Phylogeography and Demography of the Red Junglefowl and Its Domestication Process Revealed by Mitochondrial DNA Sequences

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ABSTRACT

Red jungle fowl (RJF) inhabits in South Asia and Southeast Asia, and is divided into five subspecies (*Ggallus gallus gallus, G. g. spadiceus, G. g. jabouillei, G. g. murghi* and *G. g. bankiva*). It is well known that this species is the wild progenitor of domestic chickens. To reveal the origin of chicken domestication is a topic of considerable interest to humanity. However, there are two hypotheses on the origin of domestication: a single origin situated in Thailand and its adjacent regions, or multiple origins in South and Southeast Asia. In this study, we determined mitochondrial D-loop sequences of 40 RJF specimens and 43 domestic chickens. We first estimated phylogenetic relationships among RJF (40 our own specimens and 87 Genbank data). In this analysis, RJF specimens, except for *G. g. bankiva* and Sumatran *G. g. gallus,* formed a continental super clade (CSC), in which we detected four clades (clades 1 to 4). Whereas clades 2 and 4 were composed of *G. g. murghi* only, clades 1 and 3 did not reflect taxonomic status of subspecies. Taken together, these phylogenetic relationships and estimated ancestral population sizes suggested that genetic differentiation of *G. g. bankiva*, *G. g. spadiceus*, and *G. g. gallus* were caused by i) the vicariance of their distribution area coincident with marine introgression-regression cycles in the Middle to Late Pleistocene, and ii) differentiation between *G. g. murghi* and other RJF caused by dispersal events in the Late Pleistocene. Finally, to estimate the origins of domestication, we constructed a comprehensive phylogenetic tree of RJF and domestic chickens. Our results suggest that chicken domestication occurred multiple times in South and Southeast Asia. However, Sumatran *G. g. gallus* lineage and one *G. g. murghi* lineage (clade 4) do not appear to have been involved in domestication events.

Keywords: chicken, red junglefowl, mitochondrial DNA, domestication, phylogeny

INTROCUTION

Elucidating the origins and history of domesticated animals is a subject of considerable interest to humanity, because it is closely related with development of human culture (Diamond, 2002). The domestic chicken is widely farmed around the world for purposes as diverse as food, ornamental bird, gamecock and religious affiliation. It is thought that wild progenitor of the domestic chicken is red jungle fowl (*Gallus gallus*), which is distributed in South Asia and Southeast Asia (Nishida et al., 1985, 1992) and its domestication dates back to approximately 5400 BC (Underhill, 1997). Wild populations of red jungle fowl (RJF) are morphologically divided into five subspecies; *G. g. bankiva* distributed in Java, Bali, and Lombok in Indonesia; *G. g. gallus, G. g. spadiceus* and *G. g. jabouillei* distributed in Southeast Asia; and *G. g. murghi* distributed in South Asia (Fig. 1).

