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A comparative karyological study of the blue-breasted quail (*Coturnix chinensis*, Phasianidae) and California quail (*Callipepla californica*, Odontophoridae)

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Abstract. We conducted comparative chromosome painting and chromosome mapping with chicken DNA probes against the blue-breasted quail (*Coturnix chinensis,* CCH) and California quail (*Callipepla californica,* CCA), which are classified into the Old World quail and the New World quail, respectively. Each chicken probe of chromosomes 1–9 and Z painted a pair of chromosomes in the blue-breasted quail. In California quail, chicken chromosome 2 probe painted chromosomes 3 and 6, and chicken chromosome 4 probe painted chromosomes 4 and a pair of microchromosomes. Comparison of the cytogenetic maps of the two quail species with those of chicken and Japanese quail revealed that there are several intrachromosomal rearrangements, pericentric and/or paracentric inversions, in chromosomes 1, 2 and 4 between chicken and the Old World quail. In addition, a pericentric inversion was found in chromosome 8 between chicken and the three quail species. Ordering of the Z-linked DNA clones revealed the presence of multiple rearrangements in the Z chromosomes of the three quail species. Comparing these results with the molecular phylogeny of Galliformes species, it was also cytogenetically supported that the New World quail is classified into a different clade from the lineage containing chicken and the Old World quail.

Advances in molecular cytogenetics with fluorescence in situ hybridization (FISH) and flow sorting of chromosomes provided a breakthrough for comparative chromosome studies in avian species. Comparative chromosome painting, named ZOO-FISH, is a robust approach to compare genomes of distantly related species at the whole chromosome level (Wienberg and Stanyon, 1995). Recent chromosome painting studies in birds with chicken chromosome-specific DNA probes have demonstrated chromosome homologies and interchromosomal rearrangements between chicken and 13 avian species in six orders (Shetty et al., 1999; Schmid et al., 2000; Shibusawa et al., 2002). On the other hand, intrachromosomal rearrangements, which have been speculated by conventional chromosome banding analysis, cannot be clearly identified by chromosome painting. Comparative chromosome mapping of DNA clones is a useful method to detect intrachromosomal rearrangements (Shibusawa et al., 2001, 2002). Comparative chromosome mapping provides more detailed data on chromosomal homologies and rearrangements in combination with chromosome painting.

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Quails are divided phylogenetically into two groups, the Old World quail and the New World quail. The Old World quail belongs to the Phasianidae and distributes in the Palaearctic region (Europe, North Africa and Asia). In contrast, the New World quail classified as the Odontophoridae is restricted to North and South America (Sibley and Monroe, 1990). In the New World quail, comparative chromosome studies with conventional staining have been conducted for Bobwhite quail *(Colinus virginianus)*, California quail *(Callipepla californica)*, Gambell's quail *(Callipepla gambelii)* and the scaled quail *(Callipepla squamata)* (Shoffner, 1974). All of them have the acrocentric chromosome 2, which differs from the submetacentric chromosome 2 of the Old World quail, but detailed morphological analyses by chromosome banding have not been reported except for Bobwhite quail (Stock and Bunch, 1982). Molecular phylogenetic classification has also indicated that the New World quail is classified into a different clade from the Old World quail (Sibley and Ahlquist, 1990; Kimball et al., 1999). Recently we constructed a comparative cytogenetic map between chicken and Japanese quail *(Coturnix japonica)* with chicken genomic DNA and cDNA clones (Suzuki et al., 1999a; Shibusawa et al., 2001). In the study, we demonstrated that the chromosome homologies were highly conserved between the two species, and several intrachromosomal rearrangements were found by comparative chromosome mapping. However, no karyological analyses with molecular cytogenetic techniques have been made in other quail species.

Here, we performed comparative chromosome painting and chromosome mapping with chicken DNA probes in the blue-breasted quail (*Coturnix chinensis,* CCH) and California quail (*Callipepla californica,* CCA), which are classified into the Old World quail and the New World quail respectively. By comparing the results with the chicken-Japanese quail comparative map, we defined the conserved chromosome homologies and inter- and intrachromosomal rearrangements among the three quail species and between chicken and the three quail species. In addition, we discussed the process of karyotypic evolution in the Old World quail and the New World quail.

Materials and methods

Chromosome preparation and chromosome banding

Chromosome preparations of metaphase spreads were made with the cultured fibroblast cells derived from skin of an adult female California quail and 5–7-day female embryos of the blue-breasted quail. For G-banding, chromosome slides were treated with 0.025 % trypsin (Gibco BRL) at 4 °C for 3–4 min, and then stained with 3 % Giemsa solution. C-banded patterns were obtained by the CBG (C-bands using barium hydroxide and Giemsa) technique (Sumner, 1972). Replication R-banded chromosome slides were prepared for comparative chromosome mapping as described in Suzuki et al. (1999b).

