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Centromere positions in chicken and Japanese quail chromosomes: de novo centromere formation versus pericentric inversions

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Abstract Chicken (*Gallus gallus domesticus*, GGA) and Japanese quail (*Coturnix coturnix japonica*, CCO) karyotypes are very similar. They have identical chromosome number ($2n=78$) and show a high degree of synteny. Centromere positions on the majority of orthologous chromosomes are different in these two species. To explore the nature of this divergence, we used high-resolution comparative fluorescent in situ hybridization mapping on giant lampbrush chromosomes (LBCs) from growing oocytes. We applied 41 BAC clones specific for GGA1, 2, 3, 11, 12, 13, 14, and 15 to

chicken and quail LBCs. This approach allowed us to rule out a pericentric inversion earlier proposed to explain the difference between GGA1 and CCO1. In addition to a well-established large-scale pericentric inversion that discriminates GGA2 and CCO2, we identified another, smaller one in the large inverted region. For the first time, we described in detail inversions that distinguish GGA3 from CCO3 and GGA11 from CCO11. Despite the newly identified and confirmed inversions, our data suggest that, in chicken and Japanese quail, the difference in centromere positions is not mainly caused by pericentric inversions but is instead due to centromere repositioning events and the formation of new centromeres. We also consider the formation of short arms of quail microchromosomes by heterochromatin accumulation as a third scenario that could explain the discrepancy in centromeric indexes.

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Abbreviations

BAC	Bacterial artificial chromosome
CCO	Japanese quail (<i>Coturnix coturnix japonica</i>) chromosome
CENP-A	Centromere protein-A
CNM	Chicken erythrocyte nuclear membrane repeat
DAPI	4',6-Diamidino-2-phenylindole

DOP-PCR	Degenerate oligonucleotide primed polymerase chain reaction
ENC	Evolutionary new centromere
FISH	Fluorescence in situ hybridization
GGA	Chicken (<i>Gallus gallus domesticus</i>) chromosome
LBC	Lampbrush chromosome
Mb	Megabase

Introduction

The domestic chicken (*Gallus gallus domesticus*, GGA) and Japanese quail (*Coturnix coturnix japonica*, CCO), two representative species of the order Galliformes with a significant agricultural value, diverged about 35 million years ago (van Tuinen and Dyke 2004). These species have typical avian karyotypes that consist of several pairs of relatively large macrochromosomes (chromosomes 1–10 and a pair of sex chromosomes, ZW in females and ZZ in males) and numerous tiny microchromosomes. The total number of chromosomes is the same in both species ($2n=78$). Furthermore, a high degree of syntenic conservation has been demonstrated using different approaches, such as comparative gene mapping (Shibusawa et al. 2001; Schmid et al. 2005; Galkina et al. 2006; Kayang et al. 2006), chromosome painting (Schmid et al. 2000; Guttenbach et al. 2003), and linkage analysis (Kayang et al. 2006; Sasazaki et al. 2006). The conservation of both macro- and microchromosomes suggests the absence of interchromosomal rearrangements accompanying the divergence of the chicken and Japanese quail. At the same time, the morphology of orthologous chromosomes is different in these two species. The majority of chicken microchromosomes are acrocentric whereas quail microchromosomes are primarily submetacentric (Kaelbling and Fechheimer 1983; Calderón and Pigozzi 2006; Krasikova et al. 2006, 2009). Centromere positions on some macrochromosomes are also different (Ryttman and Tegelstrom 1981; Shibusawa et al. 2001, 2004). First attempts to explain the discrepancy were done using high-resolution G-banding of metaphase chromosomes (Ryttman and Tegelstrom 1981; Sasaki 1981; Stock and Bunch 1982). It is worth noting that the extremely small size of microchromosomes, the smallest one containing only 3.4 Mb of DNA (Pichugin et al.

2001), makes it impossible to perform differential staining of microchromosomes at the metaphase stage. The comparative analysis of macrochromosome banding patterns revealed mismatches of certain chromosomal blocks between chicken and quail chromosomes 1, 2, 4, 6, and Z. Based on these observations, pericentric inversions were proposed as the most apparent mechanism of intrachromosomal rearrangements that changes centromere positions (Ryttman and Tegelstrom 1981; Sasaki 1981; Stock and Bunch 1982).

A new wave of comparative cytogenetic analysis of chicken and quail karyotypes was initiated when several libraries of chicken genome cloned fragments such as those cloned in bacterial artificial chromosomes (BACs) became available (e.g., Crooijmans et al. 2000, reviewed in Schmid et al. 2005), and integrated resources for chicken genome assembly were released (<http://www.ncbi.nlm.nih.gov/genome/111>, http://useast.ensembl.org/Gallus_gallus/Info/Index, <http://genome.ucsc.edu>). Fluorescent in situ hybridization (FISH) of the chicken-specific probes on chicken and quail metaphase chromosomes first confirmed the predicted pericentric inversions on chromosomes 1, 2, and 4 (Shibusawa et al. 2001; Schmid et al. 2005; Kayang et al. 2006). However, precise FISH mapping is tricky in birds, even on macrochromosomes. In some cases, signals for two closely located probes cannot be resolved, or they can even show an order opposite to the known one according to genome sequence assembly (Galkina et al. 2006).

FISH on giant lampbrush chromosomes (LBCs) isolated from growing oocytes (for more information about LBCs, see reviews Callan 1986; Morgan 2002; Gaginskaya et al. 2009; this issue) offers opportunities for gene mapping at a much higher resolution than would ever be possible with metaphase chromosomes (Ogawa et al. 1997; Solovei et al. 1998; Galkina et al. 2006; Krasikova et al. 2006; Deryusheva et al. 2007; Solinhac et al. 2010; Zlotina et al. 2010). When chicken BAC probes were applied to quail LBCs, despite the differences in centromere positions, the gene order identified by FISH on CCO4 was the same as the gene order on GGA4 (Galkina et al. 2006). Thus, the high-resolution mapping did not confirm the pericentric inversion proposed earlier based on G-banding analysis and comparative FISH-mapping on metaphase chromosomes. What other chromosomal rearrangements that might have occurred during karyotype evolution in Galliformes could be resolved using

the FISH on lampbrush chromosomes as an investigative procedure? On the other hand, why should we still use time-consuming methods like comparative FISH mapping on LBCs when next-generation sequencing approaches are opening up new opportunities for genome-wide analysis? Even when deep sequencing analysis becomes a routine technique in many laboratories, sequence alignment in the regions with highly repetitive sequences is challenging and in many cases unreliable. Therefore, the regions of repetitive sequences are underrepresented in sequence assemblies even for well-annotated genomes. At the same time, some functionally important chromosomal domains, such as centromeres and telomeres, mostly consist of the repetitive DNA. LBCs provide a good system for high-resolution FISH mapping when well-resolved hybridization signals can be assigned precisely to particular chromomeres and landmarks on detailed cytological maps constructed for individual chromosomes (Galkina et al. 2006; Krasikova et al. 2006; Deryusheva et al. 2007; Solinhac et al. 2010; Zlotina et al. 2010). Another major advantage of using LBCs is the possibility of identifying centromere position regardless of whether centromere-specific sequences are known. Previously, we have shown that cohesin-enriched spherical structures detectable on LBCs by immunostaining with antibodies against core proteins of the cohesin complex are reliable markers for centromere positioning (Krasikova et al. 2006; Deryusheva et al. 2007; Zlotina et al. 2010). One can argue that immunostaining for cohesin-complex proteins that we use to determine centromere position on LBCs is not a canonical centromere identifier. Recent discovery of Centromere protein A (CENP-A)-associated sequences in chicken (Shang et al. 2010) provides an additional support for our centromere mapping approach. The sequences associated with a well-established centromere marker, CENP-A, are assigned to those positions on chicken LBCs that we identified as centromere regions according to cohesin-enriched granule mapping (Krasikova et al. 2012).