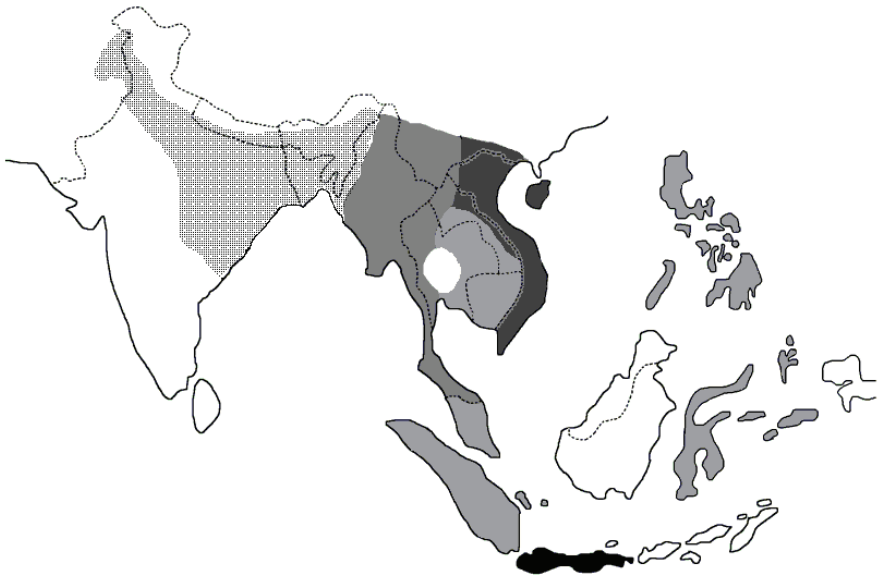

Figure 1. Distribution of subspecies of red jungle fowl (RJF) in South and Southeast Asia.

However, with regard to location and wild population, the domestication origin of chickens remains unclear, and there is still intense debate surrounding this subject (Fumihito et al., 1996; Liu et al., 2006; Kanginakudru et al., 2008). To date, two hypotheses regarding the domestication origin of the chicken have been proposed by molecular phylogenetic studies. Fumihito et al. (1996) suggested that the domestic chicken originated from a single lineage of continental RJF, which was distributed in Thailand and its adjacent regions. On the other hand, Liu et al. (2006) and Kanginakudru et al. (2008) suggested that there were multiple domestication events in South and Southeast Asia. Both studies lacked comprehensive taxon sampling for their phylogenetic analyses (for both wild populations of RJF and domestic chicken breeds). Thus, these two opposite hypotheses remain to be verified.

Mitochondrial DNA (mtDNA) is (mostly) maternally inherited and has a rapid evolutionary rate (Brown et al., 1979). Therefore, this gene is one of the most powerful genetic markers for assessing genetic differentiation within a relatively short evolutionary

time. In this study, we intended to elucidate the following subjects; 1) phylogenetic relationships among RJF subspecies, 2) phylogeographic, evolutionary and demographic history of RJF and 3) single or multiple origins of chicken domestication.

MATERIALS AND METHODS

DNA samples and sequence determination

Total genomic DNA samples were extracted from whole blood samples by Invisorb Spin Blood Mini Kit (Stratec Molecular, Germany). In this study, we analyzed D-loop sequences of mtDNA. PCR primers for amplification of D-loop were derived from Oka et al. (2007). The PCR amplification profile consisted of 35 cycles of denaturation at 94°C for 45 sec, annealing 55°C for 45 sec, and extension at 72°C for 45 sec. The PCR reaction mixture contained 2.5 units Ex *Taq* polymerase (Takara), 1×Ex *Taq* buffer, 0.2mM dNTPs, 1μM each primers, and 100 ng of genomic DNA, in a final volume 25 μl. To verify the amplified DNA fragment, we confirmed by electrophoresis in a 1.0% agarose gel (Wako) and stained with ethidium bromide for fragment characterization via ultraviolet transillumination. To remove excess primers and nucleotides, PCR products were treated with isopropyl alcohol precipitation. The precipitation mixture contained 20 μl of isopropyl alcohol and 250 mM NaCl for 20 μl of PCR product. The internal primers for sequencing were F3 (5'-GGT TCT CAA CTA CGG GAA C-3'), F2 (5'-TGG TTC CTC GGT CAG GCA CAT CC-3'), R3 (5'-CAG TGC CAT GCT TTG TGG GT-3') and R2 (5'-CGC AAC GCA GGT GTA GTC-3'). Sequence determination was performed by a sequencing service company (Macrogen Japan).