DNA probes and fluorescence in situ hybridization (FISH)

Chicken chromosome-specific DNA probes of chromosomes 1–9 and Z were used for comparative chromosome painting (Griffin et al., 1999). Each probe was amplified by DOP-PCR and hybridized to the metaphase spreads of the two species (Carter et al., 1992). Forty-three macrochromosome-specific chicken cosmid DNA clones, which had been localized to chicken chromosomes in our previous study (Shibusawa et al., 2001), were used for comparative FISH mapping. For fine ordering of DNA clones and assignment of the breakpoints of the intrachromosomal rearrangements, the following chicken cDNA clones were used for comparative mapping: G22P1 (thyroid autoantigen 70 kD; Ku70 antigen), LDHB (lactate dehydrogenase B), SH2-containing tyrosine 5-phosphatase 1, EPHA3 (ephrin receptor EphA3) and TYR (tyrosinase) on chicken (*Gallus gallus*, GGA) chromosome 1, VIPR (vasoactive intestinal polypeptide receptor), NRP1 (neuropilin 1), EGFR (epidermal growth factor receptor), PRKDC (protein kinase, DNA-activated, catalytic polypeptide), LYN (Yamaguchi sarcoma viral-related oncogene homolog), IRX5 (*Iroquois* 5) and IRX12 (*Iroquois* 12) on GGA2, and VTG1 (vitellogenin gene coding for phosvitin) on GGA8 (Suzuki et al., 1999b; Kansaku et al., 2001; Ogura et al., 2001).

Non-R-banded and R-banded chromosome slides were used for chromosome painting and chromosome mapping, respectively (Suzuki et al., 1999b). The chromosome slides were hardened at 65 °C for 2 h, denatured at 68 °C in 70 % formamide, 2× SSC and then dehydrated in 70 % and 100 % ethanol at 4 °C for 5 min each. The DNA probes were labeled by nick translation with biotin-16-dUTP (Roche Diagnostics). The labeled DNA fragments were ethanol-precipitated with an equal volume and 0–10 times volume of sonicated chicken whole genomic DNA for chromosome paint and cosmid DNA clone respectively. The labeled probes were dissolved in 100 % formamide after ethanol precipitation, and denatured at 75 °C for 10 min. After prehybridization for 15–30 min 500 ng chicken chromosome paint or 250 ng cosmid DNA clone was put on the denatured slide and covered with parafilm. The slides were incubated for 2 days for the chromosome paint and overnight for the cosmid DNA clone. After hybridization, the slides were washed for 20 min in 50 % formamide, 2× SSC at 37 °C, and in 2× SSC and 1× SSC for 20 min each at room temperature. They were incubated with fluoresceinated avidin (FITC-avidin) (Roche Diagnostics) in 1 % BSA, 4× SSC for 1 h at 37 °C. The slides were washed with 4× SSC, 0.1 % Nonidet P-40 in 4× SSC, and 4× SSC for 10 min each on the shaker, and then stained with 0.50 μg/ml propidium iodide. Kodak Ektachrome ASA100 films were used for microphotography of chromosome mapping with DNA clones. The FISH images of chromosome painting were captured with 550CW-QFISH application program of Leica Microsystems Imaging Solutions Ltd. (Cambridge, UK) using a cooled CCD camera (MicroMAX 782Y, Princeton Instruments) mounted on Leica DMRA microscope.

For two-colored FISH, two cosmid DNA clones were labeled separately by nick translation with biotin-16-dUTP and digoxigenin-11-dUTP (Roche Diagnostics). After ethanol precipitation, the labeled probes were mixed and hybridized to chromosome slides. Biotin- and digoxigenin-labeled probes were visualized with FITC-avidin and anti-digoxigenin rhodamine antibodies (Roche Diagnostics), respectively.

The slides hybridized with cDNA fragments were incubated under coverslips with goat anti-biotin antibodies (Vector Laboratories) in 1 % BSA, 4× SSC for 1 h at 37 °C. The slides were washed with 4× SSC, 0.1 % Nonidet P-40 in 4× SSC, and 4× SSC for 5 min each and then stained with FITC-labeled donkey anti-goat IgG (Nordic Immunology) for 1 h at 37 °C. The slides were then washed and stained with propidium iodide.

Results

G- and C-banded karyotypes of the blue-breasted quail and California quail

The diploid chromosome numbers of the blue-breasted quail and California quail were determined as 2n = 78–80 and 2n = 80, respectively, in this study. The G-banded karyotype of the blue-breasted quail was much the same as that of Japanese quail except for the location of the centromere on chromosome 1 and the banding pattern of chromosome 2 (Fig. 1a) (Sasaki, 1981; Shibusawa et al., 2001). The C-banded chromosomes of the blue-breasted quail are also similar to that of Japanese quail (Fig. 1b) (Stock and Bunch, 1982; de la Sena and Nestor, 1991).

