The present study aims to investigate the maternal origin and genetic diversity of laying-type Japanese quail lines based on partial sequences (453 base pairs) of a mitochondrial DNA (mtDNA) control region. A total of 478 individuals from 12 lines were sequenced and six different haplotypes with eight variable sites were identified. All haplotypes, two of which were identical to previously reported sequences, were typical for the Japanese quail (Coturnix japonica) and were distinct from those of the common quail (Coturnix coturnix) in a phylogenetic analysis including other published haplotypes. One haplotype was distributed in the majority of individuals (84.9%, 406/478) across all lines. Within each line, 72.5–100% of individuals had this predominant haplotype. The second most common haplotype was detected in 12.8% (61/478) individuals. These two haplotypes accounted for 97.7% of all individuals. The remaining four haplotypes were distributed with a low frequency; these were observed in five, three, two, and one individuals across all lines, respectively. All lines showed a low degree of haplotype diversity ranging from 0.0000 to 0.4321. Genetic differentiation indexes (FST) were not significant in approximately 80% pairwise comparisons of lines. The results suggest limited maternal origin and low mtDNA diversity of laying-type quail lines and may reflect their breeding history where the present gene pool was rooted in a small number of founders.

Key words: genetic diversity, Japanese quail, maternal origin, mitochondrial DNA

**Materials and Methods**

**Samples and Sequencing**

The samples used in the present study were the same samples used in a previously published microsatellite-based diversity study (Shimma and Tadano, 2019). One sample of Farm 5-A was excluded because reliable sequence data could not be obtained. As shown in Table 1, a total of 478 individuals from 12 laying-type lines and 40 individuals from one meat-type line were successfully sequenced using the...
following procedures. The laying-type lines were identified from nine commercial farms in five prefectures in Japan. The breeding histories of some lines are available in Shimma and Tadano (2019). The meat-type line selected for increased body weight was imported from France to Japan in September 2002.

PCR amplification of partial sequence of the mtDNA control region was performed using PHDL (5′-AGGACTACGGCTTGAAAAGC-3′) and PH-H521 (5′-TTATGTGCTTGACCGAGGAACCAG-3′) primers as described by Randi and Lucchini (1998). The 20 μL reaction volume contained 12 ng of total DNA, 1× GeneAmp PCR Buffer (Applied Biosystems, Foster City, CA, USA), 200 μM of deoxynucleoside triphosphate (Applied Biosystems), 0.25 μM of each primer, and 1.25 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems). The cycling conditions were as follows: 95°C for 10 min; followed by 35 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min; with a final extension of 72°C for 10 min. PCR products were separated on 2% agarose gels and then stained with ethidium bromide and visualized under ultraviolet (UV) light. Purification of PCR products was performed using ExoSAP-IT (Thermo Fisher Scientific, Waltham, MA). The PHDL primer and BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) were used in sequencing reactions. Sequences were determined using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

### Data Analysis

The control region sequences were edited and aligned against a published sequence of the Japanese quail (GenBank accession number AB073301; Nishibori et al., 1998). The PHDL primer and BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) were used in sequencing reactions. Sequences were determined using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

#### Table 1. Distribution of haplotypes and genetic diversity estimates within 13 commercial Japanese quail lines based on analysis of mitochondrial DNA control regions

| Line          | Location | Sample size | Number of haplotypes | Haplotype | Haplotype diversity | Nucleotide diversity |
|---------------|----------|-------------|----------------------|-----------|---------------------|---------------------|
| Farm 1-A      | Hokkaido | 40          | 2                    | Cj1       | 0.3282              | 0.0022              |
| Farm 1-B      | Hokkaido | 40          | 2                    | Cj2       | 0.2244              | 0.0015              |
| Farm 1-C      | Hokkaido | 40          | 2                    | Cj3       | 0.0500              | 0.0003              |
| Farm 2        | Saitama  | 40          | 2                    | Cj4       | 0.0974              | 0.0006              |
| Farm 3        | Shizuoka | 40          | 3                    | Cj5       | 0.4051              | 0.0024              |
| Farm 4        | Shizuoka | 40          | 3                    | Cj6       | 0.3679              | 0.0022              |
| Farm 5-A      | Aichi    | 38          | 2                    |           | 0.2347              | 0.0016              |
| Farm 5-B      | Aichi    | 40          | 3                    |           | 0.3038              | 0.0023              |
| Farm 6        | Aichi    | 40          | 3                    |           | 0.4321              | 0.0034              |
| Farm 7        | Aichi    | 40          | 3                    |           | 0.2718              | 0.0014              |
| Farm 8        | Aichi    | 40          | 4                    |           | 0.3500              | 0.0018              |
| Farm 9        | Miyazaki | 40          | 1                    |           | 0.0000              | 0.0000              |
| Meatype       | Saitama  | 40          | 2                    |           | 0.0500              | 0.0007              |
| Total         |          | 518         | 445                  |           |                     |                     |

were computed using ARLEQUIN software version 3.5.

