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FISH on avian lampbrush chromosomes produces higher resolution gene mapping

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Abbreviations: BAC – bacterial artificial chromosome; CCO – *Coturnix coturnix japonica*; cM – centiMorgan, unit of genetic distance; FISH – fluorescence *in situ* hybridization; GGA – *Gallus gallus domesticus*; LBC – lampbrush chromosome; Mb – Megabase; PAC – P1-derived artificial chromosome; PCR – polymerase chain reaction

Abstract

Giant lampbrush chromosomes, which are characteristic of the diplotene stage of prophase I during avian oogenesis, represent a very promising system for precise physical gene mapping. We applied 35 chicken BAC and 4 PAC clones to both mitotic metaphase chromosomes and meiotic lampbrush chromosomes of chicken (*Gallus gallus domesticus*) and Japanese quail (*Coturnix coturnix japonica*). Fluorescence *in situ* hybridization (FISH) mapping on lampbrush chromosomes allowed us to distinguish closely located probes and revealed gene order more precisely. Our data extended the data earlier obtained using FISH to chicken and quail metaphase chromosomes 1–6 and Z. Extremely low levels of inter- and intra-chromosomal rearrangements in the chicken and Japanese quail were demonstrated again. Moreover, we did not confirm the presence of a pericentric inversion in Japanese quail chromosome 4 as compared to chicken chromosome 4. Twelve BAC clones specific for chicken chromosome 4p and 4q showed the same order in quail as in chicken when FISH was performed on lampbrush chromosomes. The centromeres of chicken and quail chromosomes 4 seem to have formed independently after centric fusion of ancestral chromosome 4 and a microchromosome.

Introduction

The chicken (*Gallus gallus domesticus*, GGA) is a species of agricultural importance and a model species for developmental studies. Moreover, the chicken has a small genome. For these reasons many studies have focused on the chicken genome. The chicken gene map is now one of the most developed. The progress of the Chicken Genome Project (http://poultry.mph.msu.edu/) opens up possibilities for powerful gene mapping in other poultry species by fluorescence *in situ* hybridiza-

tion (FISH). Cross-species FISH also presents an opportunity for genome evolution studies. Comparative chromosome painting applied to chromosomes of avian species belonging to diverse orders (Shetty, Griffin & Graves, 1999; Schmid et al., 2000; Raudsepp et al., 2002; Shibusawa et al., 2002, 2004a, b; Guttenbach et al., 2003; Kasai et al., 2003; Derjusheva et al., 2004; Itoh & Arnold, 2005) shows high conservation of synteny. More detailed analysis indicates that this conservation is mainly due to a low number of interchromosomal rearrangements (Crooijmans et al., 2001; International Chicken Genome Sequencing Consortium, 2004). The number of intrachromosomal rearrangements, however, is much higher and their frequency (number of rearrangements/million years) seems to be similar to that in other species. The comparison of FISH patterns for a set of defined DNA probes, such as BAC, PAC, cosmid or cDNA clones, on homologous chromosomes in different species allows us to detect inversions that have accompanied genome evolution in birds.

The Japanese quail (*Coturnix coturnix japonica*, CCO) is an agricultural species of nutritional importance because quail proteins exhibit low allergenicity. Chromosomal homologies between chicken and quail have recently been shown using comparative gene mapping (Shibusawa et al., 2001; Schmid et al., 2005) and chromosome painting (Guttenbach et al., 2003).

It is worth noting that the study of quantitative traits requires that their chromosomal loci be defined. As a rule, such studies are performed by FISH to mitotic metaphase chromosomes. However, in the typical avian karyotype the majority of chromosomes are minute and difficult to identify, although, the so-called microchromosomes are well known to be enriched with genes (Schmid et al., 2000; Andreozzi et al., 2001), precise physical mapping of the genes has not been possible on the microchromosomes. Even in the case of avian macrochromosomes, the largest of which are comparable in size to medium-sized mammalian chromosomes, it is not easy to distinguish closely located FISH signals.