The karyotype of the California quail was considerably different from those of the Japanese quail and the blue-breasted quail (Fig. 2a). Chromosomes 2 and 3 were acrocentric and

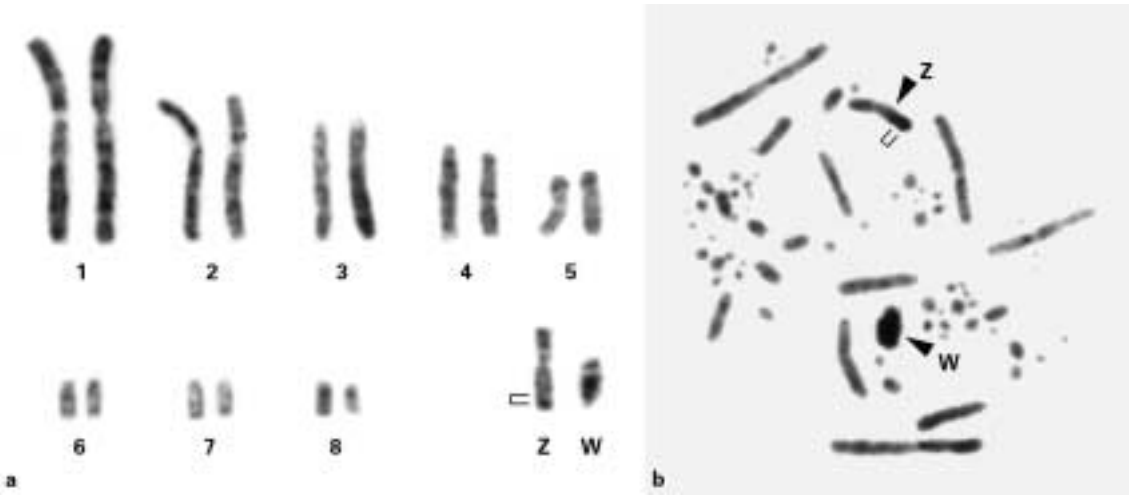


Fig. 1. (**a**) G-banded patterns of chromosomes 1–8 and ZW chromosomes and (**b**) C-banded metaphase spread of a female blue-breasted quail. The arrowheads indicate Z and W chromosomes. The brackets indicate the large G-negative band (**a**) and the large interstitial C-band (**b**) in the terminal region of the blue-breasted quail Z chromosome.

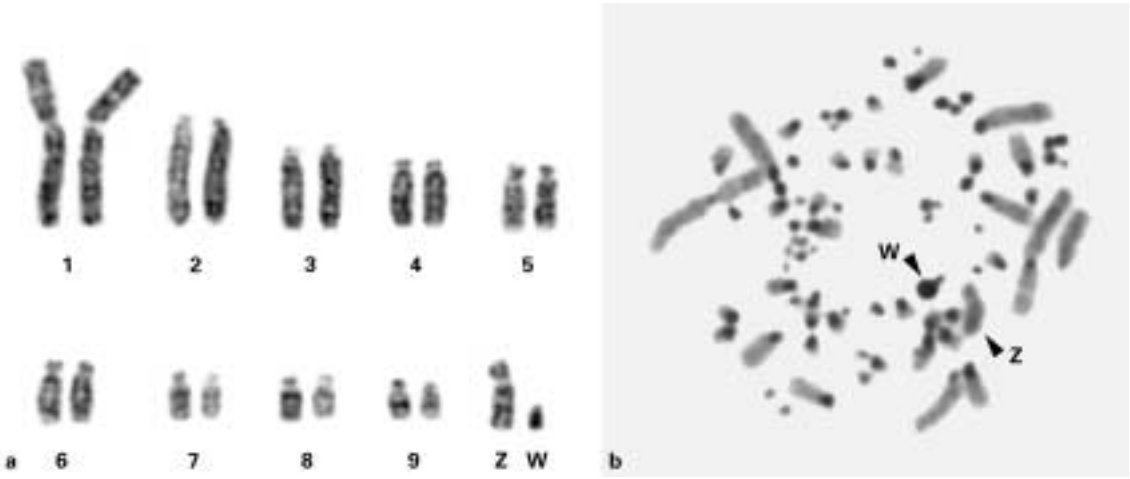


Fig. 2. (**a**) G-banded patterns of chromosomes 1–9 and ZW chromosomes and (**b**) C-banded metaphase spread of a female California quail. The arrowheads indicate Z and W chromosomes.

chromosomes 4–9 were subtelocentric in California quail, in contrast to chromosomes 3–8 being acrocentric in Japanese quail and the blue-breasted quail. It was difficult to discriminate between CCA5 and CCA6 and between CCA7 and CCA8 by G- and C-banding patterns (Fig. 2), and therefore chromosomes 5–8 were identified by comparative chromosome painting with chicken probes and ordered in sequence of the chicken chromosome number as described below. The Z chromosome of California quail was submetacentric and did not have the large interstitial C-band that corresponded to the large G-negative band on the terminal region of the q arm of the blue-breasted quail Z chromosome (Fig. 2b). The W chromosome was almost entirely heterochromatic and smaller than chromosome 9. Chromosomes smaller than chromosome 9 could not be identified individually in the two quails.