Phylogenetic relationships of haplotypes were inferred by constructing a neighbor-joining tree based on Tamura-Nei genetic distances (Tamura and Nei, 1993) with 1000 bootstrap replications using MEGA software version 7.0 (Kumar et al., 2016). Additionally, the haplotype network based on the TCS algorithm (Clement et al., 2002) was constructed using POPART version 1.7 (Leigh and Bryant, 2015). These two analyses included previously published haplotypes: four obtained from the Japanese quail (Coturnix coturnix), which are indicated with the initial “F” (GenBank accession numbers KF410832, KF410833, KF410836, and KF410837; Sanchez-Donoso et al., 2014); five obtained from the common quail (Coturnix coturnix), which are indicated with the initial “W” (GenBank accession numbers from KF410844 to KF410848; Sanchez-Donoso et al., 2014); and one obtained from the blue-breasted quail (Coturnix chinensis) (GenBank accession number AB073301; Nishibori et al., 2002).

#### Results and Discussion

### Haplotype Distribution

Partial sequences (453 base pairs) of the mtDNA control region were determined from 518 individuals from 12 laying-type lines and one meat-type line. These sequences were identified as six distinct haplotypes (Cj1–Cj6 in Table 1) defined by eight variable sites, all of which were transitions (Table 2). These six haplotypes were submitted to GenBank (accession numbers from LC492859 to LC492864). Cj1 and Cj2 found in the present study were identical to F1W1 (GenBank accession number KF410830; Sanchez-Donoso et al., 2014) and F5 (GenBank accession number KF410834; Sanchez-Donoso et al., 2014). These haplotypes were reported as the Japanese quail haplotypes, respectively, by using the Basic Local Alignment Search Tool (BLAST). The remaining four haplotypes (Cj3–Cj6) were thought to be new haplotypes because no identical sequences were found via BLAST. In a neighbor-joining tree (Fig. 1), all six hap-
lotypes were located in the Japanese quail (C. japonica) clade and were clearly different from the common quail (C. coturnix) haplotypes. Similarly, in the TCS network (Fig. 2), the six haplotypes were clustered with other Japanese quail haplotypes and sharply diverged from the common quail haplotypes with a large number of nucleotide substitutions.

Remarkably, a single haplotype was distributed with extremely high frequency in the gene pool of laying-type lines: 84.9% (406/478) of the laying-type quails had Cj1 (Table 1). When the data were stratified by farm, the Cj1 had the highest prevalence: 72.5% (29/40) in Farm 6 to 100% (40/40) in Farm 9. The second most frequent haplotype was Cj2, which was found in 12.8% (61/478) of the laying-type quails. The Cj1 and Cj2 jointly made up 97.7% (467/478) of the total. On the other hand, Cj3, Cj4, Cj5, and Cj6 were found in only five, three, two, and one individuals, respectively. The haplotype distribution indicated that the maternal origin of laying-type lines was limited. Cj1 is the main haplotype in the present gene pool, although this was obtained from partial sequences of the control region (less than 500 base pairs). This finding was also in agreement with the fact that laying-type quails were threatened with extinction during World War II, and the present gene pool was derived from a

### Table 2. Variable nucleotide sites of six haplotypes (453 base pairs) detected in the present study

| Haplotype | GenBank accession number | Variable nucleotide sites |
|-----------|--------------------------|---------------------------|
| Cj1       | LC492859                 | C A G C GAAG              |
| Cj2       | LC492860                 | T                       |
| Cj3       | LC492861                 | -                        |
| Cj4       | LC492862                 | T G A T A G G           |
| Cj5       | LC492863                 | - A - - - -              |
| Cj6       | LC492864                 | T - - - - - - - -        |

