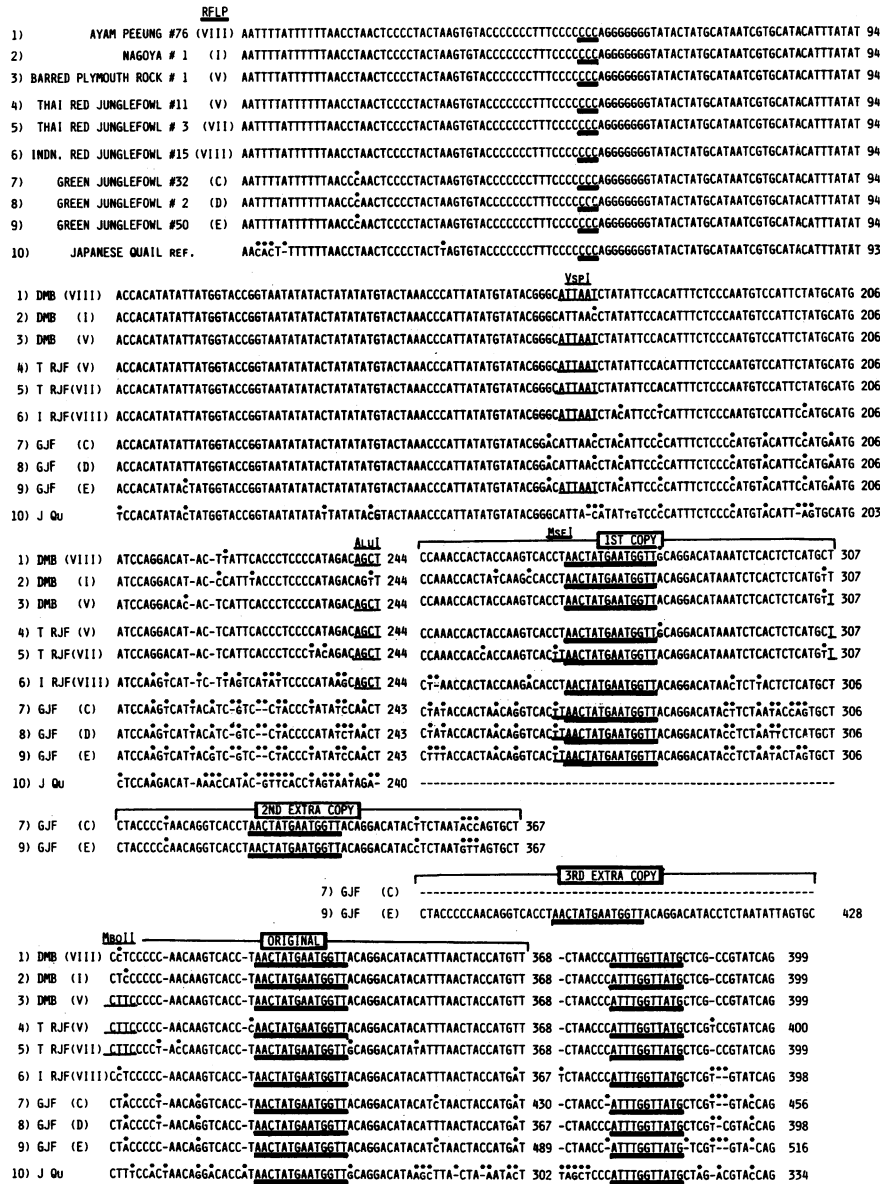
FIG. 2. L-chain sequences of the first 400 bases of the mitochondrial control region from nine gallinaceous birds representing two wild species (*G. gallus* and *G. varius*) and three domestic breeds are aligned using the published Japanese quail (*C. coturnix japonica*) sequence as the reference (7). These nine individuals and their RFLP types are identified on the left. At each polymorphic site, the majority base is shown in a large capital letter, whereas a minority base(s) is shown in small capital letters marked by asterisks. CCC base triplets underlined in the first section were bases missed in two previous publications (5, 7). Four polymorphic positions within three potential restriction sites are so indicated, *Vsp* I in the second section, *Alu* I and *Mse* I in the third section, and *Mbo* II in the bottom section. At these sites, cleavable sequences are underlined. The invariant 14-base sequence in the center of each 60-base unit is underlined and so is the invariant 10-base unit residing near the end (last section).

the sampling of >27 diverse domestic breeds. Of particular interest was type VIII. Among domesticated chickens, this type was seen only in those breeds originated in Indonesia. At the same time, 1 of the 19 red junglefowls exhibiting RFLP type VIII was also of Javanese origin. The above data appeared to have suggested the multiple sites of domestication—i.e., Indonesian breeds starting from the independent regional domestication of *G. gallus bankiva*. In the past, various population studies utilizing isozyme as well as blood group polymorphism suggested such multiple and independent sites of domestication (8).