Analysis of interchromosomal rearrangements by comparative chromosome painting

Interchromosomal rearrangements were examined between chicken and the two quail species by comparative chromosome painting with chicken chromosome-specific DNA probes. Each chicken probe painted one pair of chromosomes in the blue-breasted quail (*Coturnix chinensis*, CCH). By contrast, two interchromosomal rearrangements were found between chicken and California quail (*Callipepla californica*, CCA). Each GGA1, 3, 5, 8, 9 and Z probe painted one pair of chromosomes

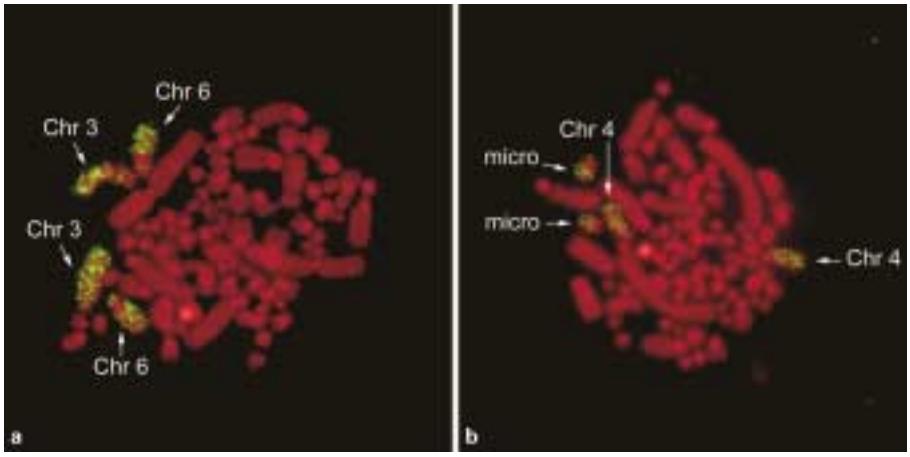


Fig. 3. Comparative chromosome painting in California quail with biotin-labeled chicken probes. (**a**) Chicken chromosome 2 probe paints chromosomes 3 and 6 of California quail. (**b**) Chicken chromosome 4 probe paints chromosome 4 and a pair of microchromosomes of California quail.

of California quail. GGA2 probe painted acrocentric CCA3 and subtelocentric CCA6 (Fig. 3a), and GGA4 probe painted CCA4 and a pair of microchromosomes (Fig. 3b). These results suggest that the p and q arms of GGA2 and GGA4 dissociated centromerically in California quail. GGAZ probe painted submetacentric CCAZ.

Comparison of cytogenetic maps between chicken and the three quail species

In order to identify intrachromosomal rearrangements, we constructed cytogenetic maps of the blue-breasted quail and California quail with 43 macrochromosome-specific chicken cosmid DNA clones, and compared them with those of chicken and Japanese quail (*Coturnix japonica*, CJA) constructed in our previous study (Shibusawa et al., 2001) (Fig. 4). The cDNA clones were mapped to the chromosomal regions, in which the presence of intrachromosomal rearrangements was presumed by G-band analysis, in CCH2 and 8, and CCA1, 3, 6 and 9. Two-colored FISH was applied to order the closely located DNA clones. Forty-two and 41 out of 43 chicken cosmid clones were mapped in the blue-breasted quail and California quail, respectively.

Eight cosmid clones, CCHOK#007, #055, #121-1, #125-4, #171, #179 and #222, and cDNA clones of four genes, *G22P1*, *LDHB*, SH2-containing tyrosine 5-phosphatase 1 and *EPHA3* were localized to CCH1 and CCA1. The order of these clones was the same as that of GGA1, therefore, no intrachromosomal rearrangements were found between these species, although a pericentric inversion has occurred between GGA1 and CJA1 (Shibusawa et al., 2001).

The locations of the DNA clones and their order on GGA2q corresponded to CCH2q and acrocentric CCA3, while the orders of the DNA clones on GGA2p was different from CCH2p. CCHOK#099 was located more distally in CCH2p than in GGA2p, and the order of CCHOK#099 and CCHOK#114 was reversed between CCH2p and GGA2p (Fig. 5). This result indicates the presence of a paracentric inversion in the region between CCHOK#087 and *EGFR*. The presence of a pericentric inversion has been reported in CJA2, therefore, the different types of inversions independently occurred in CCH2 and CJA2 after the two quail species diverged from the common ancestor. The subtelocentric CCA6 corresponded to GGA2p except for the location of *EGFR*. *EGFR* was located on the p arm across the centromere from CCHOK#099 on the q arm in CCA6, indicating the possibility that a pericentric inversion occurred between *EGFR* and CCHOK#099 in CCA6 after the p and q arms of GGA2 separated.

GGA4 probe painted the whole CCA4 and a pair of microchromosomes (Fig. 3b). The six cosmid clones, CCHOK#010, #014, #023, #104-1, #106-3 and #211, on GGA4p were mapped near the centromere on CCH4q, and localized to a pair of microchromosomes in California quail. It was confirmed by two-color FISH that all the cosmid clones were localized to the same microchromosome in California quail (data not shown). The locations and order of the six cosmid clones on CCH4 was the same as that of CJA4. The difference in the order in chromosome 4 between chicken and the two species of the Old World quail indicated that multiple rearrangements occurred between the two quail species and chicken after the event of centric fusion of acrocentric chromosome 4 and a microchromosome.

GGA8 probe painted acrocentric CCH8 and subtelocentric CCA9, while GGA8 was metacentric. CCHOK#029 and #103 on GGA8p and *VTG1* on GGA8q, were all localized to the q arms of acrocentric CCH8 and subtelocentric CCA9, and their order was the same as that of CJA8. This result revealed that metacentric GGA8 resulted from a pericentric inversion with the breakpoint between CCHOK#029 and *VTG1* on CJA8, CCH8 and CCA9. There was no difference in the order of the DNA clones on GGA3 and GGA5–7 from those on the chromosomes of the three quail species, CCH3 and CCH5–7, CJA3 and CJA5–7, and CCA2 and CCA5 and 7–8, respectively.