At the diplotene stage of meiotic prophase I in oogenesis, avian chromosomes, both macro- and micro-, transform into the lampbrush form. The lampbrush chromosomes (LBCs) are extremely long, more than 30 times longer than the corresponding mitotic metaphase chromosomes. LBCs have a distinctive chromomere-loop structure. Lateral loops associated with each chromomere are sites of intensive RNA transcription. The chromomeres consist of compact chromatin on the chromosome axes. There are five times more chromomeres on the LBCs than G/R bands on corresponding metaphase chromosomes. Chromomeric pattern, being a constant feature, characterizes each LBC and does not depend on the individual or oocyte. Thus LBCs from growing avian oocytes provide a promising system for high-resolution physical mapping of genes. Macrochromosomes at the lampbrush stage were described in detail in chicken (Chelysheva et al., 1990), Japanese quail (Rodionov & Chechik, 2002) and chaffinch *Fringilla coelebs* (Saifitdinova et al., 2003). The correspondence between specific LBCs and mitotic metaphase chromosomes has recently been confirmed in chicken using chromosome painting (Derjusheva et al., 2003) and in chaffinch using comparative mapping of an interstitial repeat GS (Saifitdinova et al., 2003).

Avian LBCs, in particular chicken LBCs, have been successfully used for FISH mapping, but most of the mapped sequences belong to highly repetitive DNA (Solovei, Gaginskaya & Macgregor, 1994; Hori et al., 1996; Solovei et al., 1996, 1998; Ogawa et al., 1997; Itoh et al., 2001; Itoh & Mizuno, 2002; Saifitdinova et al., 2003). Here we have applied chicken BAC clones (Zoorob et al., 1996; Crooijmans et al., 2000) to LBCs of chicken and Japanese quail. This approach allowed us to define the gene order in both chicken and quail genomes more precisely. Our data clearly demonstrate that the difference in centromere position in chicken and quail chromosomes 4 is not the result of an inversion.

Materials and methods

Preparation of chromosomes

Chicken and Japanese quail LBCs were isolated manually from oocytes of 1.0–1.5 mm diameter according to standard techniques (Solovei et al., 1993, 1994). After overnight fixation in 70% ethanol, preparations were dehydrated in a 70–80–96% ethanol series and air-dried.

Mitotic metaphase chromosomes were prepared from embryonic fibroblasts of chicken and Japanese quail using standard laboratory procedures.

FISH

BAC and PAC clones containing certain microsatellite markers (Figure 1) were isolated from two libraries: (1) the Wageningen chicken BAC library, which was screened with microsatellite markers from the consensus linkage map using two-dimensional PCR (Crooijmans et al., 2000) and (2) the Villejuif PAC library (Zoorob et al., 1996), which was pre-screened for the presence of a $(CA)_n$ dinucleotide repeat by hybridization with a ^{32}P-labeled $(CA)_{12}$ oligonucleotide probe. Specific information regarding the bW BAC clones and the markers can be found on the chicken genome mapping site of Wageningen (http://www.zod.wau.nl/vf/). Chicken BACs and PACs labeled with biotin−16-dUTP or digoxigenin−11-dUTP (Roche) in a DOP-PCR reaction with 6 MW primer were used as probes for FISH mapping. The labeled probes were dissolved to a final concentration of 10–20 ng/μl in hybridization buffer (30–50% formamide, 2×SSC, 10% dextran sulphate) with 10- to 50-fold excess chicken Cot−1 DNA and/or salmon sperm DNA.

In the case of FISH to metaphase chromosomes, chromosome preparations were pre-treated with RNase A (100–200 μg/ml), pepsin (0.01% in 0.01 N HCl) and formaldehyde (1% in PBS, 50 mM $MgCl_2$) according to routine procedures. Chromosomes were denatured in 70% formamide/ 0.6× SSC at 70 °C for 2 min, dehydrated in ice-cold ethanol and air-dried before applying the probe. BAC probes were denatured and then pre-annealed at 37 °C for 2 h. Hybridization was performed at 37 °C for 24–48 h. After hybridization the slides were washed in two changes of 0.2 × SSC at 58–60 °C and two changes of 2 × SSC at 42 °C. Avidin-FITC (Vector Laboratories) was used to detect biotin-labeled probes. Antibody against digoxigenin conjugated with Cy3 (Jackson ImmunoResearch Laboratories) was used to detect digoxigenin-labeled probes. Chromosomes were counterstained with 4′,6-diamidino−2-phenylindole (DAPI).

The procedure was modified for BAC mapping on LBCs. LBC preparations were pre-treated with pepsin, formaldehyde and Triton-X100 as described elsewhere (Saifitdinova et al., 2003). Two variants of FISH were carried out: (1) for the DNA/DNA hybridization protocol, chromosomes were pre-treated with RNase A; and (2) for the DNA/ (DNA + RNA) hybridization protocol, RNase A treatment was omitted allowing us to reveal RNA transcripts. In both variants, the hybridization mix with the labeled probes was applied to slides; LBCs and probes were denatured together on the slide covered with a coverslip at 82 °C for 5 min. To colocalize two or more BACs on an LBC we combined two-color FISH and ReFISH in which identically labeled probes were applied to the same slide sequentially up to three times.