In the present work, we use a combination of centromere immunodetection and high-resolution comparative FISH mapping of chicken BAC clones on chicken and quail LBCs to study in detail intrachromosomal rearrangements that must have accompanied genomic divergence during speciation. We focus here on macrochromosomes 1, 2, and 3 and the largest microchromosomes 11, 12, 13, 14, and 15. Our new

and earlier obtained data suggest that pericentric inversions are not a predominant mechanism of chromosome morphology alteration in chicken and Japanese quail karyotype evolution. The differences in centromere positions on orthologous chromosomes in these two species are rather the result of new centromere formation and/or the accumulation of heterochromatin that forms prominent short arms of quail submetacentric microchromosomes.

Materials and methods

Chromosome preparation

Chicken and Japanese quail LBCs were isolated manually from oocytes of 0.5–1.5 mm diameter according to standard protocols (<http://projects.exeter.ac.uk/lampbrush/protocols.htm>). Preparations were dehydrated in 96 % ethanol and air-dried before FISH, whereas before immunostaining preparations were kept in 70 % ethanol.

Immunofluorescent staining

To detect centromere positions on chicken and quail LBCs, preparations were stained with rabbit polyclonal antibodies K828 or K854 against STAG2 and Rad21 cohesin subunits, correspondingly, as previously described (Krasikova et al. 2005). After image acquisition, slides were washed in 4× SSC, 0.1 % Tween 20 at 42 °C, dehydrated in 96 % ethanol, air-dried, and then used for FISH.

FISH

BAC clones from the Wageningen chicken BAC library (Crooijmans et al. 2000, <http://www.bioinformatics.nl/gbrowse/cgi-bin/gbrowse>) were selected based on the genomic positions assigned for corresponding markers. The selected BAC clones are listed in Table 1. Chicken whole chromosome painting probe F12 specific for an individual microchromosome pair (Griffin et al. 1999) and a chromosome 13 painting probe (Masabanda et al. 2004) were kindly provided by Dr. F. A. Habermann (Ludwig-Maximilians-University of Munich, Germany) and Prof. D. K. Griffin (School of Biosciences, University of Kent, Canterbury, UK), respectively. DNA of BAC clones and chromosome paints were

Table 1 BAC clones mapped to chicken and Japanese quail LBCs

BAC clone name	Genetic marker or GenBank accession number	Chicken chromosome location ^a , Mb
Chromosome 1		
WAG31B10	LEI0146	53.2
WAG13E20	GCT0049	61.1
WAG69C11	MCW0007	64.0
WAG43G6	MCW0112	65.1
WAG43N11	ADL0319	65.8
WAG67J15	LDHB	67.1
WAG53E23	LEI0071	75.8
WAG25G16	LEI0101	80.6
Chromosome 2		
WAG26B13	MCW0063	38.5
WAG12M4	LEI0089	47.9
WAG21J8	CZ567603	50.6
WAG18L21	CZ566365	52.1
WAG18G1	CZ566288	52.9
WAG21J10	MCW0062	55.1
WAG14J6	MCW0358	60.7
WAG40C19	MCW0039	63.2
WAG29F23	ADL0267	85.2
WAG41C2	LEI0147	98.7
Chromosome 3		
WAG23F21	CZ568632	0.4
WAG29L12	MCW0261	0.8
WAG35O13	CZ562990	2.1
WAG54M22	MCW0141	2.5
WAG44P17	CZ564604	5.4
WAG13D11	CZ566991	5.8
WAG21I22	CZ567594	7.5
WAG40J15	CZ564186	8.1
WAG32A13	CZ562529	12.5
Chromosome 11		
WAG35F15	LEI0143	0.5
WAG12F3	LEI0110	2.1
WAG52K20	ADL0123	4.9
Chromosome 12		
WAG7E23	CZ560506	1.8
WAG40H21	CZ564147	3.4
Chromosome 13		
WAG36M12	CZ563151	0.1
WAG59J23	CZ569278	1.1
WAG40H4	BH405315	13.6

Table 1 (continued)

BAC clone name	Genetic marker or GenBank accession number	Chicken chromosome location ^a , Mb
Chromosome 14		
WAG32F10	CZ562612	3.7
WAG19G22	CZ559843	12.8
WAG42M3	CZ567168	13.8
Chromosome 15		
WAG6D21	CZ560123	0.7
WAG114F13	CZ565238	10.2
WAG21C7	CZ567457	11.8

^a All positions are given according to a new draft genome assembly (*Gallus gallus*-4.0, build 3 version 1) released on 13 January 2012 (<http://www.ncbi.nlm.nih.gov>)

labeled with biotin-dUTP or digoxigenin-dUTP in a DOP-PCR using 6MW primer (Telenius et al. 1992). Oligonucleotide probes specific for chicken CNM (Matzke et al. 1990) and quail *Bg/II*-repeat (Tanaka et al. 2000) were also used for centromere detection, namely, CNMpos, CNMneg, and CCONeg oligonucleotides (Deryusheva et al. 2007) labeled with Cy3 or Cy5. The labeled probes were dissolved in a standard formamide hybridization buffer with an excess of salmon sperm DNA as described elsewhere (Galkina et al. 2006; Krasikova et al. 2006; Deryusheva et al. 2007).

FISH was carried out according to a DNA/(DNA+RNA) hybridization protocol without any pretreatments of chromosomes. LBCs and probes were denatured together on the slide covered with a coverslip at 82 °C for 5 min followed by hybridization at 37 °C (FISH with BAC probes) or at room temperature (FISH with oligonucleotide probes) in a humid chamber for 16–20 h. After hybridization with BACs, the slides were washed in two changes of 0.2×SSC and two changes of 2×SSC at 58–60 °C. Avidin–Alexa 488 (Invitrogen) and antibody against digoxigenin conjugated with Cy3 (Jackson ImmunoResearch Laboratories) were used to detect biotin- and digoxigenin-labeled probes, respectively. After FISH with oligonucleotide probes, the slides were washed in three changes of 2×SSC at 37 °C. All preparations were dehydrated, air-dried, and mounted in antifade solution containing 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI). To co-localize two or more BACs and DNA repeats on the same LBC, we combined two-color

FISH and re-hybridization of two sets of differentially labeled probes applied to the same slide sequentially.

Microscopy

Preparations were analyzed with a $\times 100$ objective on a Leica fluorescence microscope DM4000B equipped with a monochrome digital camera DFC350 FX and appropriate filter cubes. Fluorescent and phase contrast images were acquired with Leica CW 4000 FISH software; final figures were assembled and adjusted with Photoshop (Adobe Systems).

Results

For comparative gene mapping, we applied 41 chicken BAC probes to chicken and Japanese quail LBCs. Nearly 85 % of the probes hybridized to quail LBCs in our experiments. This efficiency of heterologous hybridization corresponds to that previously observed for FISH with large-size chicken probes on metaphase chromosomes from different galliform species (Shibusawa et al. 2001, 2002; Kasai et al. 2003; Schmid et al. 2005; Kayang et al. 2006; Griffin et al. 2008). The high-resolution comparative FISH mapping combined with fine detection of centromere positions on lampbrush chromosomes allows us to determine gene order relative to centromeres most precisely. We have analyzed chromosomal rearrangements in chicken and quail by taking the three largest macrochromosomes and five microchromosomes. Because of heterochromatin accumulation, quail microchromosomes are typically much longer than corresponding chicken orthologs; the heterochromatic arms additionally vary in length (Krasikova et al. 2009). This makes it difficult to number quail microchromosomes according to their relative size. For now, we identified and described five largest microchromosomes at the lampbrush stage in chicken and assigned chicken chromosome numbers to their quail orthologs.