The green junglefowl (*G. varius*) manifested its own polymorphism composed of six allelic forms, here designated as types A, B, C, D, E, and F. However, Fig. 1 shows that while the second *Mbo* II site in all individuals of *G. gallus* was cleavable by the enzyme, the corresponding site in all 30 *G. varius* was not. If one excludes this second *Mbo* II site from consideration as reflecting a pair of species-specific traits separating *G. gallus* from *G. varius*, type I of the former now becomes the same as type A of the latter and the same applies to type II and type B. The above suggests that RFLP observed in *G. gallus* and *G. varius* has been a very ancient polymorphism antedating the separation of *G. gallus* from *G. varius*.

Tandem Duplication of 60-Base Unit Within the Control Region as the Genus-Specific Trait of *Gallus*. Before comparison of base sequences with regard to the first 400 bases of the control region L chain, tandem duplication of one 60-base unit in members of the genus *Gallus* as a genus-specific trait should be noted. The control region base sequence of White Leghorn chickens (5) and that of the Japanese quail *Coturnix coturnix japonica* (7) have been published, and it was shown that the control region of the latter was 41 bases shorter than that of the former. Our own sequencing of type I White Leghorn and Japanese quail produced only one discrepancy from the published sequences noted above. The base triplet CCC underlined in the first section of Fig. 2 was missing from the published sequences of both White Leghorn (5) and Japanese quail (7).

As shown in the fifth section of Fig. 2 (marked "Original"), the 60-base unit containing the invariant tetradecamer AAC-TATGAATGGTT in its center is present as a single unit in the quail, whereas tandem duplication of this unit was observed in all 11 *G. gallus* as well as all four *G. varius* individuals. In Fig 2, a copy of the original located immediately upstream is marked "1st copy." As shall be reported separately, we found this duplication to be present also in the third and fourth members of the genus *Gallus*: the grey junglefowl

(*Gallus sonnerati*) and Lafayette's junglefowl (*Gallus lafayettei*). Yet, their closest relatives, various pheasants of the genus *Phasianus*, were quail-like, having this 60-base unit in a solitary state. Among members of the family Phasianidae, pheasants are far more closely related to the chicken than quails are, as evidenced by the fact that pheasant–chicken hybrids are fully viable, albeit sterile, whereas only 0.15–2.0% of the incubated eggs produce live chicken–Japanese quail hybrids (9). Yet, the sequence comparison between the original and its first copy on every one of the 15 sequenced individuals of *G. gallus* and *G. varius* indicated that the average difference was 20%. Interestingly, the sequence difference between originals of the Japanese quail and of *Gallus* was 25%. It would thus appear that separation of the pheasant lineage from the chicken lineage occurred relatively soon after that of the quail lineage from the combined pheasant–chicken lineage. Tandem repeats within the control region of mitochondrial DNA have previously been reported in two papers: 79-base tandem repeats in three subspecies of the masked shrew (*Sorex sinreus*) (10) and 10-base tandem repeats in canine mitochondrial DNA (11).

Once duplication started, further duplication would have been inevitable (12). Indeed, one extra copy of the 60-base unit was found in three green junglefowls of RFLP type C, while two extra copies were found in one green junglefowl of RFLP type E (Figs. 1 and 2). One each of these individuals with one and two extra copies was sequenced. When sequence comparison was made between the original of green junglefowl nos. 32 and 50 and their own "second extra copies," the uniform sequence difference of 13.1% was noted. The above data revealed that the initial further duplication that produced a second extra copy from the original was a rather ancient affair, probably antedating the speciation of *G. varius*. Indeed, the presence of the second extra copy was also noted in certain individuals of *G. sonnerati* as well as *G. layfayettei*. This shall be reported separately. The generation of the "third extra copy" by green junglefowl no. 50, on the other hand, was a very recent event, for it differed only by a single base substitution from the second extra copy of the same individual (Fig. 2).

