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EVOLUTION

Germline-restricted chromosome (GRC) is widespread among songbirds

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An unusual supernumerary chromosome has been reported for two related avian species, the zebra and Bengalese finches. This large, germline-restricted chromosome (GRC) is eliminated from somatic cells and spermatids and transmitted via oocytes only. Its origin, distribution among avian lineages, and function were mostly unknown so far. Using immunolocalization of key meiotic proteins, we found that GRCs of varying size and genetic content are present in all 16 songbird species investigated and absent from germline genomes of all eight examined bird species from other avian orders. Results of fluorescent in situ hybridization of microdissected GRC probes and their sequencing indicate that GRCs show little homology between songbird species and contain a variety of repetitive elements and unique sequences with paralogs in the somatic genome. Our data suggest that the GRC evolved in the common ancestor of all songbirds and underwent significant changes in the extant descendant lineages.

avian genome | programmed DNA elimination | meiosis | Passeriformes | chromosome evolution

In addition to a standard chromosome set, which is present in all cells of an organism, cells of many animal, plant, and fungi species contain additional, so called B-chromosomes. Their origin, evolution, and adaptive significance remain obscure. B-chromosomes show erratic phylogenetic distribution indicating their independent occurrence in different species. The fact that B-chromosomes vary in number between different individuals of the same species or even between cells of the same individual suggests they are not critical for survival and may be considered as selfish genomic elements (1, 2). In birds, additional chromosomes were described so far in two related species of the family Estrildidae: zebra and Bengalese finches (3, 4). However, these chromosomes behave differently from a typical B-chromosome.

In the germline cells of these two species, a large additional acrocentric chromosome is found, which is absent from somatic cells. In oocytes, this germline-restricted chromosome (GRC) is usually present in two copies, forming a bivalent that undergoes recombination. In spermatocytes, one copy of this chromosome forms a round heterochromatic body, which is eliminated from the nucleus during the first meiotic division (4, 5). Camacho et al. (2, 6) indeed suggested that the GRC is a genomic parasite, a bird variant of supernumerary B-chromosome. Recent studies revealed that the zebra finch GRC contains multiple copies of genes paralogous to the genes from the somatic genome (7, 8). Some of these genes are amplified and importantly could be expressed at both the RNA and protein levels in the testes and ovaries. Thus, GRC contains genetic material, which could be important for germline cells, but not essential for the majority of the body (somatic) cells.

The origin of GRC remains unclear. Itoh et al. (9) found that the zebra finch GRC contains sequences homologous to an interval of chromosome 3 as well as repetitive elements absent from the sequenced somatic genome. Phylogenetic analysis of GRC-derived sequences, together with the zebra finch and chicken somatic cell counterparts, suggests that the GRC was formed after the galliform–neoaves split (9). A recent study hypothesized that the GRC is evolutionarily old and could be present in other birds as well (7).

To answer the questions about the origin, architecture, and distribution of GRCs in avian lineages, we performed a comprehensive comparative cytogenetic study of the germ cell chromosomes from 24 avian species representing eight orders. To further examine the degree of GRC conservation between distinct species, we made a sequence-based comparison of the microdissected GRC probes from four passerine species.

Results and Discussion

Using antibodies to the core proteinaceous structure of meiotic chromosomes, the synaptonemal complex (SC), we found that GRCs are present in all 16 songbird species examined (14 in this study and two in the previous studies). These species represent nine families of Passeri (Fig. 1). In 10 species, the GRCs were large acrocentric macrochromosomes (macro-GRCs) absent from bone marrow cells (Fig. 2 *A* and *B* and *SI Appendix*, Fig. S1). In oocytes, the macro-GRCs were usually present as a bivalent, containing one or two terminally located recombination sites visualized by antibodies to MLH1, a mismatch repair protein. In spermatocytes, the GRC usually occurred as a univalent

Significance

We discovered that contrary to other bird species and most other animals, all examined songbird lineages contain a different number of chromosomes in the somatic and germline genomes. Their germ cells have an additional germline-restricted chromosome (GRC). GRCs contain highly duplicated genetic material represented by repetitive elements and sequences homologous to unique regions of the somatic genome. Surprisingly, GRCs even in very closely related species, vary drastically in size and show little homology. We hypothesize that the GRC was formed as an additional parasitic microchromosome in the songbird ancestor about 35 million years ago and subsequently underwent significant changes in size and genetic content, becoming an important component of the germline genome.