Phylogenetic and demographic analyses

The nucleotide sequence data determined in this study and those retrieved from previous studies (Fumihito et al. 1996, Liu et al. 2004, Oka et al. 2007, Kanginakudru et al. 2008, Silva et al. 2009, Berthouly-Salazar et al. 2010) were automatically aligned using Clustal W (Thompson et al. 1997) and carefully verified by eye. All gaps were retained and treated as missing data (total length of the alignment was 1229 bp). Phylogenetic trees were inferred by RAxML ver. 7.2.6 (Stamatakis et al. 2008) with the GTR+I+□ model. The confidences of the internal branches were evaluated by the rapid bootstrap method (Stamatakis et al. 2008) with 1000 replications.

Ancestral population sizes were estimated by the Bayesian Skyline Plot method with BEAST ver. 1.7.4 (Drummond and Rambaut 2007), using an HKY+□ nucleotide substitution model. All gaps were eliminated, and an alignment of 350 bp in length was retained for these analyses. A strict clock model with a substitution rate of 7.0 x 10^{-8} /site/year was assumed (Sasaki et al. unpublished). The MCMC was conducted under the following conditions: The total length was 100,000,000 generations, with trees and parameters sampled every 1000 generations. The first 10,000,000 generations were discarded as burn-in. Verification of MCMC convergence and summarization of posterior parameters were carried out with TRACER ver. 1.5 (http://evolve.zoo.ox.ac.uk/software/ 2003).

Table 1. Specimens used in this study.

Breeds/Species	No. of individuals	Locality/Source
Ayam	2	Republic of Indonesia
Bangkok		
Ayam Kate	2	Republic of Indonesia
Brahma	7	United States of America*1
Cochin	3	United States of America*1
Cornish	4	United States of America*1
Jersey	8	United States of America*1
Giant		
Langshan	6	United States of America*1
Spanish	5	United States of America*1
Wyandotte	6	United States of America*1
Gallus	1	Thailand
gallus sp.*2		
Gallus	1	Unknown
gallus sp.*2		
Gallus	38	Bangladesh
gallus sp.*2		

*1=Murray McMurray Hutchery. *2=subspecies name is unknown.

RESULTS AND DISCUSSION

Phylogenetic relationships among red jungle fowls

Figure 2 shows the unrooted maximum likelihood (ML) tree among the specimens of RJF based on mitochondrial D-loop sequences. *G. g. bankiva* was relatively far from the other RJFs in genetic relationship as demonstrated previously (Fumihito et al., 1996; Liu et al., 2006). We also confirmed that the three *G. g. gallus* lineages derived from South Sumatra of Indonesia formed a distinct, independent clade with relatively distant position from the continental specimens as mentioned by Fumihito et al. (1996) (Fig. 2). The phylogenetic tree indicated a difference in geographical distribution of RJFs between islands and the continent. Therefore, we designated the clade constituted by continental RJFs as the "continental super clade" (CSC) (Fig. 2). In the CSC, four major clades of continental RJFs could be recognized, except for one Bangladeshi RJF and one Yunnan *G. g. spadiceus*. In addition, the clade 1 was subdivided into four subclades (Fig. 2; Table 2 shows a summary of the clade classification). Previously, Fumihito et al. (1996) raised questions about taxonomic status of *G. g. gallus* and *G. g. spadiceus* subspecies in particular. Our phylogenetic tree also indicated nested and highly intermingled phylogenetic relationships with respect to subspecies classification (see clade 1 and 3 in Fig. 2 and Table 2). However, it appeared that some populations showed evidence of genetic differentiation, as shown in *G. g. murghi* single clades (see clade 2 and 4 in Fig. 2 and Table 2).