Japanese quail LBCs were also probed with biotinylated oligonucleotides CCOpos and CCOneg, which were designed according to the consensus sequence of a 41 bp tandem repeat isolated from the Japanese quail genome and described by Tanaka et al. (2000). Oligonucleotides were as follows:

CCOpos, 5′-biotin-ATGGGGCAGGAGCTG CTGTGGGGCAGATGT-3′
CCOneg, 5′-biotin-ACATCTGCCCCACAGC AGCTCCTGCCCCAT-3′.

The CCOpos and CCOneg oligonucleotides were mixed and dissolved in the hybridization buffer with 50-fold excess of salmon sperm DNA to a final concentration of 5 ng/μl. LBCs with the applied oligonucleotide probe were denatured as described above and then incubated overnight at room temperature. Biotin was detected with avidin-Cy3 (Jackson ImmunoResearch Laboratories).

Preparations were examined using a Leica DMRXA or Zeiss fluorescence microscope equipped with a black and white CCD camera and appropriate filters. QFISH (Leica) or Cytovision software (Applied Imaging) was used to capture and process color images.

Results and discussion

Thirty-five chicken BAC and four PAC clones were mapped by FISH to chicken LBCs and metaphase chromosomes. Maps of chicken metaphase and lampbrush chromosomes 1–6 and Z summarizing the FISH data are shown in