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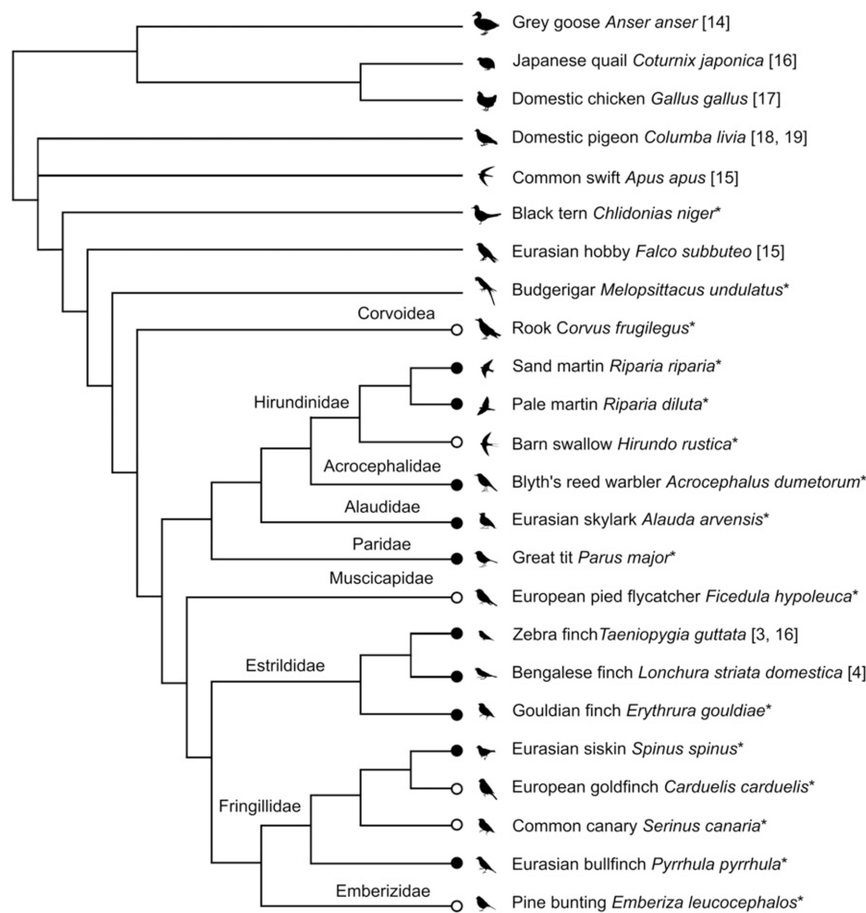

Fig. 1. Topology of the bird species examined. Our sampling covers all major passerine groups (except for the basal oscines, suboscines, and acanthisittides) as well as Galloanserae, Columbaves, Apodiformes, Charadriiformes, Falconiformes, and Psittaciformes. Black circles indicate species with a macro-GRC, and white circles indicate species with a micro-GRC. Numbers after the species' names indicate references for SC studies; asterisks indicate new data. A consensus topology of bird orders is based on the cladogram from Reddy et al. (10). Position of the common swift is defined according to Prum et al. (11). Topology of passerine birds is shown according to Roquet et al. (12). Positions of species within the Estrildidae lineage is established according to Hooper and Price (13).

lacking recombination sites, and was diffusely labeled with centromere antibodies (Fig. 2*A* and *SI Appendix*, Fig. S1). At the end of male meiotic prophase, this GRC was transformed into a dense round body and ejected from the nucleus (*SI Appendix*, Fig. S2). A similar meiotic behavior has been described for GRCs in zebra and Bengalese finches (3, 4).