A remarkable morphological difference was found in the Z chromosomes between the blue-breasted quail and California quail. GGAZ, CJAZ and CCHZ were metacentric, in contrast, CCAZ was submetacentric. CCHOK#052 and #054 in the terminal regions of the p arms of GGAZ, CJAZ and CCHZ were

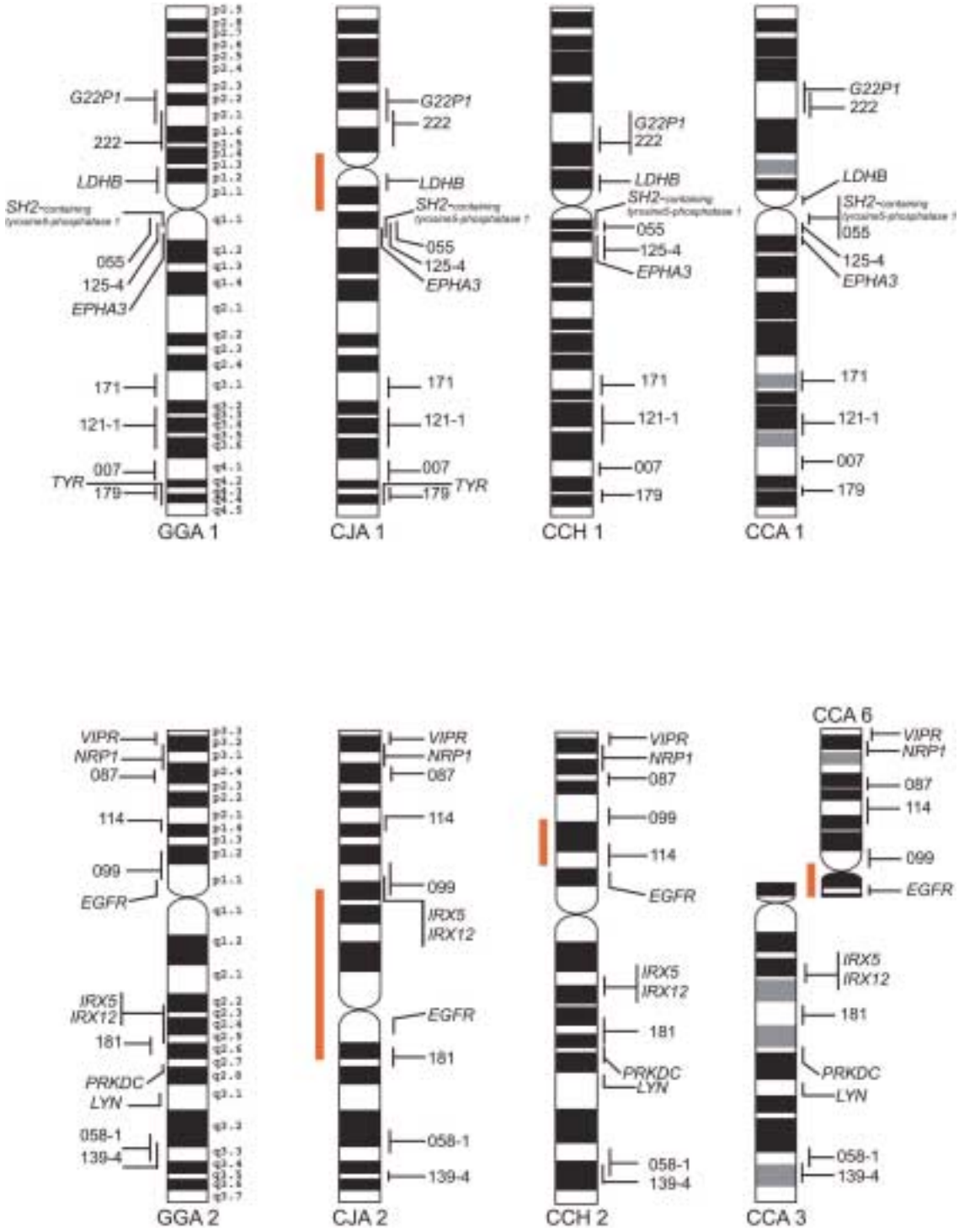
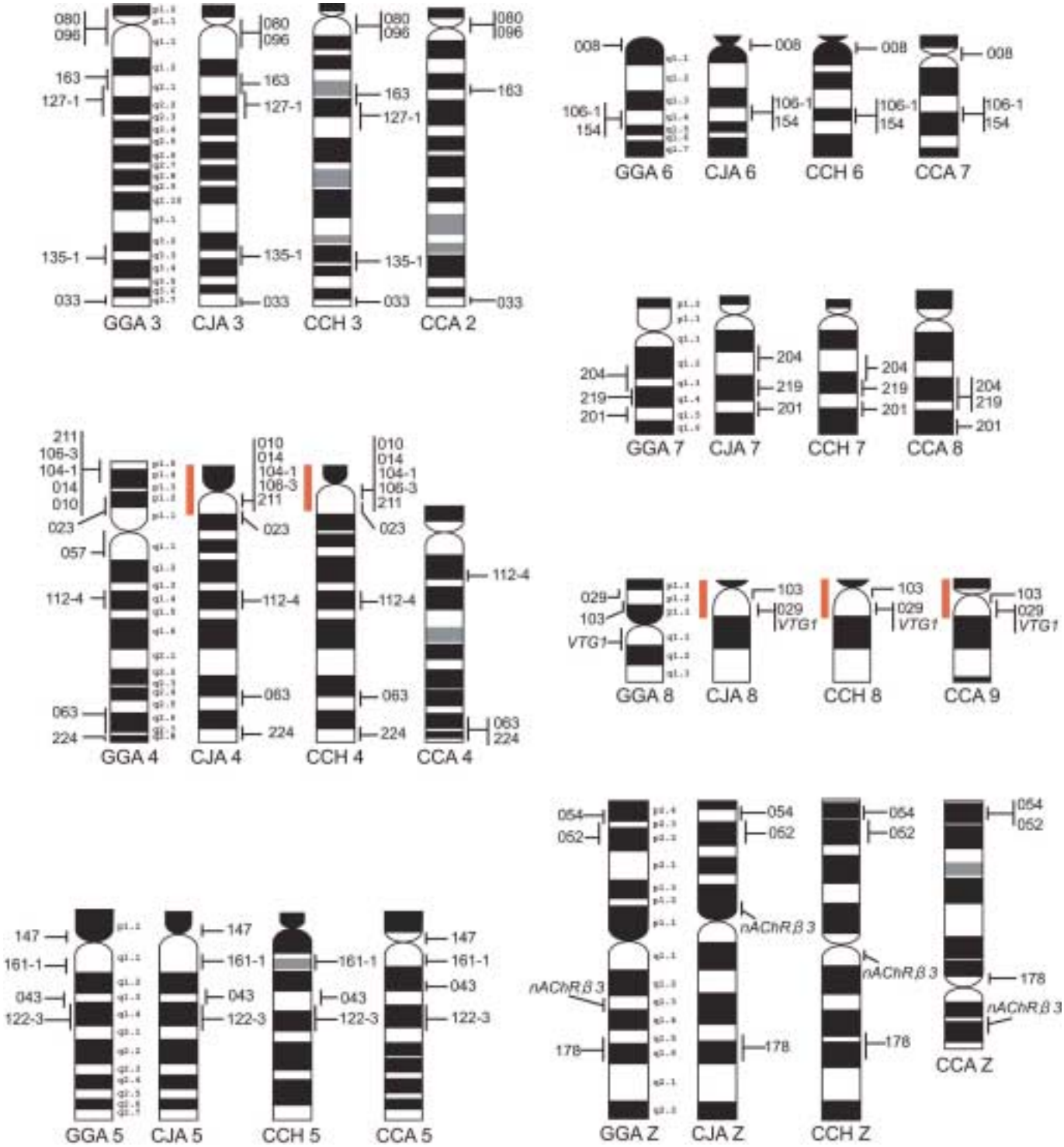