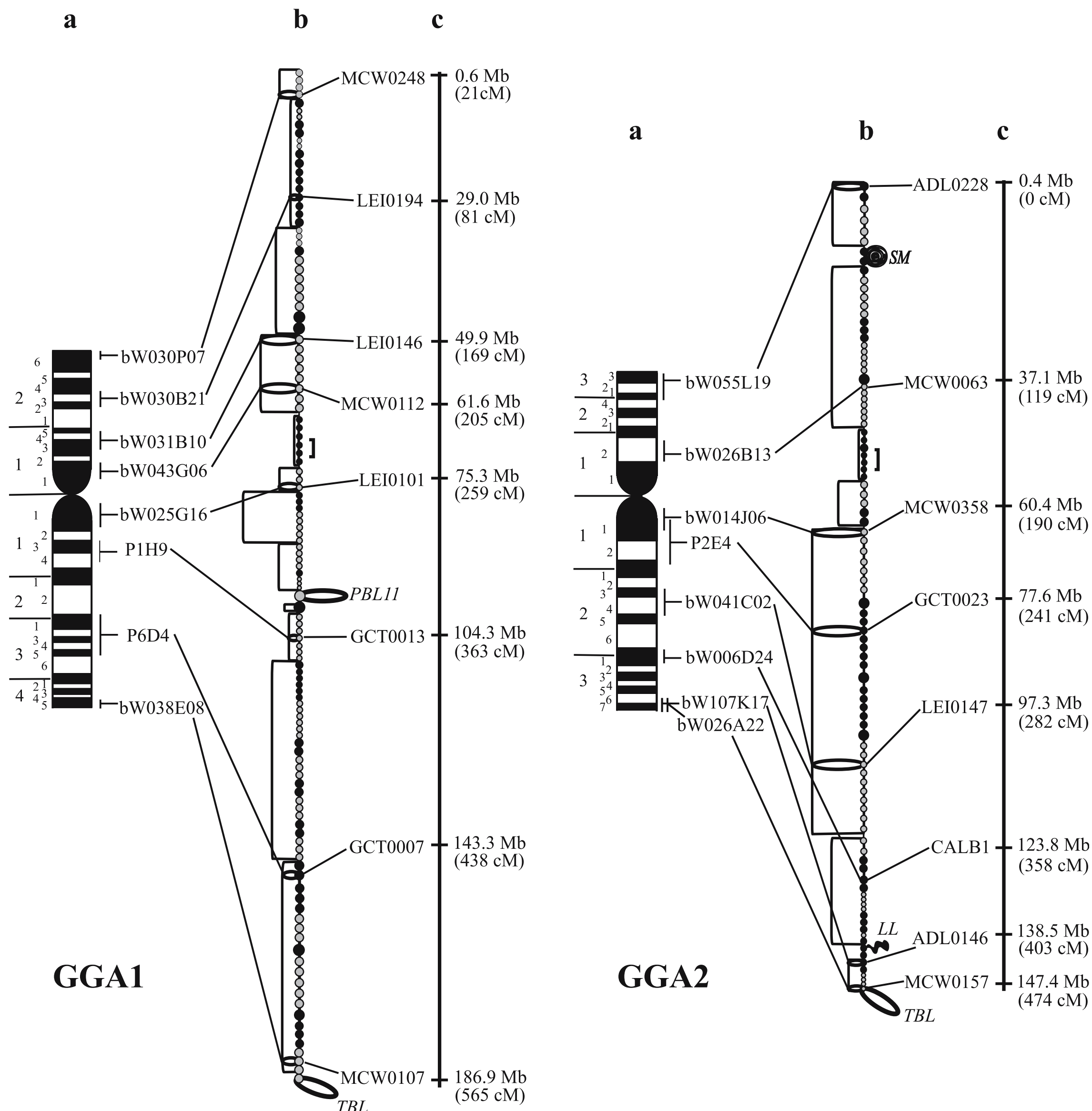

Figure 1. Localization of chicken BAC clones on chromosomes 1–6 and Z in chicken. (a) Idiograms of R-banded metaphase chromosomes. (b) Schemes of chromomeric-loop patterns of lampbrush chromosomes. Black and gray axial dots represent DAPI-stained chromomeres. Long brackets show the average loop lengths over each region. BAC-bearing loops are depicted. Brackets indicate putative centromeric regions; LL – non-transcribing lumpy loops; ML – marker loop; PBL11 – marker loop on LBC1, which is known to bind C-rich single-stranded nucleic acids (Solovei, Macgregor & Gaginskaya, 1995); SM – spaghetti marker (Solovei et al., 1992); TBL – telomere bow-like loops, which are known to contain Z chromosome macrosatellite DNA transcribed intensively during the lampbrush stage (Hori et al., 1996); TGL – non-transcribing terminal giant loops. (c) Positions of BAC-linked microsatellite markers in the chicken genome sequence assembly (Mb) and genetic map (cM). Curly brace indicates the region of LBCZ containing the Z chromosome macrosatellite; n/d – non-detected position in GGA DNA sequence assembly.

Figure 1. FISH mapping on LBCs is shown so that closely located sites of hybridization can be distinguished (Figures 1 and 2a, b).

The location of the BAC clones on LBCs showed the best correlation with the positions reported in the chicken DNA sequence database. A simple calculation (chromosome size in Mb divided by the number of chromomeres in the corresponding LBC) allows us to estimate the average amount of DNA per chromomere with