Chromosome 1

Seven BAC clones (Table 1), covering the region from 53.2 to 80.6 Mb in the current GGA1 sequence assembly (build 3.1, <http://www.ncbi.nlm.nih.gov>), successfully hybridized on both chicken and quail

LBCs. The FISH signals showed exactly the same order on GGA1 and CCO1 (Figs. 1a and 2). At the same time, their positions relative to centromeres are different in the two species. In chicken, five BAC clones hybridized to the short arm and two BACs hybridized to the long arm, so that the centromere on GGA1 is flanked by WAG67J15 and WAG53E23. In Japanese quail, all seven BACs hybridized to the long arm only; the centromere is located next to WAG31B10. This means that centromere regions on GGA1 and CCO1 are surrounded by different genetic material. These results suggest that the centromere repositioning was not induced by a pericentric inversion, as earlier proposed to explain the difference between two species (Shibusawa et al. 2001; Schmid et al. 2005; Kayang et al. 2006).

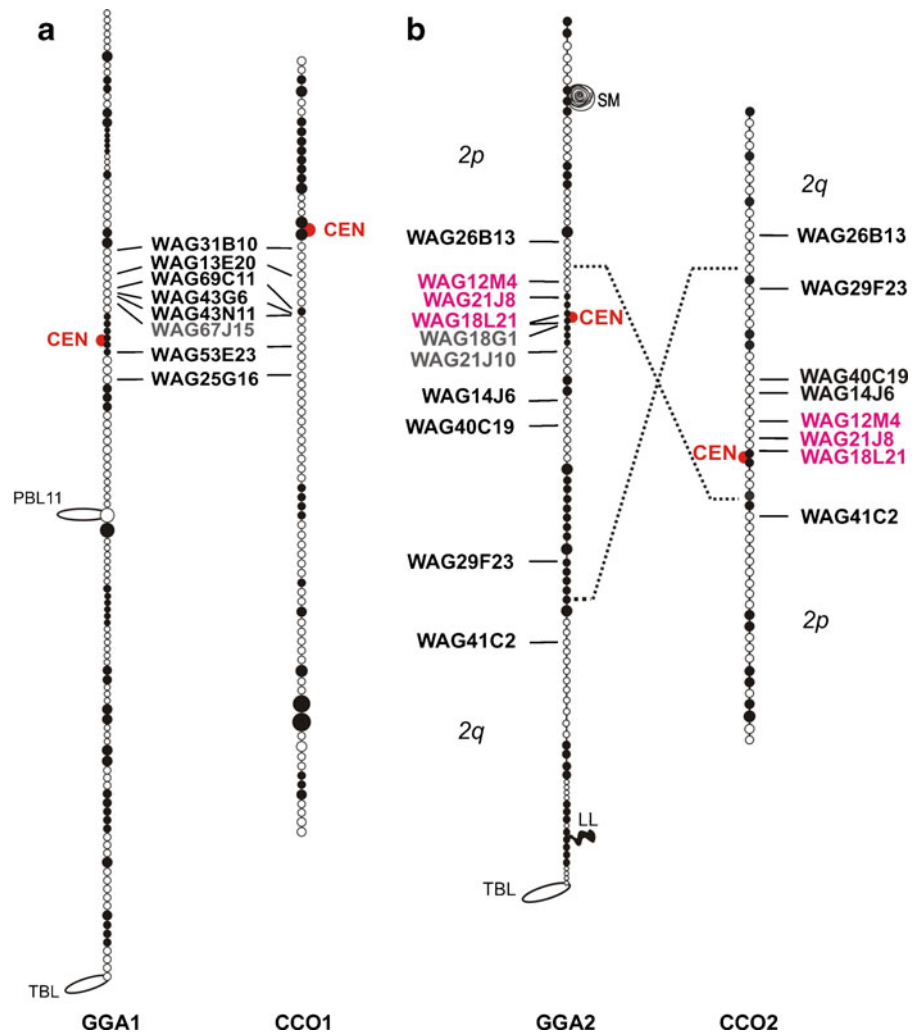
Chromosome 2

The existence of large-scale inversion that distinguishes chromosomes 2 in chicken and Japanese quail karyotypes has been repeatedly demonstrated by genetic and physical gene mapping (Shibusawa et al. 2001; Schmid et al. 2005; Kayang et al. 2006; Sasazaki et al. 2006). High-resolution FISH mapping on LBCs confirmed a true pericentric inversion in the case of chromosome 2 (Fig. 1b). Collectively, our data allowed us to narrow down breakpoints of the large-scale pericentric inversion to the following regions in the current GGA2 sequence assembly: One corresponds to a position between 38.5 Mb (WAG26B13) and 47.9 Mb (WAG12M4) and the other, to a position between 85.2 Mb (WAG29F23) and 97.0 Mb (a marker in gene *Spir-1* previously mapped by Sasazaki et al. 2006). Furthermore, detailed analysis of near-centromeric regions in GGA2 and CCO2 showed the centromeres to be located in the same genetic context in both species (Fig. 1b); BAC WAG18L21 seems to comprise pericentromeric sequences. Intriguingly, the pericentric BAC, WAG18L21, and two adjoined ones (WAG21J8 and WAG12M4) showed identical hybridization pattern in chicken and Japanese quail, as if this small region within the large-scale pericentric inversion was inverted again.

Chromosome 3

Comparison of linkage maps previously constructed for chicken and quail genomes has revealed a potentially

Fig. 1 Comparative mapping of chicken BAC clones to chicken (GGA) and Japanese quail (CCO) lampbrush chromosomes 1 (a) and 2 (b). Positions of BAC clones are assigned to cytological maps of chromomeric pattern of corresponding lampbrush chromosomes. DAPI-stained prominent chromomeres are indicated as *black axial dots*. The *red circles* indicate the centromeric cohesin-enriched granules (CEN). To stress the homology between GGA2p and the distal part of CCO2q as well as the distal part of GGA2q and CCO2p, quail LBC2 map is inverted. The *dashed lines* indicate the boundaries of a large-scale pericentric inversion between GGA2 and CCO2. BAC clones within the large inverted region that have identical order in both species are depicted with *magenta*. BAC clones hybridized only to chicken LBCs are depicted with *grey*. Cytological landmarks: *TBL* telomere bow-like loops, *PBL11* marker loop on chicken LBC1, *SM* spaghetti marker, *LL* lumpy loops



inverted region on chromosome 3 (Sasazaki et al. 2006). Comparative FISH mapping of nine BAC clones on chicken and quail LBCs allowed us to verify this inversion and to define its breakpoints precisely (Fig. 3). BAC clones WAG13D11, WAG21I22, WAG40J15, and WAG32A13 had an identical order in GGA3 and CCO3, while WAG23F21, WAG29L12, WAG35O13, WAG54M22, and WAG44P17 showed an inverted order in CCO3 as compared with GGA3 (Fig. 3c). WAG44P17 and WAG13D11 hybridized very close together on GGA3 but were separated by six chromomeres on CCO3 with WAG44P17 located terminally (Fig. 3). In chicken, WAG23F21 is a terminally hybridized probe on LBC3. Thus, the breakpoint of the inversion appears to localize in a very narrow region at position 5.4–5.8 Mb. It is worth noting that, in the current GGA3 sequence assembly, a gap exists at

position 5.6 Mb. This gap was previously shown to contain a CNM repeat cluster (Zlotina et al. 2010).