Fig. 1. Neighbor-joining tree using Tamura-Nei genetic distances among mitochondrial DNA control region haplotypes of quails. Coturnix chinensis was used as an outgroup. Six haplotypes identified in the present study are indicated by “Cj.” “F” and “W” are published haplotypes of the Japanese quail (Coturnix japonica) and the common quail (Coturnix coturnix), respectively. GenBank accession numbers of haplotypes are shown in parentheses. Bootstrap values >50% are shown at each node.
small number of individuals after World War II (Yamashina, 1961; Wakasugi, 1984). The uniformity of the haplotype distribution in domestic Japanese quail lines has been observed in other studies. For example, Barilani et al. (2005) reported that 12 individuals of a Japanese quail line maintained by the University of Renne in France were analyzed, and all of them showed a single haplotype of the mtDNA control region. This haplotype (374 base pairs) (GenBank accession number DQ087957) showed 100% similarity to the prevalent Cj1 haplotype (473 base pairs) in the present study. Therefore, these two haplotypes are possibly the same.

Sanchez-Donoso et al. (2014) also reported that 22 of 29 (75.9%) individuals of farm quail in Spain, which have Japanese quail maternal origin, had a single haplotype “F1W1” identical to the Cj1 haplotype in the present study. Therefore, these two haplotypes are possibly the same. Sanchez-Donoso et al. (2014) also reported that 22 of 29 (75.9%) individuals of farm quail in Spain, which have Japanese quail maternal origin, had a single haplotype “F1W1” identical to the Cj1 haplotype in the present study. Furthermore, Nunome et al. (2017) reported a predominant mtDNA haplotype of the control region across various experimental lines of domestic Japanese quail. The Cj2 haplotype had a relatively high frequency (12.8%) in the present study. However, Sanchez-Donoso et al. (2014) reported that a haplotype “F5” identical to Cj2 was distributed with low frequency (one of 29 individuals of farm quail in Spain). This may result from the differences in the number of samples analyzed among the two studies. In the present study, meat-type line, which was bred in France, showed the same haplotype distribution as that shown by laying-type lines, and no haplotypes unique to the meat-type line were detected. This observation may reflect that meat-type quails in Europe have their roots in domestic Japanese quails (i.e., laying-type quails) introduced from Japan after World War II.

**Genetic Diversity and Differentiation**

Within each laying-type line, the number of haplotypes varied from one (Farm 9) to four (Farm 8) (Table 1). As mentioned above, all lines had Cj1 with high frequency. In contrast, Cj5 and Cj6 were unique to Farm 8 and Farm 4, respectively, although these occurred with low frequency. Haplotype diversity and nucleotide diversity ranged from 0.000 (Farm 9) to 0.4321 (Farm 6), and from 0.000 (Farm 9) to 0.0034 (Farm 6), respectively. The results indicate low mtDNA diversity of laying-type lines. As mentioned in a previous paper (Shimma and Tadano, 2019), each farm generally incorporates quails from other farms every three or five years in order to avoid inbreeding depression. For example, Farm 2 in the present study introduces quails from three different farms into its breeding stocks every three years. However, the number of distinct haplotypes is low: only two haplotypes (Cj1 and Cj2) were found in Farm 2, despite the existence of gene flows from other farms. This may suggest that there are only a small number of maternal lineages throughout the entire gene pool of laying-type lines.
No significant $F_{ST}$ values were obtained in 78.8% (52/66) pairwise comparisons of lines (Table 3). This result indicates that laying-type lines are genetically close to each other in terms of mtDNA variations. This lack of differentiation may result from a small number of common founders of these lines. A similar tendency was observed in a previous study with the same samples that were used in the present study, which was based on nuclear microsatellite variations (Shimma and Tadano, 2019) in which the 42.4% (28/66) $F_{ST}$ values between pairs of lines were not significant.

In the present study, most individuals of different laying-type quail lines shared a single mtDNA haplotype. In contrast to relatively high nuclear microsatellite diversity reported in a previous study (Shimma and Tadano, 2019), mtDNA diversity of the laying-type lines is low. Genetic differentiation was not estimated in most pairwise comparisons of the laying-type quail lines. These observations are thought to be associated with the history that the laying-type lines were restored from a small number of individuals after World War II. In the future, it may be necessary to implement strategies for preventing loss of genetic diversity and/or introducing novel genetic diversity in the gene pool of laying-type lines.

Conflicts of Interest
The authors declare no conflict of interest.

References
Barilani M, Deregnaucourt S, Gallego S, Galli L, Mucci N, Piombo R, Puigcerver M, Rimondi S, Rodriguez-Teijeiro JD, Spanò S and Randi E. Detecting hybridization in wild (*Coturnix c. coturnix*) and domesticated (*Coturnix c. japonica*) quail populations. Biological Conservation, 126: 445–455. 2005.