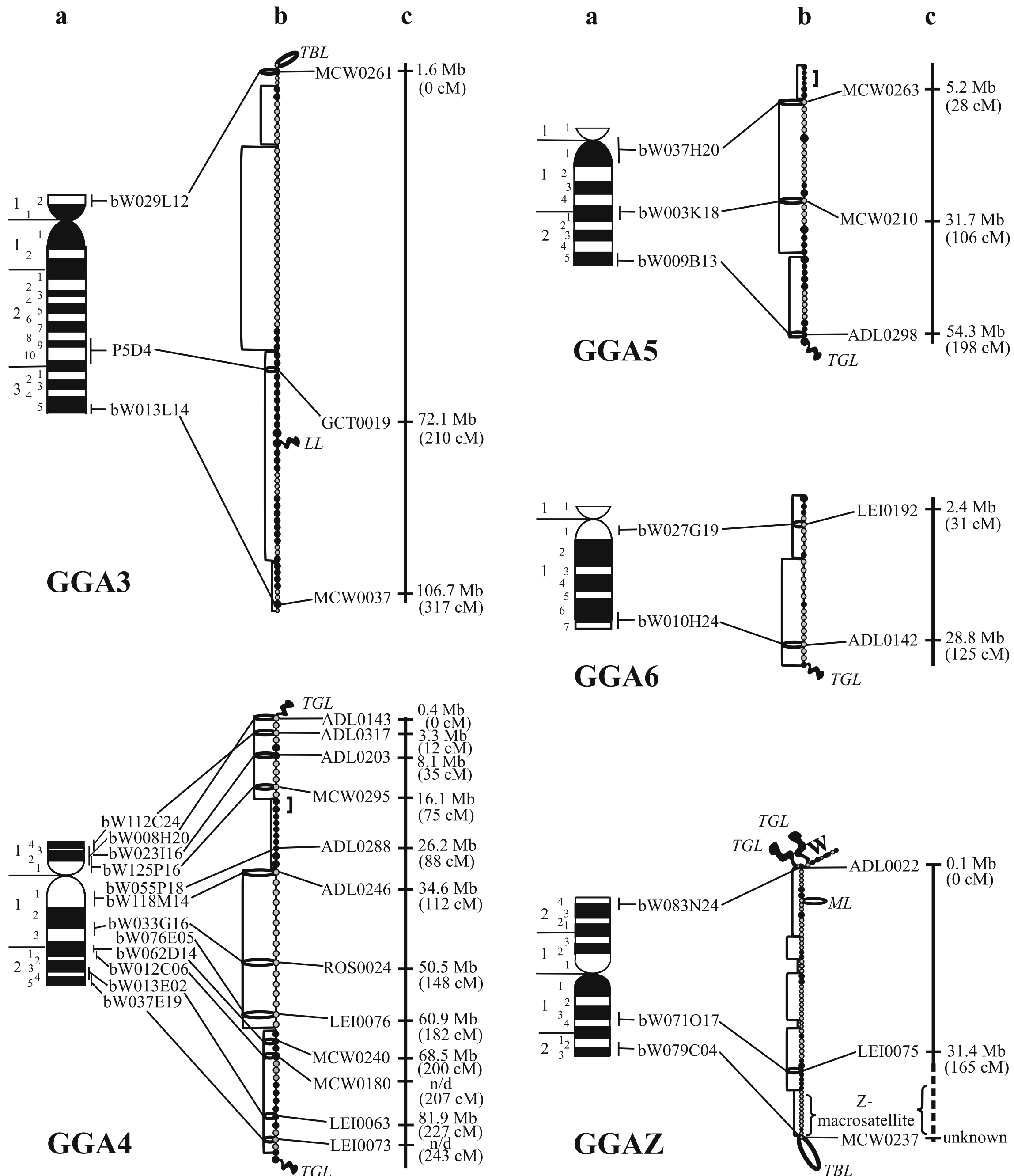

Figure 1. (Contd.)

extended lateral loops as 1.5–2 Mb. The average amount of DNA per chromomere multiplied by the number of chromomeres between the labeled sites corresponds to the distance between the genetic markers detected in the BACs (Figure 1). This makes it possible to predict the position of markers that have not yet been added to the current version of the chicken genome. Moreover, because LBCs are actively transcribing chromosomes on which individual transcription units can

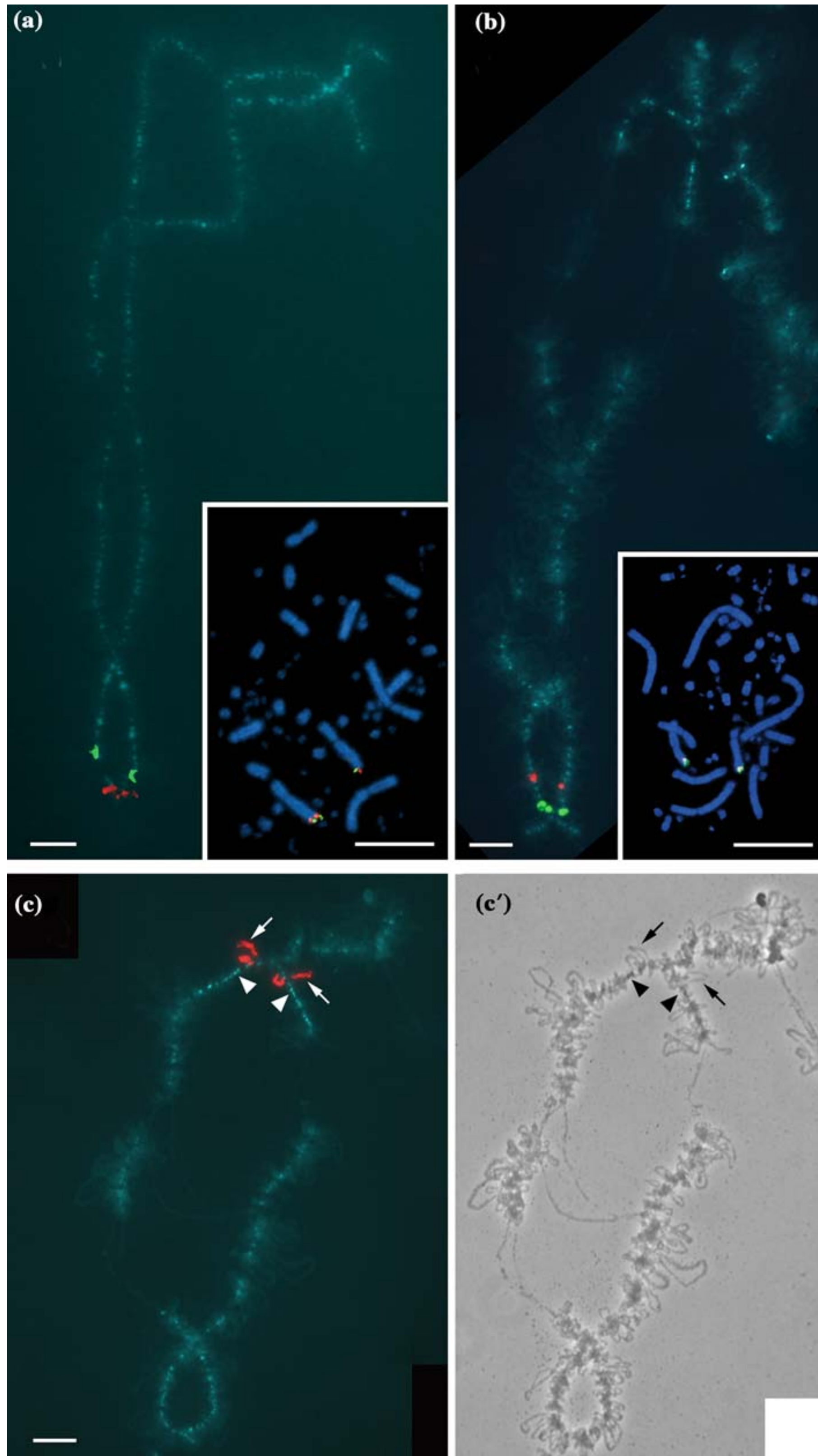
(a)
(b)
(c)
(c′)