When we determined the inversion of centromere bearing region in chromosome 3, we decided to look closely at the sequences surrounding centromeres in GGA3 and CCO3. In chicken, the centromere position marked with a cohesin-enriched granule on LBC3 was assigned to position 2.4 Mb in the GGA3 sequence assembly (Zlotina et al. 2010). This position was shown to associate with WAG35O13- and WAG54M22-positive chromomeres. In the case of quail chromosome 3, centromere is located at the most terminal prominent chromomere (Krasikova et al. 2006, Fig. 3); the other flank centromere-adjacent chromomere hybridizes with BAC WAG44P17 whereas WAG35O13- and WAG54M22-positive region is two chromomeres away (Fig. 3). Thus, different genetic markers flank centromeres on

Fig. 2 High-resolution two-color FISH on chicken (**a, b**) and Japanese quail LBC1 (**a', b'**). FISH signals for chicken BAC clones WAG31B10 (*red*), WAG43G6 (*green*), WAG53E23 (*green*), and WAG25G16 (*red*) are shown on *top* of LBC phase contrast images and pointed out with *white arrows*. *White arrowheads* indicate centromere positions. Scale bars=10 μ m

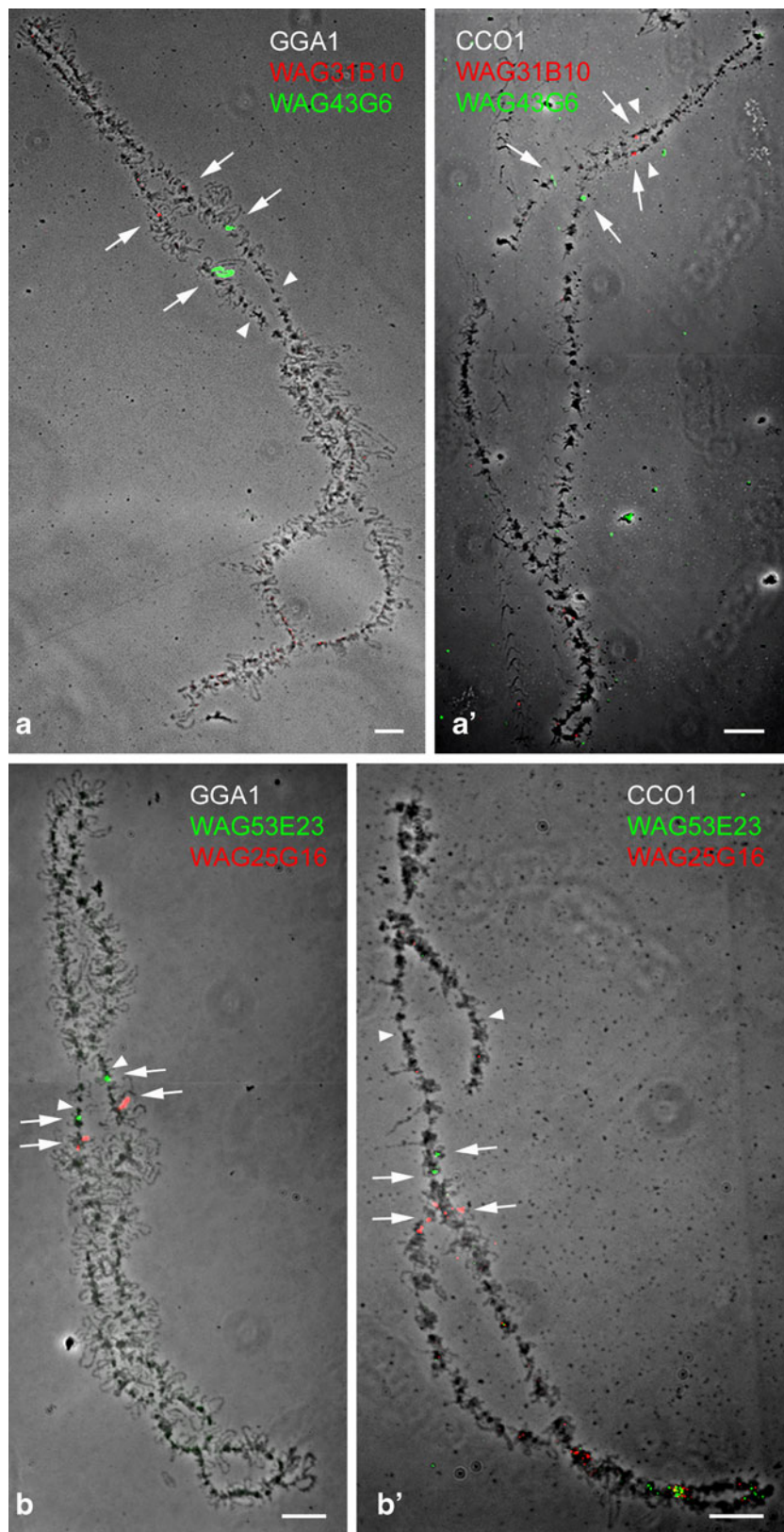
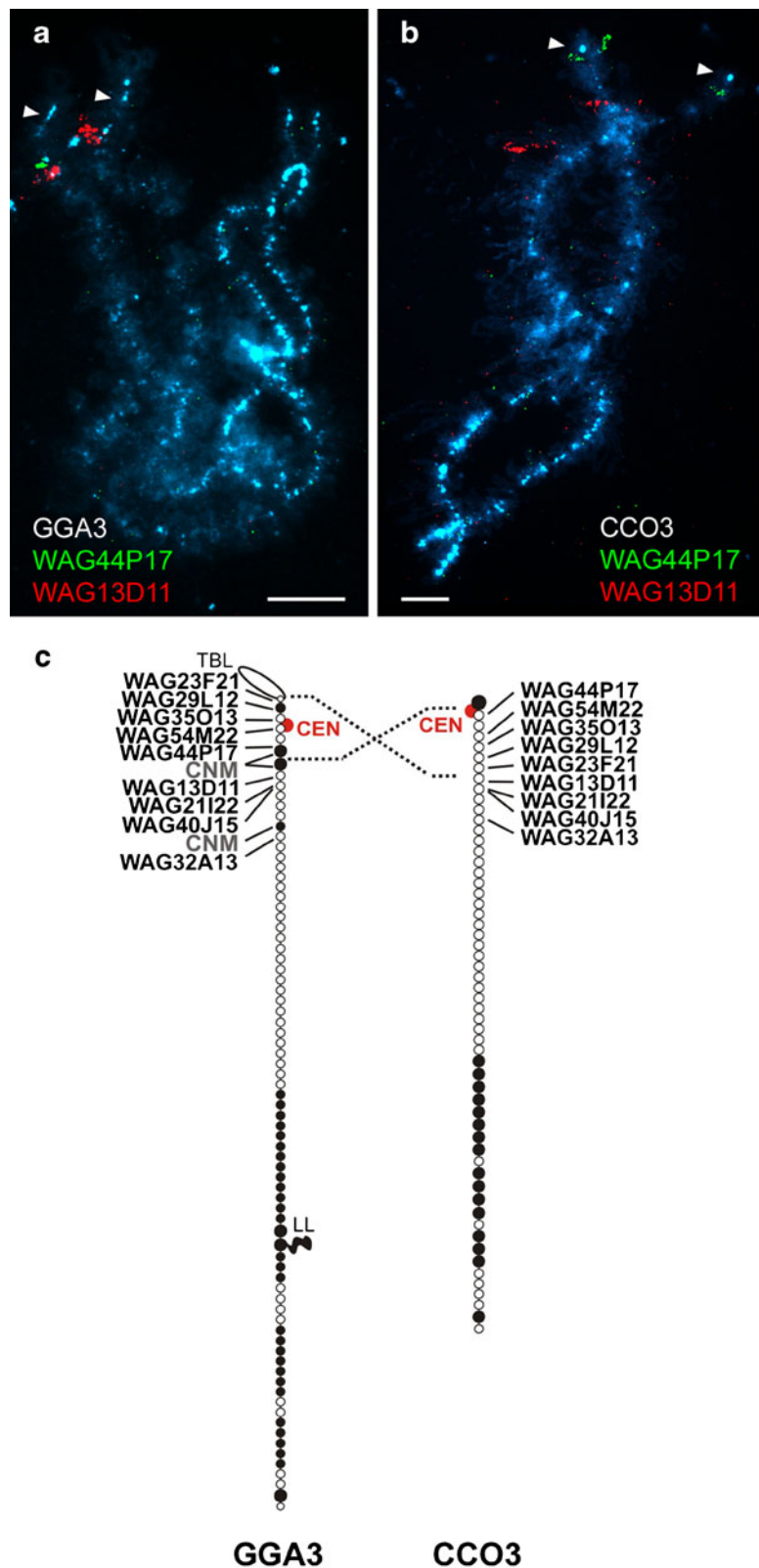