In male germline cells of six other species, we detected micro-GRCs, which appeared as a univalent surrounded by a cloud of centromere antibodies and lacking recombination sites, similar to the behavior of macro-GRCs in the 10 other species. In the oocytes of these species, the GRCs formed a bivalent indistinguishable from the standard microchromosomes. We did not observe any phylogenetic clustering for the GRCs by size. Both macro- and micro-GRCs were present within the families Fringillidae and Hirundinidae (Fig. 1).

Every examined primary spermatocyte of the 16 songbird species contained a GRC. This suggests that the GRC is an important component of the germline genome. However, no GRCs were observed [by reanalyzing our own data (14, 15) and published SC images (16–19)] in eight species of nonpasserine birds from seven separate lineages (Fig. 1). This implies a monophyletic origin of the GRC. The estimated time since songbird divergence from other avian lineages is 35 million years (20). However, basal oscines, suboscines, and Acanthisittidae species have not been examined yet, so we cannot exclude the possibility that GRCs formed in the common ancestor of all Passeriformes, about 60 million years ago (20).

To estimate the sequence homology between GRCs of different species and to get insight into their genetic content, we prepared DNA probes of macro-GRCs for four representatives of three families: Estrildidae (zebra and Bengalese finches), Fringillidae (Eurasian siskin), and Hirundinidae (pale martin). We microdissected the round dense bodies (*SI Appendix*, Fig. S2) containing the GRC from spermatocyte spreads and carried out whole genome amplification of the dissected material. The resulting probes were used for fluorescent in situ hybridization (FISH) and for sequencing.

Reverse FISH with these GRC probes produced strong specific signals on the GRCs of each species, indicating that the round dense bodies are indeed the ejected GRCs (*SI Appendix*, Figs. S2*C* and S3). In cross-species FISH experiments, the intensity of specific GRC signals was much lower. Interestingly, micro-GRCs were painted with DNA probes derived from macro-GRCs of closely related species, indicating that GRCs of related species share at least a part of their genetic content. In both reverse and cross species FISH, we also detected GRC signals on somatic chromosomes. Some signals remained visible after suppression of repeated sequences with Cot-1 DNA. This indicates that GRCs contain multiple copies of sequences homologous to genomic repeats, as well as sequences homologous to unique regions present in the somatic genomes.

To identify these sequences, we aligned the reads resulted from next-generation sequencing (NGS) of the GRCs to the repeat-masked