Figure 2. Fluorescence *in situ* hybridization to chicken chromosomes at the lampbrush stage. (a) FISH mapping of two closely located BACs bW107K17 (green) and bW026A22 (red) using two-color FISH to LBC2 and metaphase spread (insert). (b) FISH mapping of two closely located BACs bW013E02 (red) and bW037E19 (green) using two-color FISH to LBC4 and metaphase spread (insert). On the LBCs, the fluorescent signals, which could not be distinguished on mitotic chromosomes, are separated by two chromomeres. (c) FISH with BAC bW125P16 (red signal) to LBC4; (c′) phase contrast image of the same chromosome. Only one of the two visible transcription units on each labeled lateral loop hybridizes with the BAC bW125P16 (arrows). Arrowheads indicate loopless chromomeres predicted to be the centromeric region on LBC4. Chromosomes are counterstained with DAPI. Scale bars = 10 μm.

be visualized under an ordinary light microscope (Figure 2c, c′), they can be regarded as a 1.5 Mb ''microarray''.

Our data appear to refute the suggestion recently made by Sazanov et al. (2005) on the basis of BAC mapping on chicken metaphase chromosomes, that the chicken chromosome 4 centromere is near the 16 Mb position in the GGA4 sequence assembly. Unfortunately, since centromeric repeats specific for chicken macrochromosomes are not available, we could not directly detect centromeric chromomeres on LBCs by FISH. Nevertheless, the centromeric region on LBC1 was predicted according to the pattern of interstitial telomeric sites (Rodionov et al., 2002). The centromere has been assigned to a loopless bar. One of the BACs we mapped on LBC4, is BAC bW125P16 containing microsatellite MCW0295, which is located at 16,085,269 on the sequence map of GGA4. If the centromere of chicken chromosome 4 were assigned to the 16 Mb position, than the centromeric region of chicken LBC4 would be located where there are small chromomeres with long transcribing loops – loops that hybridize with the BAC bW125P16 (Figure 2c, c′). It seems more probable that the loopless chromomeres at the 18–21 Mb position on LBC4 correspond to the centromeres (Figure 2c, c′), which agrees with the position of putative centromeric sequences in the GGA4 sequence assembly (www.ensembl.org).