Chazara O, Minvielle F, Roux D, Bed’hom B, Feve K, Coville JL, Kayang BB, Lumineau S, Vignal A, Boutin JM and Rognon X. Evidence for introgressive hybridization of wild common quail (*Coturnix coturnix*) by domesticated Japanese quail (*Coturnix japonica*) in France. Conservation Genetics, 11: 1051–1062. 2010.

Clement M, Snell Q, Walker P, Posada D and Crandall K. TCS: estimating gene genealogies. Proceedings of 16th International Parallel and Distributed Processing Symposium, 2: 184. 2002.

Excoffier L and Lischer HEL. ARLEQUIN suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources, 10: 564–567. 2010.

Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series, 41: 95–98. 1999.

Kumar S, Stecher G and Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular Biology and Evolution, 33: 1870–1874. 2016.

Leigh JW and Bryant D. POPART: full-feature software for haplotype network construction. Methods in Ecology and Evolution, 6: 1110–1116. 2015.

Nishibori M, Tsuzuki M, Hayashi T, Yamamoto Y and Yasue H. Complete nucleotide sequence of the *Coturnix chinensis* (blue-breasted quail) mitochondrial genome and a phylogenetic analysis with related species. Journal of Heredity, 93: 439-

| Table 3. Genetic differentiation index ($F_{ST}$) between pairs of commercial Japanese quail lines based on analysis of mitochondrial DNA control regions |
|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
| Line                | Farm 1-A              | Farm 1-B              | Farm 1-C              | Farm 2                | Farm 3                | Farm 4                | Farm 5-A              | Farm 5-B              | Farm 6                | Farm 7                | Farm 8                | Farm 9                | Meat-type              |
| Farm 1-A            | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     |
| Farm 1-B            | 0.0047 NS             | 0.0474 NS             | 0.0025 NS             | 0.0073 NS             | −                     | 0.0019 NS             | −                     | −                     | −                     | −                     | −                     | −                     | −                     |
| Farm 1-C            | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     |
| Farm 2              | −                     | 0.0118 NS             | 0.0174 NS             | 0.0025 NS             | −                     | 0.0019 NS             | −                     | −                     | −                     | −                     | −                     | −                     | −                     |
| Farm 3              | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     |
| Farm 4              | 0.0162 NS             | 0.0032 NS             | 0.0090 NS             | 0.0083 NS             | −                     | 0.0019 NS             | −                     | −                     | −                     | −                     | −                     | −                     | −                     |
| Farm 5-A            | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     |
| Farm 5-B            | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     |
| Farm 6              | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     |
| Farm 7              | 0.0029 NS             | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     |
| Farm 8              | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     |
| Farm 9              | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     | −                     |
| Meat-type           | 0.1097 NS             | 0.0038 NS             | 0.0001 NS             | 0.0001 NS             | 0.0070 NS             | 0.0001 NS             | 0.0001 NS             | 0.0001 NS             | 0.0001 NS             | 0.0001 NS             | 0.0001 NS             | 0.0001 NS             | 0.0001 NS             |

* $P<0.05$, ** $P<0.01$, *** $P<0.001$, NS Not significant.
Nunome M, Nakano M, Tadano R, Kawahara-Miki R, Kono T, Takahashi S, Kawashima T, Fujiwara A, Nirasawa K, Mizutani M and Matsuda Y. Genetic divergence in domestic Japanese quail inferred from mitochondrial DNA D-loop and microsatellite markers. PLoS ONE, 12: e0169978. 2017.

Randi E and Lucchini V. Organization and evolution of the mitochondrial DNA control region in the avian genus *Alectoris*. Journal of Molecular Evolution, 47: 449–462. 1998.

Sanchez-Donoso I, Huisman J, Echegaray J, Puigcerver M, Rodriguez-Teijeiro JD, Hailer F and Vilà C. Detecting slow introgression of invasive alleles in an extensively restocked game bird. Frontiers in Ecology and Evolution, 2: 1–17. 2014.

Shimma K and Tadano R. Genetic differentiation among commercial lines of laying-type Japanese quail. Journal of Poultry Science, 56: 12–19. 2019.

Tamura K and Nei M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution, 10: 512–526. 1993.

Wakasugi N. Japanese quail. In: Evolution of Domesticated Animals (Mason IL ed.). pp. 319–321. Longman Inc., New York. 1984.

Yamashina Y. Quail breeding in Japan. Journal of the Bombay Natural History Society, 58: 216–222. 1961.