Fig. 4. Cytogenetic maps of the blue-breasted quail and California quail constructed with chicken cosmid DNA clones and cDNA clones. The chromosome maps are compared with those of chicken and Japanese quail reported in Shibusawa et al. (2001). The red bars on the left side of chromosomes indicate the regions where chromosomal rearrangements are defined between chicken and the three quail species by comparative mapping. GGA: chicken, CJA: Japanese quail, CCH: blue-breasted quail, and CCA: California quail.



localized to the q arm of submetacentric CCAZ. In addition, the large G-negative band on the q2.1 region of GGAZ was not present in CCAZ, indicating that the large G-negative q2.1 region was deleted in CCAZ or added in GGAZ, CJAZ and CCHZ after the two lineages diverged from the common ancestor. CCHOK#178 was located near the centromere of CCAZq, in contrast, the clone was localized to the interstitial regions on the q arms of GGAZ, CJAZ and CCHZ. The chicken BAC clone containing the *CHRNB3* (*nAChRβ3,* nicotinic acetylcholine receptor β3) gene, which was mapped to the interstitial

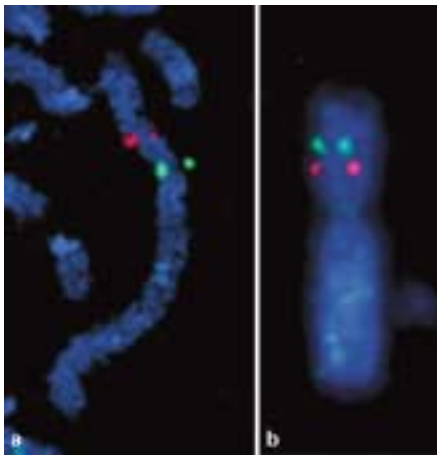


Fig. 5. Comparison of the order of DNA clones on the short arms of chromosomes 2 in (**a**) chicken and (**b**) blue-breasted quail. Green signals: biotin-labeled CCHOK#099, red signals: digoxigenin-labeled CCHOK#114.

region of GGAZq (Kuroda et al., 2001), was localized near the centromere on the q arm of CCHZ and to the p arm in CCAZ. Furthermore, this clone was located near the centromere on the p arm of CJAZ, indicating the locations of the *CHRNB3* gene being all different among the three species. These results suggest that paracentric or pericentric rearrangements independently occurred in the chromosomal region containing the *CHRNB3* gene and/or CCHOK#178 in the three quail species.