Sequence Differences Between *G. varius* and *G. gallus* and Affinity of All the Domestic Breeds to Thai Red Junglefowl (*G.*

g. gallus). Of the four *G. varius* individuals sequenced, two (nos. 6 and 32) were of the same RFLP type C. In spite of the fact that the latter was endowed with the second extra copy, these two demonstrated the least sequence divergence of 1.50%. Furthermore, all the substitutions were transitions (Fig. 3). In view of the considerable antiquity of the second extra copy already discussed, this probably means the recent loss of the second extra copy by the lineage represented by no. 6. The difference between these two RFLP type C individuals and no. 2 of RFLP type D increased to 2.25%, while a 3.20% sequence difference separated no. 50 of RFLP type E from the rest. Furthermore, these differences included a few transversions (Figs. 2 and 3).

In contrast to the green junglefowl, which is a local species confined to the Indonesian Islands, the red junglefowl (*G. gallus*) inhabits a very large area: the Asian mainland stretching from northeastern India in the west to the western coast of China to the east. In addition, its range includes various Indonesian Islands where it is sympatric with *G. varius* as well as Hainan Island in the South China Sea. It is no surprise that *G. gallus* has often been subdivided into five subspecies (13).

As shown in Fig. 2, when dealing with different subspecies, the same RFLP type was no indication of genetic similarity. Both red junglefowl no. 15 and the domestic breed ayam pelung no. 76 typed as RFLP type VIII and they were from the same Indonesia island. Yet, 5.75% sequence divergence separated the two. Furthermore, 9 of the 23 substitutions were transversions (Fig. 2). The above clearly excluded the involvement of *G. gallus bankiva* in the domestication event. In sharp contrast, all three Thai red junglefowls (two *G. g. gallus* and one *G. g. spadiceus*) were very close to all breeds of domestic chicken. The closest affinity of only 0.5% (one each of transition and deletion) difference was seen between Thai red junglefowl no. 11 of RFLP type V and a member of the Indonesian breed, ayam cemani, of the same RFLP type (Fig. 4). Of three subspecies of the red junglefowl, *G. g. gallus* (Thai nos. 8 and 11) was far more closely related to *G. g. spadiceus* (Thai no. 3) from the adjacent area than to *G. g. bankiva* from Java (Indonesian no. 15). Nevertheless, a transversion was involved in a difference between the first two and RFLP type VII was unique to *G. g. spadiceus*.
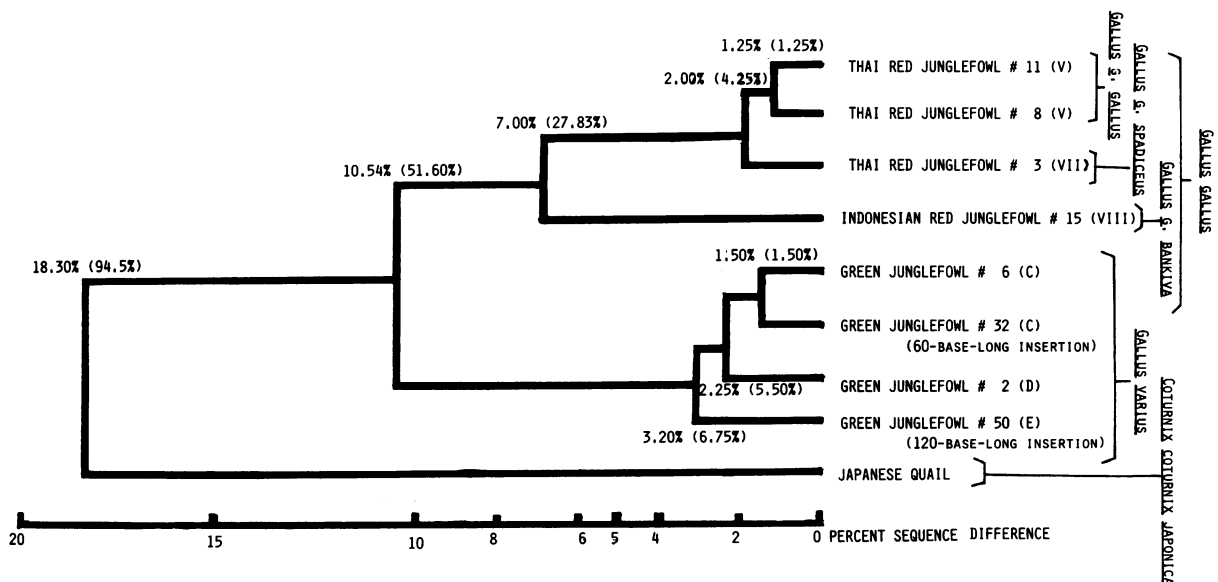