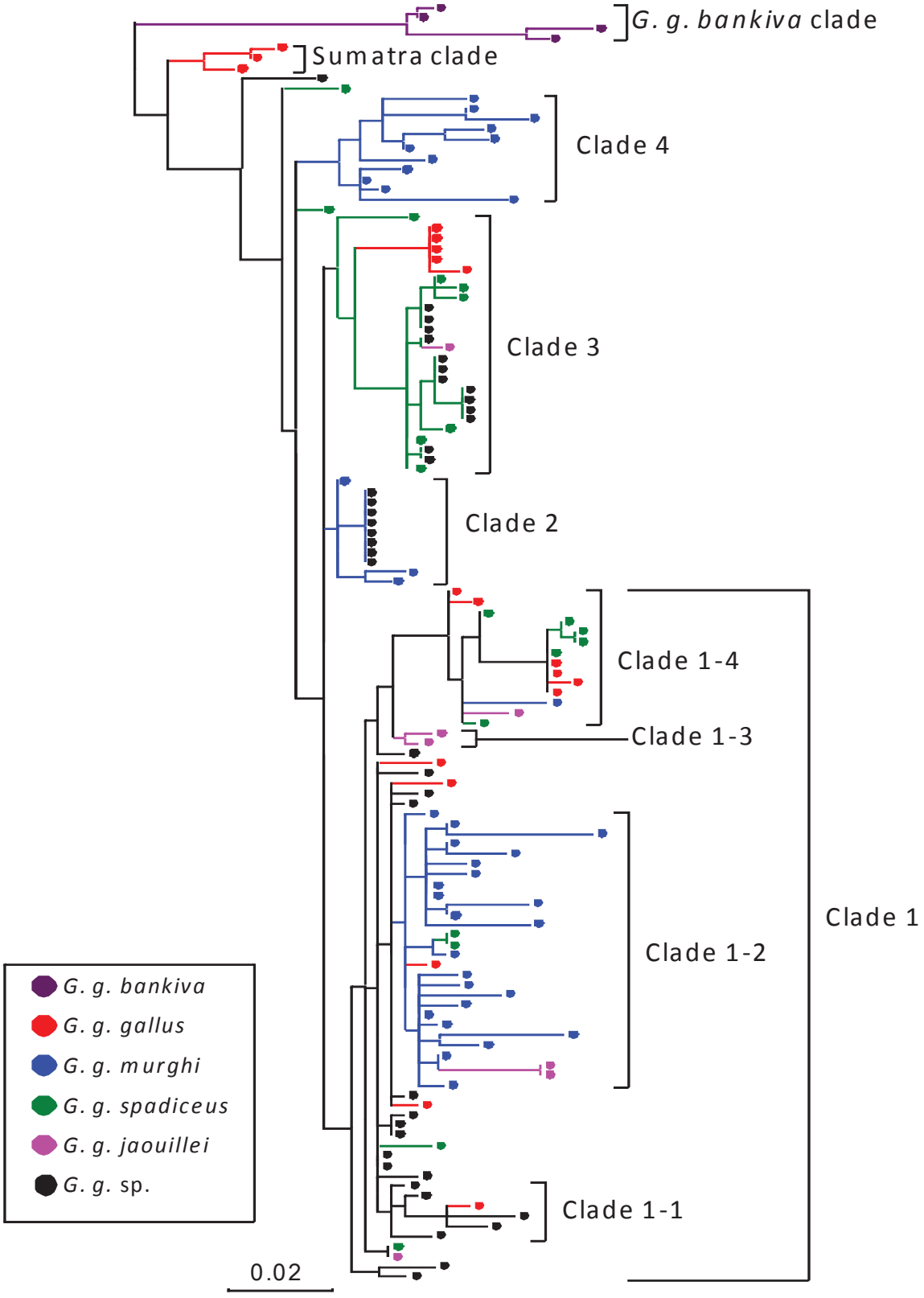

Figure 2. Unrooted maximum likelihood tree among RJFs. See text for details of estimation.

Table 2. Taxonomy, locality and number of individual contents of each clade in Fig.2.