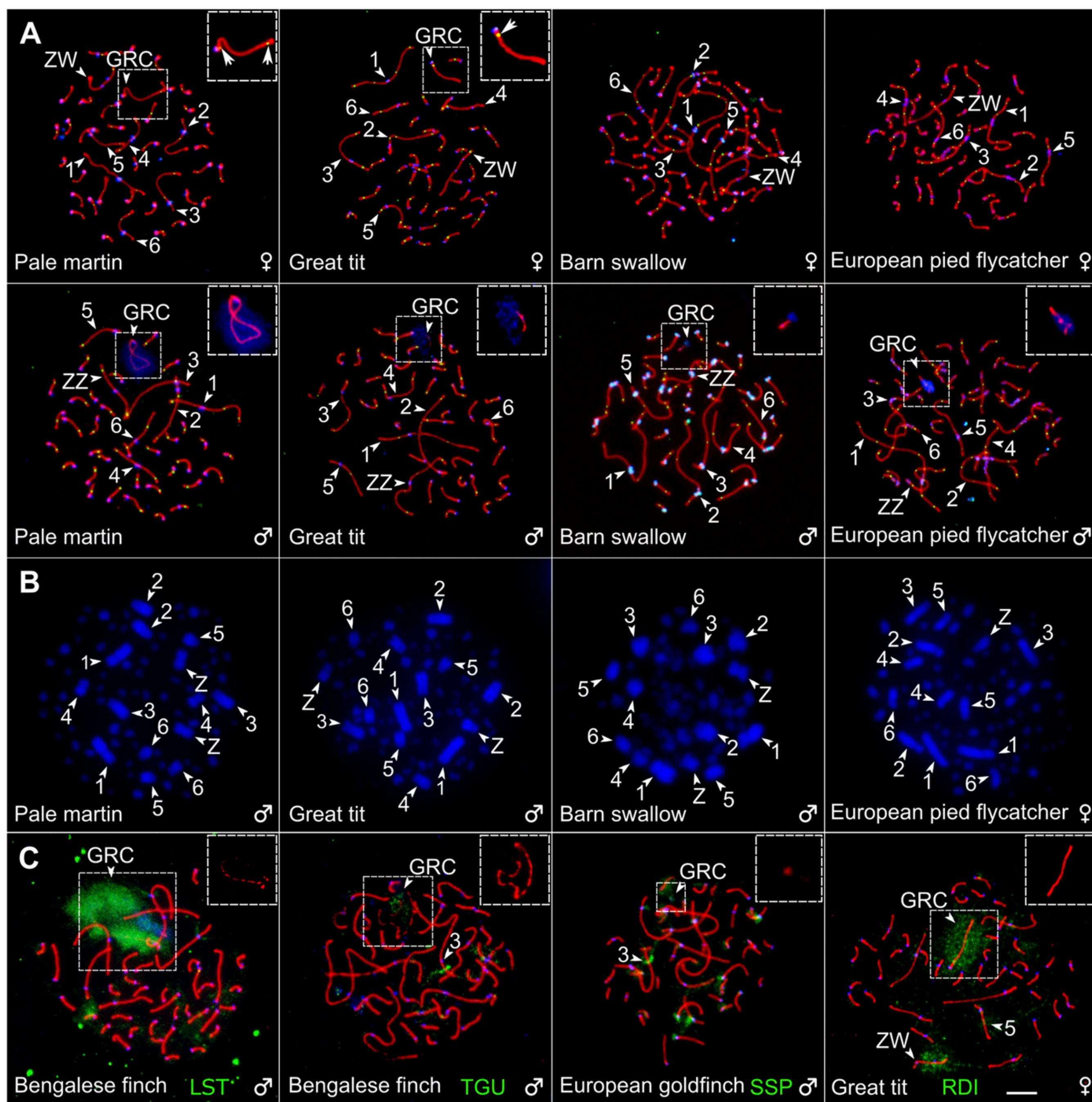

Fig. 2. Discovery of GRCs in bird species. (*A*) SC spreads of four oscine species immunolabeled with antibodies against SYCP3, the main protein of the lateral element of SC (red), centromere proteins (blue) and MLH1, mismatch repair protein marking recombination sites (green). Arrowheads point to the largest chromosomes ordered according to their size ranks, ZZ (identified by its size and arm ratio), ZW (identified by heteromorphic SC and misaligned centromeres), and GRCs. Arrows in the *Insets* point to MLH1 foci in GRCs. Micro-GRC bivalents in female barn swallow and European pied flycatcher are indistinguishable from the microchromosomes of the somatic chromosome set. (*B*) DAPI-stained bone marrow cells. (*C*) Reverse and cross-species FISH of GRC DNA probes (green) derived from Bengalese finch (LST), zebra finch (TGU), Eurasian siskin (SSP), and pale martin (RDI) with SC spreads, immunolabeled with antibodies against SYCP3 (red). Centromeres are labeled with antibodies against centromere proteins (blue). Arrowheads point to GRCs and regions on the somatic chromosome set intensely painted with GRC probes in cross-species FISH. *Insets* show GRCs. The Bengalese finch GRC-specific DNA probe gives a strong signal on the Bengalese finch GRC and slightly paints some regions of the somatic chromosome set. The zebra finch GRC probe paints the distal area of the Bengalese finch GRC and a region of the short arm of SC3. The Eurasian siskin GRC probe paints a micro-GRC of European goldfinch, a region on the long arm of SC3 and some pericentromeric regions. The pale martin GRC probe gives a dispersed signal on the great tit GRC, the ZW bivalent and on SC5. Scale bar: 5 μm.