Fig. 3 High-resolution FISH mapping on chicken and Japanese quail LBC3. **(a, b)** Two-color FISH with BAC clones WAG44P17 (green) and WAG13D11 (red) to chicken **(a)** and quail LBC3 **(b)**. Chromosomes are counterstained with DAPI (blue). Arrowheads indicate centromere positions. Scale bars = 10 μ m. **c** Localization of chicken BAC clones and CNM-repeat clusters on cytological maps of chicken and quail LBC3. All indications are the same as in Fig. 1. Dashed lines depict the inversion between GGA3 and CCO3



GGA3 and CCO3. We suggest that the centromere repositioning to a different genetic environment cannot be explained by the inversion we detected.

Microchromosomes

It is worth noting that, except for chicken NOR-bearing chromosome 16 (Solinhas et al. 2010), none of chicken and quail microchromosomes at the lampbrush stage have been identified so far and studied in detail. The first essential step in this comparative mapping study was identification and morphological description of corresponding LBCs.

BAC clones specific for GGA11 hybridized to a single relatively large microbivalent (Fig. 4). Surprisingly, in chicken, it was the same LBC that was previously characterized as chromosome 10 (Krasikova et al. 2006) using painting probe F12 (Griffin et al. 1999). This chromosome has unique features: It is the only submetacentric microchromosome in chicken that additionally lacks microchromosomal centromere-specific CNM-repeat (Krasikova et al. 2006). To verify whether we are dealing with the same chromosome, we applied F12 paint and GGA11-specific BAC probes to LBCs simultaneously. They hybridized together on the same LBC (Fig. 4a). We assume F12 painting probe was misannotated because of difficulties with microchromosome

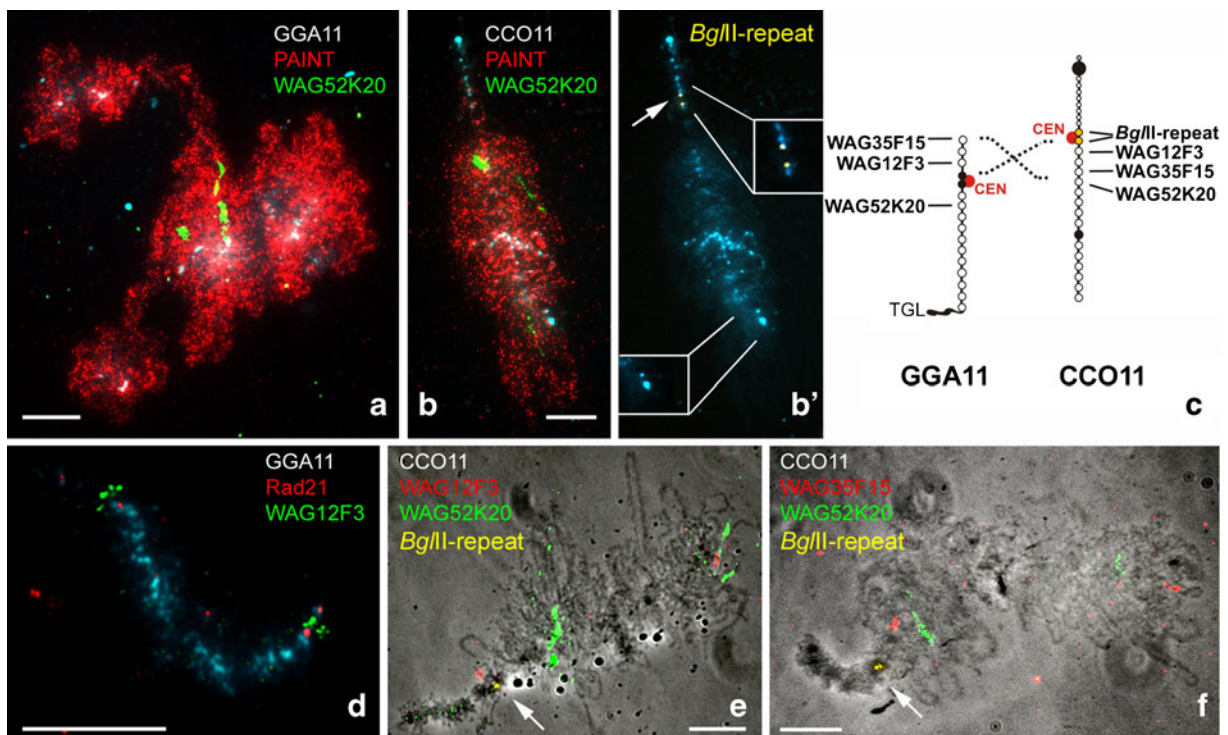


Fig. 4 Comparative cytogenetic analysis of chicken and Japanese quail chromosome 11 at the lampbrush stage. **a, b** Identification of chicken (**a**) and quail (**b**) LBC11 using FISH with chicken whole chromosome paint F12 (red) and BAC clone WAG52K20 (green). (**b'**) Re-FISH with a *Bg/III*-repeat probe (yellow) on the same quail chromosome shown in **b**. This quail chromosome represents asymmetric bivalent 11 with polymorphic heterochromatic short arms. One of the homologs is submetacentric with *Bg/III*-repeat cluster (arrow) in the centromeric region (insert on the right); the other homolog is acrocentric with *Bg/III*-repeat undetectable in centromeric region (insert on the left). **c, d, e, f** Centromere positioning on chicken and quail LBC11 relative to chicken molecular markers. **c** Cytological maps of chicken and quail LBC11 with chicken BAC clones

depicted. Submetacentric variant of CCO11 is shown as a map; yellow chromomeres indicate *Bg/III*-repeat-positive chromomeres. TGL terminal giant loop. The rest of indications are the same as in Fig. 1. **d** FISH with BAC WAG12F3 (green) after immunofluorescent detection of a centromeric granule (red) using antibodies against Rad21 on chicken LBC11. **e, f** FISH with BAC clones WAG12F3 (red), WAG52K20 (green), and WAG35F15 (red) followed by FISH mapping of centromere-specific *Bg/III*-repeat (yellow) to quail LBC11. White arrows point to centromeric *Bg/III*-repeat clusters. Chromosomes are counterstained with DAPI (**a, b, b', d**) or hybridization signals are shown on the corresponding phase contrast images (**e, f**). Scale bars=10 μm

identification. When we identified GGA11 and its quail ortholog (Fig. 4b), we constructed cytological maps for chicken and quail LBC11 (Fig. 4c). Though chicken and quail LBC11 are poor with landmarks, these working maps are useful for further high-resolution comparative gene mapping.