Discussion

A molecular cytogenetic approach with comparative chromosome painting and comparative mapping of DNA clones allows us to delineate chromosome homologies between phylogenetically distant species (Wienberg and Stanyon, 1995; De-Bry and Seldin, 1996; O'Brien et al., 1997; Serikawa et al. 1998). Chicken painting probes of chromosomes 1–9 and Z

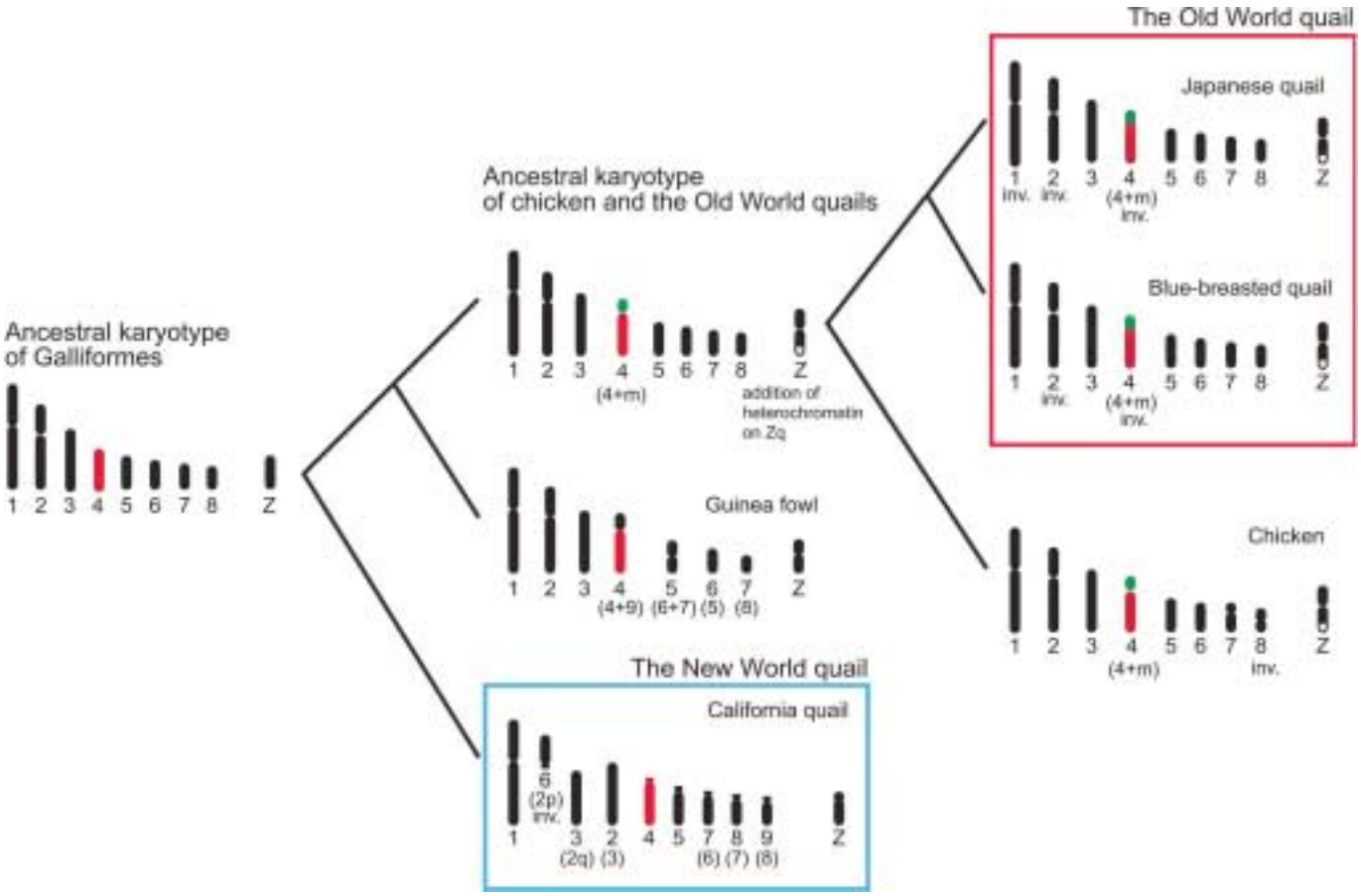


Fig. 6. Schematic representation of chromosomal changes in the macrochromosomes in chicken, Japanese quail, blue-breasted quail, California quail and guinea fowl. This scheme is described based on the cytogenetic maps constructed in the present study and Shibusawa et al. (2001, 2002). The ancestral karyotype that is presumed by Belterman and de Boer (1984) and us is demonstrated in the base of the phyletic tree. The ancestral chromosome 4 and a microchromosome that are centromerically fused in the Phasianidae species are shown in red and green, respectively. Chromosome numbers of the ancestral karyotype are shown in parenthesis under each chromosome. The chromosome numbers of California quail, which are ordered in sequence of the chicken chromosome number, are shown in parentheses. m: microchromosome, inv: inversion.

efficiently hybridized to chromosomes of the blue-breasted quail and California quail, and two interchromosomal rearrangements were identified between chicken and California quail. In addition, the comparative cytogenetic maps with the chicken cosmid and cDNA clones defined intrachromosomal rearrangements between chicken and the two quail species.