The chicken BAC clones efficiently hybridized to quail mitotic and lampbrush chromosomes (Figure 3). FISH results showed no rearrangements in quail chromosomes 3 and 5 and conformed with the inversions described earlier on quail chromosomes 1 and 2 as compared to the homologous chicken chromosomes (Shibusawa et al., 2001, 2004a, b; Schmid et al., 2005). Previous data obtained using FISH to mitotic chromosomes suggested that chicken and quail chromosomes 4 differ by a pericentric inversion. This inversion was thought to have occurred after centric fusion of ancestral chromosome 4 and a microchromosome (Shibusawa et al., 2004a, b; Schmid et al., 2005). The comparative gene mapping on LBCs demonstrated absolutely identical gene order in chicken and Japanese quail chromosomes 4 (Figure 3a). Interestingly, both the order of the BAC positive loops on LBC4 and the distances between loops in Japanese quail are not different from those in chicken. This regularity was observed on all BAC clones mapped to the chicken and quail LBC4. It is worth noting that the two BAC clones bW055P18 and bW118M14 mapped at chicken 4q1.1 (Figure 1) hybridized to quail chromosome 4 (Figure 3a, b), in contrast to the cosmid clone used by Shibusawa et al. (2001), which was mapped at 4q1.1 in chicken and 1qter in Japanese quail.

The pattern of hybridization to chicken and quail chromosomes 4 requires special discussion. The discrepancy between the present data and the earlier reported data (Shibusawa et al., 2001, 2004a, b; Schmid et al., 2005) can be easily explained. Since the short arm of chicken chromosome 4 represents an ancestral microchromosome (Shibusawa et al., 2002; Guttenbach et al., 2003; Derjusheva et al., 2004; Itoh & Arnold, 2005), precise assignment of chicken 4p molecular markers by FISH to metaphase chromosomes seems to be a bit tricky. On the LBCs four BAC clones assigned to chicken 4p were mapped to lateral loops extending from four clearly separated chromomeres. However, on mitotic chromosomes three of these clones hybridized very close together and in an order (Figure 1) different from their position in the sequence database and location on LBC4.

The most intriguing observation is the FISH pattern of the BAC clones specific for the short arm of chicken chromosome 4. In Japanese quail the locations of the clones on the mitotic chromosome look quite different from their locations