Clade-subclade	Subspecies	Locality	No. of individuals
1-1	*G. g. gallus*	Thailand	1
	Gallus gallus sp.*1	Bangladesh	5
1-2	*G. g. gallus*	Thailand	2
	G. g. spadiceus	Yunnan, China	1
		Myanmar	1
	G. g. jaouillei	Unknown	1
	G. g. murghi	India	23
1-3	*G. g. jaouillei*	Hainan, China	2
1-4	*G. g. gallus*	Thailand	4
		Vietnam	2
	G. g. spadiceus	Yunnan, China	3
		Myanmar	3
	G. g. murghi	India	1
	G. g. murghi	India	2
		Nepal	1
	Gallus gallus sp.*1	Bangladesh	8
3	*G. g. gallus*	Vietnam	6
	G. g. spadiceus	Yunnan, China	5
		Myanmar	2
	Gallus gallus sp.*1	Bangladesh	13
4	*G. g. murghi*	India	11

*1=subspecies name is unknown spcimen.

Estimated ancestral demography of the red jungle fowl

Our estimated fluctuations in the ancestral population sizes of the each subspecies of RJFs are also shown in Figure 2. The population sizes of *G. g. gallus*, *G. g. spadiceus,* and *G. g. bankiva* (Fig. 3a, b, c, respectively) have been basically stable, or shown slight increase or decline from the TMRCA (time of the most recent common ancestor). By contrast, that of *G. g. murghi* (Fig. 2 d) shows rapid population expansion beginning around 50,000 years ago. The significant negative Tajima's D of this subspecies also supported an ancestral recent population expansion event (-1.834: P-value < 0.05).

We also estimated fluctuations of the ancestral population size of RJF as a whole, excluding the subspecies *G. g. murghi* (Fig. 3e). Interestingly, while the population sizes of each subspecies have been stable, those of the whole RJF clade show moderate increase. Taking these demographic analyses and phylogenetic relationships together, we have constructed the following evolutionary scenario for RJF.