Here, we compared centromere positions relative to molecular markers in chicken and quail LBC11 (Fig. 4c, d, e, f); centromeres were mapped by immunostaining for centromeric cohesin-enriched granules and using FISH with a quail centromeric *Bg/II*-repeat probe. Though chromosome 11 is submetacentric both in chicken and quail, the nature of the short arm seems to be different in the two species. The short arm of GGA11 appears to be euchromatic: BACs WAG35F15 and WAG12F3 hybridized to the short arm. The short arm of CCO11 is formed by partially decondensed heterochromatin previously described to be typical of quail microchromosomes (Krasikova et al. 2009); all GGA11-specific BACs we used hybridized to the long arm of CCO11 (Fig. 4c, e, f). In the previous studies of quail LBCs (Deryusheva et al. 2007; Krasikova et al. 2009), we observed polymorphism in the length of the short arms of certain microchromosomes. In some extreme cases, one homolog in a bivalent was metacentric while the other, acrocentric with centromeric *Bg/II*-repeat being lost along with heterochromatic short arm. Now, we can identify this atypical chromosome pair as CCO11 (Fig. 4b').

The hybridization pattern of three BACs mapped on GGA11 and CCO11 suggests an inversion that involves the genetic material of the short arm of GGA11 (Fig. 4). Interestingly, a similar inversion was revealed between GGA11 and its turkey ortholog MGA13 (Dalloul et al. 2010; Zhang et al. 2011).

The next LBC we identified and analyzed in chicken and Japanese quail was chromosome 13, for which a well-characterized whole-chromosome paint (Masabanda et al. 2004) and BAC clones were available. Cytological maps of chicken and quail LBC13 with assigned BACs are shown in Fig. 5. In the current GGA13 sequence assembly, centromere position relative to molecular markers is ambiguous. Chicken LBC13 represents a typical acrocentric chromosome with a centromeric cohesin-enriched granule detectable near prominent terminal DAPI-positive chromomeres comprising a CNM repeat cluster. BAC clone WAG59J23 (1.1 Mb) hybridized very close to the centromeric granule (Fig. 5d) and the CNM-repeat cluster (Fig. 5e). Contrarily, on quail LBC13,

WAG59J23 was mapped to the most terminal chromomere on the long arm; centromere position was identified by FISH with a *Bg/II*-repeat probe specific for quail centromere (Fig. 5f). BAC clone WAG40H4 (13.6 Mb) hybridized as expected to the terminal region of chicken LBC13 whereas, in quail, this probe hybridized to the proximal region of the long arm, only few chromomeres away from centromere (Fig. 5g). Thus, the mapped BAC clones show an inverse order relative to centromere in chicken and quail chromosome 13; centromeres in GGA13 and CCO13 appear to be flanked by different genetic markers.

Similarly, two BAC clones from near-centromeric region of chicken chromosome 12 hybridized to quail LBC12 in an inverted order at the distal region of the long arm (Fig. 5a, b, c). This suggests that centromeres of GGA12 and CCO12 are in a different genetic context as in the case of chromosome 13.

Chromosome 14 is another example of microchromosomes whose morphology is different in chicken and quail. Working cytological maps of chicken and quail LBCs 14 are shown in Fig. 6c. Three BAC clones were mapped to chicken LBC14. Surprisingly, BAC clones WAG19G22 and WAG42M3, assigned to positions 12.8 and 13.8 Mb, respectively, in the current GGA14 sequence assembly, hybridized next to a CNM-positive chromomere, which corresponds to the position of centromere (Fig. 6a, c). BAC WAG32F10 (3.7 Mb) from the other end of GGA14 linkage group hybridized to the distal region of LBC14 (Fig. 6a, c). This hybridization pattern suggests that the actual gene order relative to centromere position in GGA14 is opposite to the annotation in the current chicken genome sequence assembly.

FISH with two BACs WAG19G22 and WAG32F10 to LBCs allowed us to identify quail ortholog of GGA14. These BACs hybridized to the long arm of CCO14 but in reverse order, as compare with GGA14 (Fig. 6b, c). Despite the detected intrachromosomal rearrangement, the difference in centromeric index between GGA14 and CCO14 is the result of heterochromatic short arm formation in quail.

The last chromosome we analyzed was microchromosome 15. Three markers from GGA15 were mapped to chicken LBC15. Similarly to GGA14, BAC WAG21C7 assigned to position 11.8 Mb in the current GGA15 sequence assembly hybridized to lateral loops at the CNM-positive centromeric

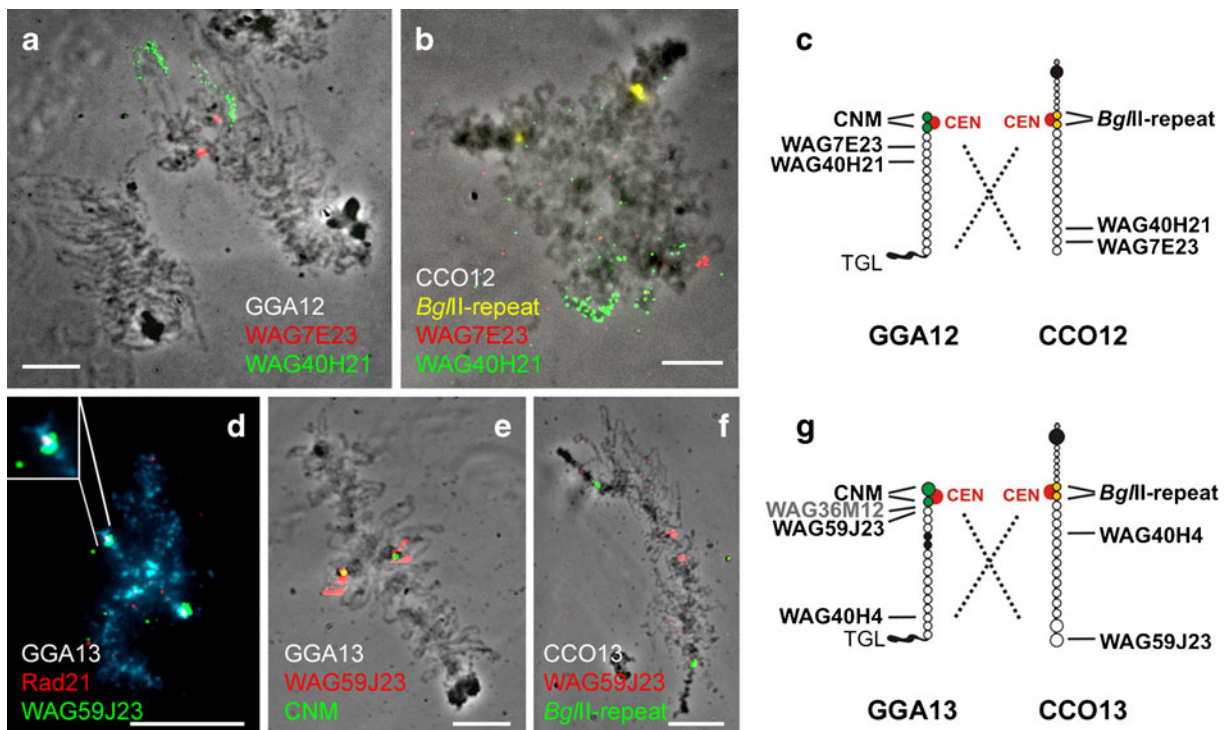


Fig. 5 Comparative cytogenetic analysis of chicken and Japanese quail chromosomes 12 and 13 at the lampbrush stage. **a, b, c** Comparative FISH mapping of chicken BAC clones WAG7E23 (red) and WAG40H21 (green) to chicken (**a**) and quail (**b**) LBC12. Centromere positions on quail LBC12 are detected by FISH with a *Bg/II*-repeat probe (yellow). **c** Localization of the mapped BAC clones is depicted on GGA12 and CCO12 cytological maps. **d, e, f, g** Comparative FISH mapping of chicken BAC clones to chicken and quail LBC13. **d** FISH with BAC clone WAG59J23 (green) to chicken LBC13 after immunofluorescent detection of a centromeric granule (red) using antibodies against Rad21. *Insert* shows enlarged centromeric region with fluorescent