zebra finch reference genome (Taeniopygia_guttata-3.2.4) using BLAT (21) with a 90% identity setting. Average genome coverage estimated in 10-kb windows was 0.15 ± (SD) 4.60, 0.12 ± 3.29, 0.03 ± 1.16, and 0.01 ± 0.25 for reads of zebra finch, Bengalese finch, Eurasian siskin, and pale martin GRC libraries, respectively. The coverage was highly uneven. GRCs of different species showed homology to different regions of the reference genome. Using the four GRC libraries, we characterized 27 regions

longer than 10 kb, covered in at least 30% of their length and with an excess of two SD from the genome average (*SI Appendix*, Table S1). In some regions, where the GRC of one species showed a high coverage, GRCs of other species showed lower, but still above average, coverage. This may indicate that the unique sequences located in these regions have been copied from the ancestral somatic genome into the ancestral GRC and have then subsequently become diverged at the sequence level and/or in copy number.

The longest of such excessively covered genomic regions were also detected by FISH at the SCs of the corresponding species. Some regions partially overlapped sequences of zebra finch genes (22) or sequences homologous to nonzebra finch RefSeq genes (23) (*SI Appendix*, Table S1). For example, the zebra finch GRC probe gave a strong hybridization signal on the short arm of the zebra finch SC3 (corresponding to TGU1) and on one of the largest SCs of other species examined (Fig. 2*C* and *SI Appendix*, Fig. S3). In the corresponding region of TGU1, we found a 2.5-Mb long cluster of several regions with ~70 fold coverage excess (*SI Appendix*, Table S1). This cluster overlapped with two genes: completely with *ROBO1*, a gene involved in vocal learning (24); and partially with *GBE1*, a gene encoding 1,4-alpha-glucan branching enzyme 1. The homology between the zebra finch GRC and a part of the genomic interval on TGU1 has been detected earlier by the random amplification of polymorphic DNA technique (9) and recently confirmed by Kinsella et al. (7).

Besides functional genes, GRCs also contain multiple repeated sequences. We estimated their representation in the GRC reads and in the somatic genome of zebra finch using RepeatMasker (25) with the RepBase avian library (26) (*SI Appendix*, Table S2). This revealed both simple and low complexity repeats. The fraction of transposable elements (TEs) in the GRCs was typical for avian genomes (27). The majority were long terminal repeats (LTRs) and long interspersed nuclear elements (LINEs), while short interspersed nuclear elements (SINEs) and DNA TEs were represented in lower fractions than in the somatic genome. Overall abundance of LTRs and LINEs and their ratio varied between different species' GRCs, reflecting their different evolutionary trajectories. It has been shown that although activity of TEs in avian genomes was rather low and ancient (especially for SINEs), avian species differed for the timing of TE family activities. Interestingly, the zebra finch genome shows a peak of LTR activity from 5 to 20 million years ago (27). This is a likely reason why LTRs are more abundant in zebra finch GRC than in other GRCs. On the other hand, SINEs are rare in avian genomes and they did not show any activity during last 30 million years, yet they are present in the GRCs of all four examined species, likely being inherited from the GRC ancestor. This provides further evidence for the formation of GRCs in the songbird genome rather than in older avian ancestors, because GRCs had a chance to accumulate at higher rate LTRs active in songbirds but not older SINEs. Therefore, a few SINEs found in the GRCs likely represent copies transferred from the somatic genome and amplified in the GRCs rather than those inserted during the actual activity of SINEs.