Stock and Bunch (1982) and Belterman and de Boer (1984) speculated the ancestral karyotype and the process of the karyotypic evolution in the Galliformes by comparing the Giemsa-stained or G-banded karyotypes of many species. With reference to their studies and our previous data on Japanese quail and guinea fowl *(Numida meleagris)* (Shibusawa et al., 2001, 2002), we examined the phyletic relationship and the process of karyotypic evolution in the five species, chicken, guinea fowl, the blue-breasted quail, Japanese quail and California quail. The scheme of the possible evolutionary changes of macrochromosomes represents that there are three lineages derived from the common ancestral karyotype; 1) California quail of the Odontophoridae, 2) guinea fowl of the Numidae, and 3) chicken and the two Old World quail species of the Phasianidae (Fig. 6). Four different types of interchromosomal rearrangements are found in the three lineages; 1) a centric fission of the ancestral submetacentric chromosome 2 in California quail, 2) a centric fusion between the ancestral acrocentric chromosome 4 and chromosome 9 in guinea fowl, 3) a centric fusion between acrocentric chromosome 4 and microchromosome in the ancestral karyotype of the three Phasianidae species, chicken, Japanese quail and the blue-breasted quail, and 4) a centric fusion between chromosomes 6 and 7 in guinea fowl. Several types of intrachromosomal rearrangements have also occurred independently in the three quail species; 1) a pericentric inversion in chromosome 6 in California quail, 2) pericentric inversions in chromosomes 1 and 2 in Japanese quail, 3) a paracentric inversion in chromosome 2p in the blue-breasted quail, and 4) multiple rearrangements including a pericentric inversion in Japanese quail and the blue-breasted quail. In addition, a pericentric inversion is observed in chromosome 8 of chicken. These rearrangements have occurred independently after the three lineages, the Phasianidae, Numidae and Odontophoridae, diverged from the common ancestor of the Galliformes.

The rearrangements on the Z chromosome are all different among the three lineages. Recently we defined that the Z chromosomes are acrocentric in the species of the two different families, plain chachalaca *(Ortalis vetula)* of the Cracidae and maleo *(Macrocephalon maleo)* of the Megapodiidae (Shibusawa et al., unpublished data), indicating that the acrocentric Z chromosome is the ancestral type of the Z chromosome in the Galliformes (Belterman and de Boer, 1984). Comparative mapping of the Z-linked DNA clones reveals that different types of inversions have occurred independently in the Odontophoridae, Numidae and Phasianidae after divergence from the lineage of the Cracidae and Megapodiidae. In the scheme of karyological evolution, the different morphology of the submetacentric Z chromosomes of California quail and guinea fowl has resulted from different types of pericentric inversions that occurred in the ancestral acrocentric Z chromosomes of the Cracidae and Megapodiidae. G-banded karyotypes and comparative mapping reveal that guinea fowl has the primitive type of the Phasianidae Z chromosomes, and that an addition of a large G-negative band to the terminal region of the p arm has yielded metacentric Z chromosomes of the three Phasianidae species, whose p arms correspond to the q arm of guinea fowl Z chromosome (Shibusawa et al., 2002). In California quail, rearrangement has independently occurred in the ancestral Z chromosome, and different orders of DNA clones on the Z chromosomes among the three Phasianidae species have also arisen from the rearrangements that independently occurred in the three species. The highly synteny conservation of X chromosomes across mammalian species, in which interchromosomal rearrangements with autosomes have been restricted, is attributable to a mechanism of gene dosage compensation via X chromosome inactivation obtained during mammalian evolution. However, interspecific comparative mapping on mouse and rat X chromosomes demonstrated that many intrachromosomal rearrangements have accumulated within the X chromosomes by frequent occurrence of chromosome inversions between the two species (Kuroiwa et al., 1998). There have been several findings supporting the presence of a bird-specific gene dosage compensation other than Z-inactivation, which was acquired after Z and W chromosomes differentiated from the ancestral pairs of autosomes in the carinates (McQueen et al., 2001; Kuroiwa et al., 2002). It is probable that this limitation of interchromosomal rearrangements between autosomes and the Z chromosome by gene-dosage compensation causes the accumulation of intrachromosomal rearrangements within Z chromosomes in these Galliformes species.

The comparison of the chromosome rearrangements and the molecular phylogeny allows us to speculate on the process of karyotype evolution. The phyletic relationships of karyotypes among the three families, Odontophoridae, Numidae and Phasianidae, and between the three families and the outer groups of families, Cracidae and Megapodiidae, are consistent with the molecular phylogenetic relationships determined by DNA-DNA hybridization (Sibley and Ahlquist, 1990). This consistency between the phyletic relationship of the karyotypes and the molecular phylogeny suggests that the comparative molecular cytogenetic approach is a useful tool for clarifying the process of the genome evolution in avian species.

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