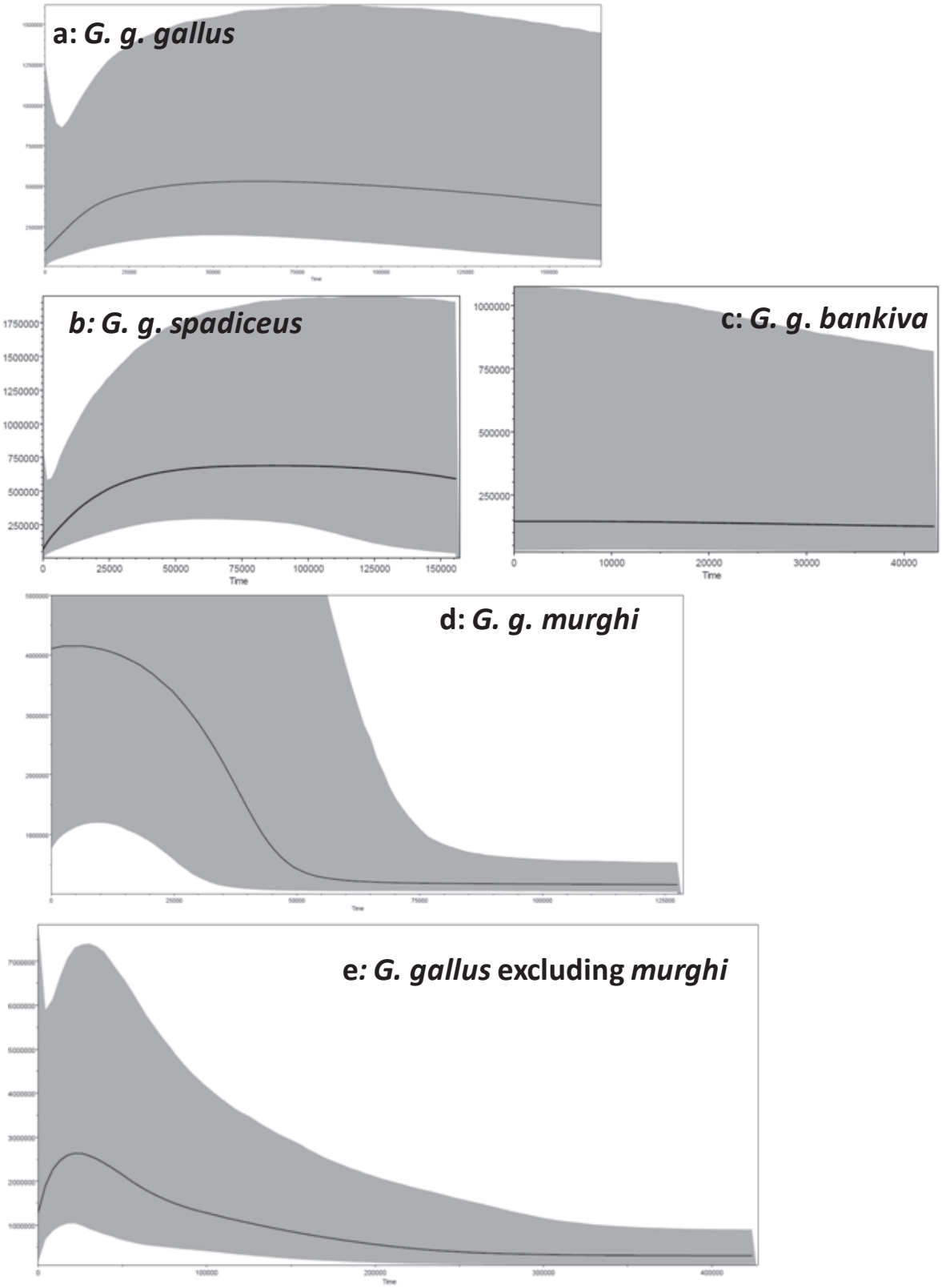

Figure 3. Bayesian Skyline Plot of each RJF subspecies. The X axis represents time (years before present) and Y axis indicates the effective female population size multiplied by generation interval. Blue belt indicates range of 95% confidence intervals.

i) The subspecies *bankiva* branched earliest from other subspecies. The divergence time between *G. g. bankiva* and other subspecies was estimated to be about 625 Ka (kilo annum or thousands of years), assuming a substitution rate $7.0*10^{-8}$/site/year (data not shown). This would have been during the transition between Glacial C (the glacial period) and Cromerian V (the inter-glacial period) in the Middle Pleistocene.

ii) After the divergence of *bankiva*, the total population size of other RJF shows a moderate increase. As we discuss below, the geographical distribution area of the ancestral RJF seems to be almost the same as that of extant *G. g. spadiceus*. This increase in ancestral population size accelerated beginning around 100,000 years ago (Fig. 3 e), and its timing is almost coeval with a marine regression that occurred during the Wisconsinan period (the last glacial period). It is well known that "Sundaland" formed during this period. Sundaland was a broad area encompassing the present-day Sunda shelf and Asian continental shelf, and was exposed by marine regression coincident with the glacial period. The inferred increase in population size during this time may be related to the expansion of their distribution area caused by the formation of Sundaland. The stable or even declining population size of *G. g. spadiceus* may suggest that this subspecies has maintained its ancestral distribution area, and has not expanded its range.

iii) By contrast, even though it was very moderate, the increasing population size of *G. g. gallus* in this period may imply that this subspecies is descended from the pioneers that colonized the modern distribution area of RJF. Taking into account the distribution area of this subspecies, expansion first occurred eastward and then southward. Finally, the ancestor of *G. g. gallus* expanded throughout the whole of Sundaland. The last glacial maximum was 20000 years ago. Afterward, sea-level rose due to global warming, and resulted in the reduction of the land mass of Sundaland, leading naturally to a reduction of the distribution area of *G. g. gallus.*