signals. **e, f** FISH with BAC clone WAG59J23 (red) to chicken (**e**) and quail (**f**) LBC13 followed by FISH mapping of centromere-specific CNM (green) and *Bg/II*-repeat (green), respectively. **g** Localization of the mapped BAC clones is depicted on GGA13 and CCO13 cytological maps. CNM and *Bg/II*-repeat-bearing chromomeres are shown in green and yellow, respectively. *Dashed lines* indicate the inverted gene order in Japanese quail chromosomes as compared with chicken chromosomes. The rest of indications are the same as in Fig. 1. Chromosomes are counterstained with DAPI (**d**) or fluorescent signals are shown on the corresponding phase contrast images (**a, b, e, f**). Scale bars=10 μ m

chromomere (Fig. 6d), while BAC WAG6D21 (0.7 Mb) hybridized to the q terminus of LBC15 (Fig. 6f). Quite predictably, BAC WAG114F13 (10.2 Mb) hybridized to the proximal region of LBC15, between WAG6D21 and WAG21C7, in the close proximity to the latter one. Thus, similar to GGA14, gene order in GGA15 relative to centromere position is misannotated in the current chicken genome sequence assembly. Centromere positions determined on radiation hybrid maps of GGA14 and GGA15 (Alain Vignal, personal communication) also conform to our findings. Therefore, we suggest that gene order in the current GGA14 and GGA15 sequence assembly

should be flipped to make it consistent with other annotated chromosomes.

Only one out of three GGA15-specific BACs hybridized successfully to quail LBCs. This allowed us to identify quail ortholog of GGA15. Similar to FISH on GGA15, BAC WAG21C7 hybridized to the long arm of CCO15 close to centromere position, namely, at the chromomere adjacent to a centromeric *Bg/II*-repeat cluster (Fig. 6e, f). Thus, we assume that centromeres in chicken and quail chromosome 15 co-localize with similar genetic markers, and the difference in chromosome morphology between these two species is triggered by the formation of heterochromatic short arms, which is typical of quail microchromosomes.

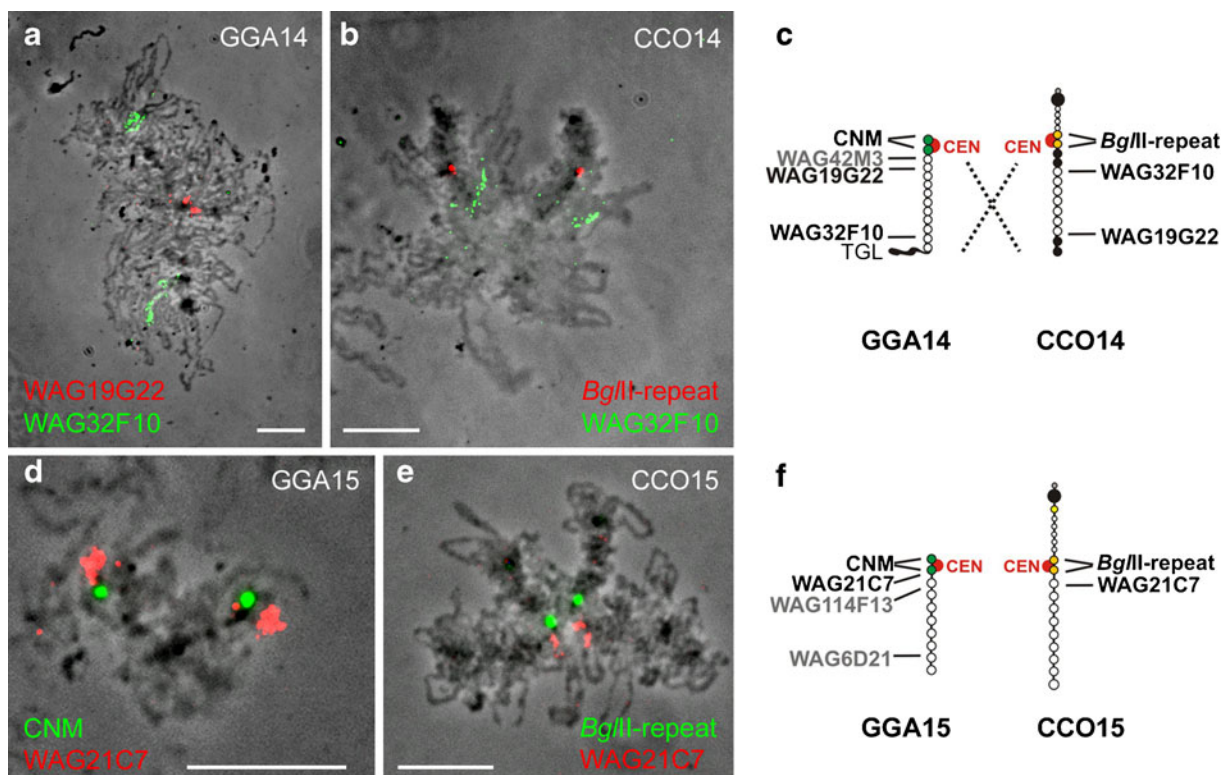


Fig. 6 Comparative cytogenetic analysis of chicken and Japanese quail chromosomes 14 (a, b, c) and 15 (d, e, f) at the lampbrush stage. **a** FISH with chicken BAC clones WAG19G22 (red) and WAG32F10 (green) to chicken LBC14. **b** FISH with BAC WAG32F10 (green) and a *Bgl*II-repeat probe (red) to quail LBC14. **c** Localization of the mapped BAC clones is depicted on GGA14 and CCO14 cytological maps. **d, e** Comparative

FISH mapping of BAC WAG21C7 (red) to chicken (d) and quail (e) LBC15 followed by FISH with CNM (green) or *Bgl*II-repeat (green) probes, respectively. **f** Localization of the mapped BAC clones is depicted on GGA15 and CCO15 cytological maps. All indications are the same as in Figs. 1 and 5. Hybridization signals are shown on the corresponding phase contrast images. Scale bars=10 μm

Discussion

Very high conservation of synteny demonstrated for the chicken and Japanese quail (Shibusawa et al. 2001; Guttenbach et al. 2003; Schmid et al. 2005; Kayang et al. 2006; Sasazaki et al. 2006) is quite typical of bird karyotypes (Derjusheva et al. 2004; Schmid et al. 2005; Griffin et al. 2007; Nanda et al. 2008). At the same time, the rate of intrachromosomal rearrangements that change gene order seems not to be low. We verified and extended previous data on chromosomal rearrangements between chicken and Japanese quail using high-resolution comparative FISH on LBCs. The main conclusion emerging from our observations is that pericentric inversions are not the major mechanism of chromosome morphology alteration in chicken and quail karyotypes. In fact, microchromosomes in quail must have become biarmed through

heterochromatin accumulation, which forms entire short arms, as compared with corresponding chicken acrocentric microchromosomes (Krasikova et al. 2009, this study). Centromere repositioning in chromosomes 1 (Fig. 1) and 4 (Galkina et al. 2006) has not been accompanied by gene reshuffling; centromeres of these chromosomes in chicken and Japanese quail appear to have formed de novo. Another example is chromosome 3 rearrangement. Although the chromosomal rearrangement between GGA3 and CCO3 we identified should be considered as a pericentric inversion, an independent event, namely centromere repositioning to a different genetic location, must have driven the change of chromosome morphology. De novo centromere formation has been earlier proposed for chromosome 4 in red-legged partridge (Kasai et al. 2003) and chromosome Z in Pekin duck as compare with chicken orthologs (Skinner et al. 2009).