To examine the general pattern of GRC transcription in oogenesis, we analyzed lampbrush GRCs isolated from zebra finch oocytes at the previtellogenic growth phase. The lampbrush GRC exhibited a typical chromomere-loop pattern, with several pairs of transcriptionally active lateral loops extending from all chromomeres except for those located in a prominent DAPI-positive region. Antibodies against RNA-polymerase II labeled the whole GRC except for this region (*SI Appendix*, Fig. S4). Thus, lampbrush GRCs display a pattern of transcription typical for somatic chromosomes (28).

Indeed, recent studies demonstrated that many GRC-linked genes are transcribed (7, 8) and at least some of them are translated in the zebra finch germline (7). The evolutionary history of some of these genes points to the songbird origin of the proto-GRC. This is an excellent complementary confirmation of our own findings which drove us to the same conclusion based on a direct (cytogenetic) observation and indirect (transposable element composition) analysis of our sequenced GRC libraries. Our study, however, also points to the existence of both micro- and macrochromosome versions of GRCs in avian lineages, suggesting that this chromosome is highly dynamic in songbird evolution.

Thus, GRCs are present in all of the songbirds studied, but are absent from germlines of birds from other orders. These chromosomes vary drastically in size and show a low sequence similarity between different species. GRCs contain various highly duplicated regions represented in the somatic genome by both unique and repetitive sequences. The spectrum of transposable elements found in our sequenced GRC libraries suggests that the GRC was more likely formed in the ancestral songbird lineage followed by an extensive sequence divergence in the descendent species genomes rather than to appear in the avian ancestor and then being lost in the nonsongbirds.

Therefore, we propose that the GRC has formed as an additional "parasitic B-like" microchromosome in the ancestral songbird genome likely due to a whole-chromosome duplication (Fig. 3). If this proto-GRC already contained some copies of somatic genes contributing to reproductive and developmental processes, it could become beneficial due to providing a higher dosage of these genes and therefore escape purifying selection pressure in the germline. Its presence in the germline only could also relax selection for the functional integrity of the GRC's genetic content.

This in turn could make the GRC a target for selfish genetic elements active during its evolutionary history. Additional copies of unique sequences (e.g., genes) from the somatic genome could also populate the GRC through nonallelic recombination process, using its own and somatic genome transposable elements as templates. Suppression of recombination along GRCs (except for their termini in female meiosis) could facilitate their divergence and the degradation of their original genetic content via Muller's ratchet (29). This could lead to a rapid and massive loss of homology between various species' GRCs.

However, as the contemporary GRCs contain expressed and transcribed genes and persist in the germline of all songbirds studied, it likely has changed its original "parasitic" state to a more "symbiotic" one, providing evolutionary benefits to the representatives

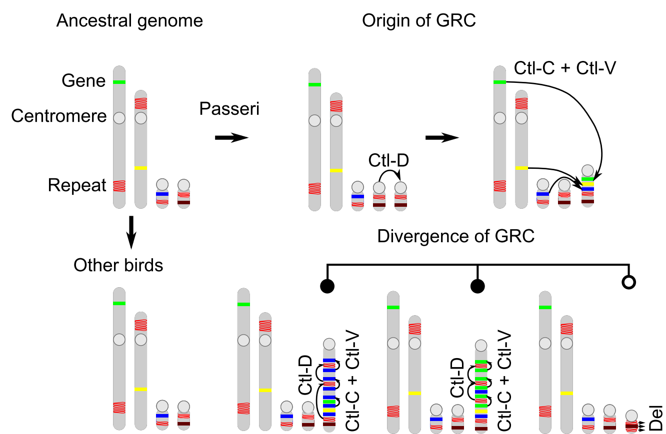

Fig. 3. Scenario of GRC origin and evolution. A proto-GRC forms due to duplication (Ctl-D) of a microchromosome likely containing genes involved in germ cell development. Copies of unique somatic cell sequences and repetitive elements invade the GRC (Ctl-C+Ctl-V). Divergence of GRCs in different songbird lineages occurs due to amplification and deletion (Del) of their sequences.