FIG. 3. Dendrogram based on sequence divergence with regard to the first 400 bases of the mitochondrial control region of four *G. varius* and two *G. g. gallus* and one each of *G. g. spadiceus* and *G. g. bankiva*. Japanese quail (7) was chosen as the outgroup. Sequence difference is shown as percentage at each branch point. Often larger percentages in parentheses are derived by regarding each transversion as an equivalent of 10 transitions.

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                                      167 171 210 217 220 221 225 243 246 254 256 261 265 281 282 306 309 310 315 317 327 342 391' 394'
         THAI RED JUNGLEFOWL # 8 (V)   T   C   C   T   T   C   C   C   C   T   C   T   C   G   T   C   T   T   C   A   T   G   -    -
         THAI RED JUNGLEFOWL #11 (V)   T   T   C   T   T   C   C   C   C   T   C   T   C   G   C   C   T   T   C   A   C   A   T    -
                   AYAM CEMANI   (V)   T   T   C   T   T   C   C   C   C   T   C   T   C   G   C   C   T   T   C   A   T   A   -    -
            BARRED PLYMOUTH ROCK (V)   T   T   C   C   T   C   C   C   C   T   C   T   C   A   C   T   T   T   C   A   T   A   -    -
  WHITE LEGHORN(HIROSHIMA VAR.)  (V)   T   T   C   C   T   C   C   C   T   T   C   T   C   A   C   T   T   T   C   A   T   A   -    T
  WHITE LEGHORN(HIROSHIMA VAR.) (IV)   T   T   C   C   T   C   C   T   C   T   C   A   C   T   T   T   C   A   T   A   -    T
         THAI RED JUNGLEFOWL # 3 (VII) T   C   C   T   T   C   C   C   C   C   C   T   T   A   C   T   T   T   T   C   T   G   -    -
                   AYAM PELUNG (VIII)  T   T   C   T   T   T   C   C   C   T   C   T   C   G   C   C   C   T   C   A   T   A   -    -
                   WHITE LEGHORN (I)   C   T   T   T   T   C   T   T   C   T   T   C   C   A   C   T   T   C   C   A   T   A   -    -
                         NAGOYA  (I)   C   T   C   T   C   C   T   T   C   T   T   C   C   A   C   T   T   C   C   A   T   A   -    -
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THAI RED JUNGLEFOWL # 8 (V)                THAI RED JUNGLEFOWL #11 (V)            WHITE LEGHORN (I)
                            ]1.25% (1.25%)                            ]0.50% (0.50%)              ]0.50% (0.50%)
THAI RED JUNGLEFOWL #11 (V)                AYAM CEMANI            (V)            NAGOYA (I)

THAI RED JUNGLEFOWL # 8  (V)               THAI RED JUNGLEFOWL #11 (V)            AYAM CEMANI      (V)
                            ]1.75% (4.00%)                            ]1.25% (1.25%)              ]0.50% (0.50%)
THAI RED JUNGLEFOWL # 3 (VII)              BARRED PLYMOUTH ROCK    (V)            AYAM PELUNG (VIII)

THAI RED JUNGLEFOWL #11   (V)              THAI RED JUNGLEFOWL # 8 (V)            AYAM CEMANI      (V)
                            ]2.25% (4.50%)                            ]1.25% (1.25%)              ]0.75% (0.75%)
THAI RED JUNGLEFOWL # 3 (VII)              AYAM PELUNG         (VIII)            BARRED PLYMOUTH ROCK (V)