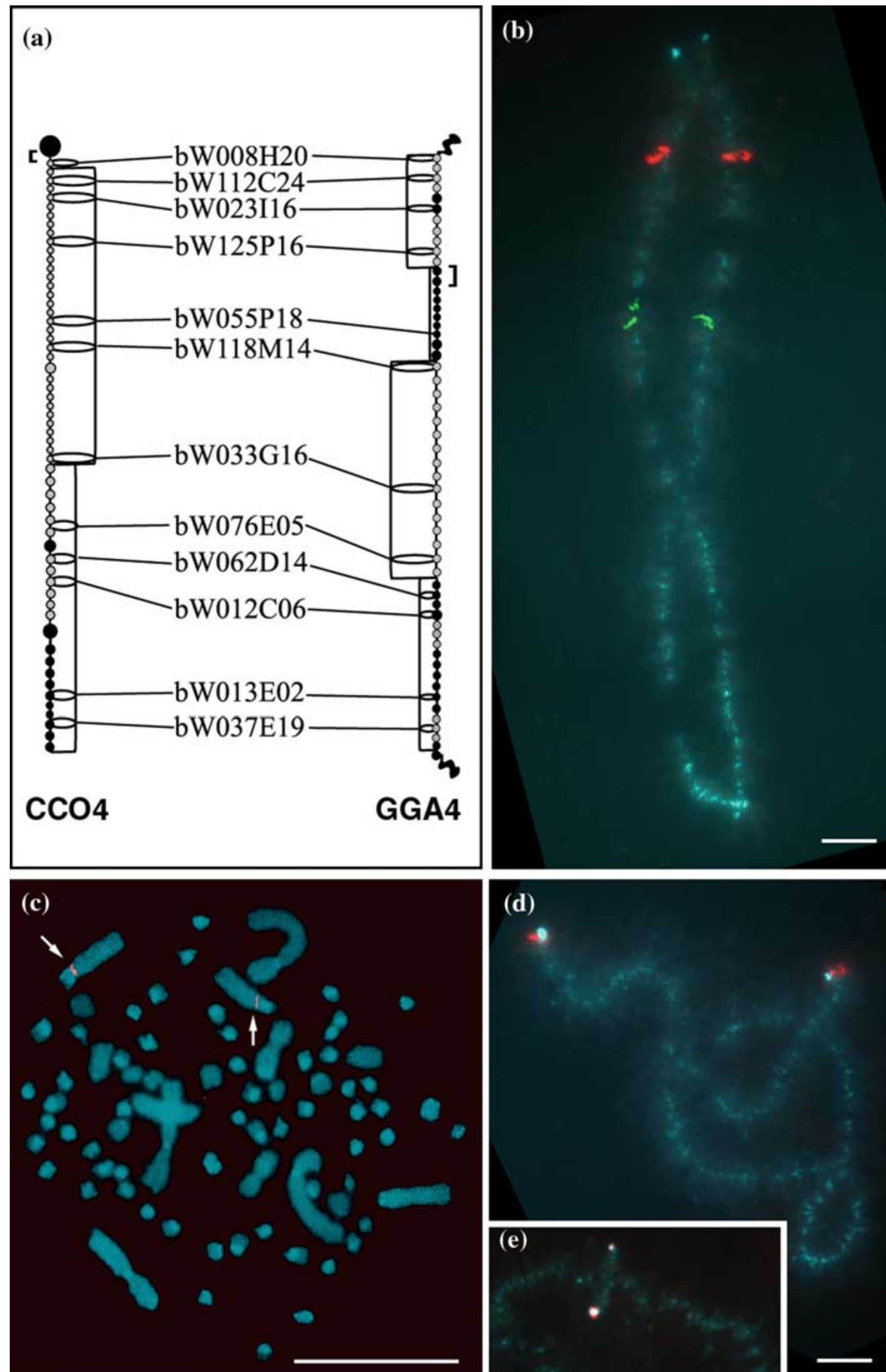

Figure 3. High-resolution FISH mapping of 12 chicken BAC clones on chromosome 4 of Japanese quail. (a) The order of the BAC clones in Japanese quail (left scheme, CCO4) is identical to that in chicken (right scheme, GGA4). All notations are the same as in Figure 1. Brackets indicate putative centromeric regions. (b) Two-color FISH with BACs bW125P16 (red) and bW118M14 (green) to quail LBC4. (c, d) FISH with BAC bW008H20 (red signal) to quail metaphase chromosomes (c) and LBC4 (d). (e) Fragment of quail LBC4 after FISH with biotinylated oligonucleotides CCOpos and CCOneg as a probe (red signals cover over the prominent terminal chromomeres). Chromosomes are counterstained with DAPI. Scale bars = 10 μm.

on the LBC. The clone bW008H20 is located near the primary constriction on the long arm of mitotic CCO 4 (Figure 3c), whereas on the LBC the signals are found at the lateral loops extending from the chromomere next to the terminal one (Figure 3d). How can we explain this discrepancy? The prominent terminal chromomeres near the bW008H20-positive loops intensively hybridized with the oligonucleotides CCOpos and CCOneg homologous to a species-specific 41 bp tandem repeat (Figure 3e) that was shown to concentrate in the centromeric heterochromatin of microchromosomes and on the short arm of chromosome 4 (Tanaka et al., 2000). It is interesting to note that the short arm of chromosome 4 in Japanese quail is variable and C-band positive, i.e. represents constitutive heterochromatin. The stable variants of the short arm of chromosome 4 are not the result of chromosome rearrangements during cell cultivation and correlate with the size of the corresponding C-block (de la Sena, Fechheimer & Nestor, 1991). As can be seen in Figure 3, the absolute size of the terminal chromomeres containing satellite DNA on quail LBC4 is variable and very similar to that of the short arm of mitotic CCO 4 at the metaphase stage. In the case of quail LBC4, it appears we have observed an example of unequal condensation/decondensation of chromosomal arms at the lampbrush stage. Earlier, this phenomenon was defined on sex chromosomes Z and W, which, in the lampbrush form, remain more condensed than other macrochromosomes (Solovei et al., 1993; Saifitdinova et al., 2003). Thus we can hypothesize that the terminal chromomere near the hybridization site of BAC clone bW008H20 corresponds to the heterochromatic short arm of quail chromosome 4. Therefore centromere position on quail LBC4 is different from chicken and can be predicted at the region between the terminal heterochromatic chromomere and the chromomere with bW008H20-positive loops, while the gene order remains identical (Figure 3a). Recently, the identical orientation of the six chicken BAC clones has been shown between chromosome 4 in the chicken and red-legged partridge (Kasai et al., 2003). The centromere position of chromosome 4 in the red-legged partridge just as in Japanese quail is different from that in the chicken. The centromeres seem to form *de novo* during karyotype evolution in Galliformes.

In conclusion, we have shown here that FISH to chromosomes at the lampbrush stage allows us to map genes more precisely than on mitotic chromosomes. At the same time learning the sites of genes on chicken LBCs provides an opportunity to understand the lampbrush phenomenon itself. Notwithstanding many years of exploration into lampbrush structure and function, the biological significance of these chromosomes remains uncertain and the spectrum of DNA sequences transcribing on the lampbrush loops is not yet known.

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