The centromere repositioning during karyotype evolution, the so-called evolutionary new centromere (ENC) formation, is rather a common phenomenon. It has been repeatedly described in different taxonomic groups as diverged as mammals, birds, and plants (Kasai et al. 2003; Nagaki et al. 2004; O'Neill et al. 2004; Galkina et al. 2006; Han et al. 2009; Skinner et al. 2009; Rocchi et al. 2012). Though mechanisms of ENC formation are obscure, an attractive hypothesis implies the following events: old centromere inactivation and neocentromere formation in a new euchromatic locus, with fixation of these first steps in population. The next events are the disappearance of satellite DNA at the old inactivated centromeres and accumulation of new satellite DNA at the new centromere positions with an evident elimination of their neocentromeric nature (Amor et al. 2004; Marshall et al. 2008; Piras et al. 2010). Does centromere repositioning in birds fit this model? Can we trace steps of ENC formation?

For a long time, the information about centromere-specific sequences in birds was limited to a few satellites identified in several species (Matzke et al. 1990; Solovei et al. 1996; Tanaka et al. 2000; Saifitdinova et al. 2001; Wang et al. 2002; Krasikova et al. 2006; Deryusheva et al. 2007). Even in extensively studied galliform species, such as chicken and quail, primarily microchromosome-specific centromeric repeats were well characterized (Matzke et al. 1990; Tanaka et al. 2000; Krasikova et al. 2006; Deryusheva et al. 2007). A recent comprehensive analysis revealed elusive centromere DNA specific for chicken chromosomes (Shang et al. 2010). We expected to see a difference in centromere organization between chromosomes with centromere position preserved in the same genetic environment during karyotype evolution (chromosome 2) and chromosomes with centromeres shifted along chromosomes (chromosomes 1, 3, and 4). Surprisingly, these chromosomes have similar genome organization of their centromere regions: Centromeric sequences are organized in typical tandem repeat arrays and must have derived from a common ancestor, at least partially (Shang et al. 2010). Given the fact that more than 30 million years separate the chicken and Japanese quail, chicken centromeres could accumulate satellite repeats being transformed into “mature centromeres.” On the other hand, chicken centromeres might represent ancestral centromeres whereas ENC formation occurred in quail karyotype evolution. Without

extensive comparative analysis including centromere positioning on orthologous chromosomes from out-group karyotypes, the directionality of ENC formation is ambiguous. Sex chromosome Z is the most frequently and extensively rearranged chromosome in bird karyotypes (Griffin et al. 2007; Nanda et al. 2008). Centromere repositioning was predicted for chromosome Z when Pekin duck and chicken karyotypes were compared (Skinner et al. 2009). In fact, non-tandem-repeat sequences were found to form functional centromere of chicken chromosome Z (Shang et al. 2010). This type of centromere organization is expected for early events of centromere formation. Another candidate for finding of newly formed centromere is chromosome 5. The centromere of this chromosome in chicken also does not contain tandem repeats typical of well-established centromeres (Shang et al. 2010).

Microchromosomal counterparts of chicken and quail karyotypes are designated by conserved centromeric repeats CNM and *Bg/II*-repeat, respectively, which must have originated from a common ancestral sequence. Does it indicate that centromere repositioning is characteristic of macrochromosomes only? The pattern of comparative FISH mapping on chicken and quail LBCs 12, 13, and 14 (Figs. 5 and 6) poses a question whether these chromosomes are examples of centromere repositioning events in microchromosomes or examples of large-scale paracentric inversions. Intriguingly, a subset of chicken microchromosomes show the presence of CNM repeat at both termini: centromeric and q arm-terminal clusters (Krasikova et al. 2006; Deryusheva et al. 2007). Three possibilities could be hypothesized based on this observation: (1) CNM repeat marked originally both chromosomal ends that allowed stochastic fixation of centromere position, (2) recent centromere repositioning occurred via CNM duplication, and the telomeric CNM cluster represents a residual array at the old inactivated centromere, (3) paracentric inversions spanning the whole arm with breakpoints in the CNM cluster occurred in chicken karyotype evolution.

Molecular mechanisms underlying chromosomal rearrangements in birds are poorly understood. Although little is known about the sequences from breakpoint regions, the speculations that repetitive DNA is involved in genome reshuffling in birds sound reasonable. In fact, satellite DNA is believed to have promoted chromosomal rearrangements in mammals

(Slamovits and Rossi 2002; Ruiz-Herrera et al. 2006; Adega et al. 2009). In birds, the breakpoints of the inversion, which distinguish GGA3 and CCO3, were mapped to positions occupied by long clusters of tandem repeats: CNM repeat at position 5.6 Mb in the current GGA3 sequence assembly (Fig. 3, Zlotina et al. 2010) and Z chromosome-specific macrosatellite at telomere bow-like loops (TBL) (Hori et al. 1996). Strikingly, the position 5.6 Mb in GGA3 was identified as a breakpoint of an inversion between GGA3 and its turkey ortholog MGA2 with the other breakpoint located in GGA3 at position 11.6 Mb (Dalloul et al. 2010; Zhang et al. 2011), which is another CNM-enriched region (Zlotina et al. 2010). The second inversion between GGA3 and MGA2 was predicted to have a breakpoint at position 2.4 Mb in GGA3 (Dalloul et al. 2010; Zhang et al. 2011). This position corresponds to the centromere of GGA3 (Zlotina et al. 2010) and also comprises of satellite repeats (Shang et al. 2010). Numerous repetitive sequences of different classes have been revealed in evolutionary breakpoints in chicken, turkey, and zebra finch (Itoh et al. 2011; Skinner and Griffin 2011). One third of the identified breakpoint regions are thought to have been “reused” during avian genome evolution. These results are consistent with a widespread “fragile-breakage” model of chromosomal evolution implying a nonrandom breakpoint distribution and the existence of chromosomal regions prone to breakage in mammalian genomes (Pevzner and Tesler 2003; Murphy et al. 2005; Kemkemer et al. 2009). However, what is a primary event in chromosomal rearrangements: Accumulation of repeated sequences produces a fragile site, or repetitive sequences accumulate at sites of chromosome breaks remains an open question.

A strong association between structural genomic variations and increased recombination rates has been recently revealed; non-allelic homologous recombination is proposed to play a role in chromosomal rearrangements in avian genome evolution (Völker et al. 2010; Skinner and Griffin 2011). Indeed, CNM clusters on GGA3 that are associated with chromosomal breakpoints may be considered as recombination hot spots. A high-density SNP-based linkage map of the chicken genome revealed elevated recombination rates in these chromosomal regions (Groenen et al. 2009). Analysis of chiasma distribution along chicken LBC3, which also allows us to estimate frequency of recombination, supports this assumption (Figure S1). We do

not exclude that recombination activity could promote chromosomal reshuffling. On the other hand, high frequency of recombination may be a consequence of tandem repeat accumulation at the breakpoints. Similarly, a recombination hot spot was identified in chicken LBC16 at position of the long arrays of PO41 tandem repeat (Solinac et al. 2010). Despite intensive studies of the genomic organization in a wide variety of different species, with many genomes fully sequenced, the main trigger(s) of evolutionary genome rearrangements remains obscure.

Based on our findings and earlier data, we suggest that intrachromosomal rearrangements that accompanied avian karyotype evolution are more diverse than previously thought. In conclusion, we demonstrated again that giant lampbrush chromosomes represent a reliable system for high-resolution physical gene mapping and the detail comparative cytogenetic analysis of avian karyotypes.

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