                                          THAI RED JUNGLEFOWL # 3 (VII)          WHITE LEGHORN     (I)
                                                                     ] 3.00% (5.25%)              ]2.75% (2.75%)
                                          WHITE LEGHORN           (I)            AYAM PELUNG (VIII)
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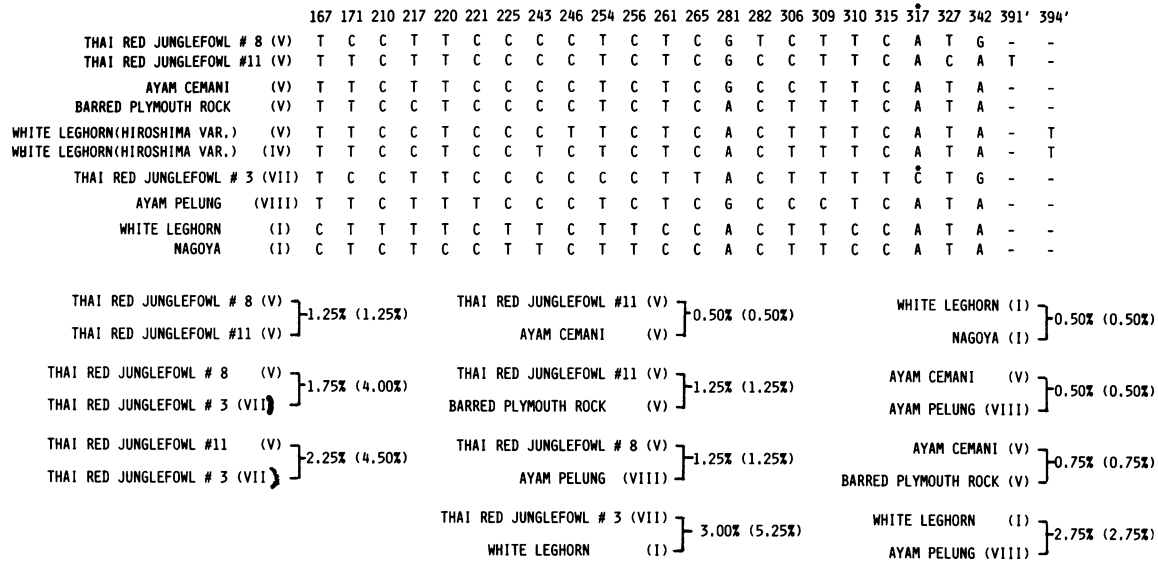
FIG. 4.   With regard to two sequenced members of *G. g. gallus* (Thai nos. 8 and 11) and one of *G. g. spadiceus* (Thai no. 3) and seven individuals representing six domestic breeds and four RFLP types, individual bases at 24 polymorphic positions are identified. Asterisk at position 317 marks a single instance of transversion (A to C). All other substitutions are transitions. Shown below alignments are sequence differences in percentages of pertinent pairs.

DISCUSSION

Because of the well-known Mohenjo-Doro site in Pakistan, it was held for a long time that the original domestication of the chicken occurred in the Indus Valley only 4000 years ago (13). Subsequently, however, earlier signs of domestication were found in unlikely places far removed from the habitat of junglefowls—e.g., Ukraine and Spain (14). Indeed, the earliest domestication of the chicken had been pushed back to nearly 8000 years ago. Remains of domesticated chickens were evident in 16 neolithic sites along the Yellow River in Northeast China, and some of these sites were dated to be at least 7500 years old (14). Inasmuch as the semiarid steppe environment of these loess highlands of North China has never been a suitable habitat for red junglefowls, the time and place of the original domestication should be sought still earlier, further to the south and the west. The present finding places the original site of domestication in the area inhabited by a single subspecies of the red junglefowl (*G. g. gallus*).

In studying sequence divergence of the human mitochondrial DNA control region, Vigilant *et al.* (2) noted that while all intraspecific base substitutions were transitions, the difference between humans and chimpanzees involved a number of transversions. Accordingly, the observed sequence divergence of 13.6% between the two species was converted to 42% difference by regarding each transversion as an equivalent of 10 transitions in accordance with Nei (15). In the present study, we found that not only interspecific differences but also intraspecific differences within *G. varius* involved transversions. This was also true of differences between three subspecies of *G. gallus*. In sharp contrast, all differences among one subspecies *G. g. gallus* and diverse domestic breeds were transitions (Fig. 4).

Among the diverse domestic breeds presently studied, the greatest sequence divergence of 2.75% separated two breeds of RFLP type I from that of RFLP type VIII. This was considerably more than 1.25% that separated two individual *G. g. gallus* from Thailand. The sampling of this subspecies from distant areas such as Sumatra Island is expected to expand the scope of intrasubspecific diversity. It would thus appear that this subspecies alone had been sufficient to yield all the diverse breeds of domestic chicken, provided that not

only the mainstream RFLP type V but also a number of hens of other RFLP types contributed to the domestication event.

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