Interestingly, their population size also turned to decline. During this process, the populations of Sumatra, Java, and the islands of Philippines should have become geographically isolated. The maximum marine introgression was 7000 years ago. During this period, the sea-level was about 10 m higher than today, and both the Kura Isthmus that connects the Asian continent and Malay Peninsula, as well as a broad area of the Chaophraya River plain, were both submerged undersea. This process would have led to the isolation of *G. g. gallus* populations of the Malay Peninsula from those of the area comprising modern Thailand, Laos, and Vietnam. The genetic differentiation of *G. g. gallus* and *G. g. spadiceus* might be caused by these "vicariance" events accompanying marine introgression. Since these events should be very recent, genetic differentiation between *G. g. gallus* and *G. g. spadiceus* remains incomplete; for this reason, the ancestral polymorphisms of mitochondrial DNA can be still observed.

iv) The genetic differentiation of *G. g. murghi* and others may result from a different process. The original ancestral population size of *G. g. murghi* (Indian population) was small and then increased rapidly from 50000 Ka (the Late Pleistocene). However, the population size of the RJF from Bangladesh was stable. A geological feature such as the Ganges River could have served as a geographical barrier, restricting the migration of the RJF. We hypothesize that relatively small populations of the ancestral RJF occasionally moved westward across this barrier, and then rapidly expanded in this new territory. This small population evolved to *G. g. murghi.* Thus, genetic differentiation of *G. g. murghi* seems to

have been be caused by a dispersal event. Concerning the origin and evolution of *G. g. jabouillei*, available data remains too limited to construct an hypothesis of their evolutionary process.

Multiple origins of domestic chickens

To examine the origin of domestic chickens, we analyzed phylogenetic relationships among RJF and 262 chicken specimens (Fig. 4). Domestic chickens were found to be widely distributed in clades that were defined by the RJF phylogenetic tree (Fig. 2). Liu et al. (2006) had suggested previously that domestic chickens occurred in multiple continental RJF lineages based on their phylogenetic tree constructed from mtDNA D-loop sequences.

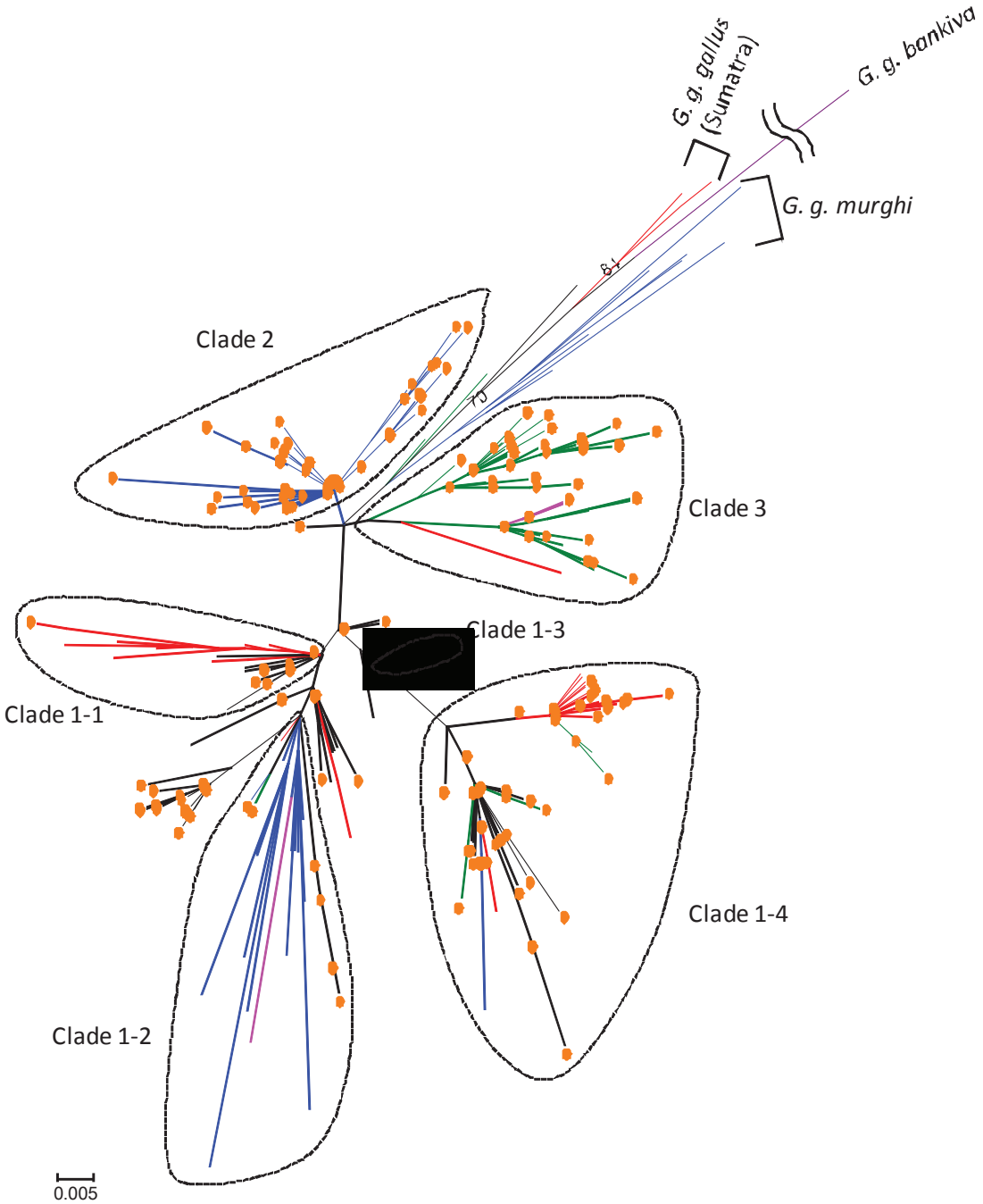

Figure 4. Unrooted maximum likelihood tree among RJF and domestic chickens.

Phylogenetic positions of each domestic chicken are indicated by orange circles.

The present study's clade composed of Sumatran *G. g. gallus,* seemingly corresponds to their clade H. It is probable that the RJFs in clade A have never been domesticated in their history, as suggested previously (Fumihito et al., 1996; Liu et al., 2006). Notably, Clade 4 was also constituted by RJF (*G. g. murghi*) only. This clade was newly discovered as a nondomesticated lineage in the present study. Our phylogenetic tree indicated that chicken domestication occurred in three of four clades composed of continental RJF, namely clades 1 (except for clade 1-3), 2 and 3. Clade 1-3 did not contain a cluster with domestic chickens. This phylogeny may indicate that clade 1-3 was not involved in chicken domestication. The RJF belonging to clades 1 and 3 were distributed in South and Southeast Asia, whereas the RJF belonging to clade 2 were exclusively distributed in South Asia (Table 2). This result indicates that chicken domestication occurred multiple times in various areas in South and Southeast Asia. In conclusion, we suggest that chicken domestication occurred multiply in a broad area of South Asia and Southeast Asia. Our study supported the hypothesis suggested by Liu et al. (2006).

ACKNOWLEDGEMENT

We wish to express our special thanks to Prof. Yoshio Yamamoto for helping us to collect samples and providing many valuable comments. Lucien A (Bud) Wood, Chris Huseman and Curt McCoskey of Murray McMurray Hatchery provided the precious chicken samples, and helped us collecting specimens. Prof. Susan J. Lamont, Dr. Michael G. Kaiser, and students of Department of Animal Sciences, Iowa State University carried out DNA extraction. Ms. Saki Kaneko collected sequence date as her graduation thesis. Dr. Gabriel Yedid read our manuscript and improved it. We would like to take this opportunity to deeply thank for all of their kind supports. We also thank to Prince Akishinonomiya Fumihito for the stimulate discussions and valuable comments. This work was supported by JSAC.

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