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of the most speciose group of birds. We believe that a detailed comparison of micro- and macro-GRCs, phylogenetic studies of shared and lineage-specific GRC sequences, and detailed analysis of their stratification within each GRC will shed further light on the origin and evolution of this highly dynamic and surprising chromosome.

Materials and Methods

Experimental Model and Subject Details. Adult males of pale martin, great tit, barn swallow, European pied flycatcher, Blyth's reed warbler, and black tern were captured at the beginning of breeding season. Nestling females of sand martin, pale martin, barn swallow, great tit, and European pied flycatcher were collected from nests ∼3–6 d after hatching.

Adult male zebra finch, Gouldian finch, Bengalese finch, Eurasian siskin, European goldfinch, Eurasian skylark, pine bunting, Eurasian bullfinch, common canary and budgerigar were purchased from a commercial breeder. Sexually mature zebra finch females were provided by the Leningrad Zoo (Saint Petersburg, Russia). An adult male rook with fatal accident trauma was provided by the Bird Rehabilitation Centre of Novosibirsk.

Capture, handling, and euthanasia of birds followed protocols approved by the Animal Care and Use Committee of the Institute of Cytology and Genetics SD RAS (protocol #35 from 26.10.2016) and by the Saint Petersburg State University Ethics Committee (statement #131–03-2). Experiments described in this manuscript were carried out in accordance with the approved national guidelines for the care and use of animals. No additional permits are required for research on nonlisted species in Russia.

Mitotic Metaphase Chromosomes. Mitotic chromosome preparations were obtained from short-term bone marrow cell cultures incubated for 2 h at 37 °C with 10 μg/mL colchicine in culture Dulbecco's Modified Eagle's medium with UltraGlutamine. Hypotonic treatment was performed with 0.56% KCl solution for 15 min at 37 °**C** and followed by centrifugation for 5 min at 500 × *g*. Fresh cold fixative solution (methanol: glacial acetic acid, 3:1) was changed three times. Cell suspension was dropped on cold, wet slides (76 mm × 26 mm, 1 mm thick). The slides were dried for 2 h at 65 °C and stained for 4 min with 1 μg/mL solution of DAPI in 2× SSC. Then slides were washed in deionized water, dried at room temperature, and mounted in Vectashield antifade mounting medium (Vector Laboratories) to reduce fluorescence fading.

SC Spreading and Immunostaining. Chromosome spreads for SC analysis were prepared from spermatocytes or juvenile oocytes according to Peters et al. (30). Immunostaining was performed according to the protocol described by Anderson et al. (31) using rabbit polyclonal anti-SYCP3 (1:500; Abcam), mouse monoclonal anti-MLH1 (1:50; Abcam), and human anticentromere (ACA) (1:100; Antibodies Inc) primary antibodies. The secondary antibodies used were Cy3-conjugated goat anti-rabbit (1:500; Jackson ImmunoResearch), FITC-conjugated goat anti-mouse (1:50; Jackson ImmunoResearch), and AMCA-conjugated donkey anti-human (1:100; Jackson ImmunoResearch). Antibodies were diluted in PBT (3% BSA and 0.05% Tween 20 in PBS). A solution of 10% PBT was used for blocking. Primary antibody incubations were performed overnight in a humid chamber at 37 °C; and secondary antibody incubations, for 1 h at 37 °C. Slides were mounted in Vectashield antifade mounting medium (Vector Laboratories) to reduce fluorescence fading.

Lampbrush Chromosome Preparations. Zebra finch lampbrush chromosomes were manually dissected from previtellogenic or early vitellogenic oocytes using the standard avian lampbrush technique described by Saifitdinova et al. (32). After centrifugation, preparations were fixed in 2% paraformaldehyde, then in 50% and in 70% ethanol, air-dried, and kept at room temperature until used for FISH. For immunostaining experiments, lampbrush chromosome preparations were kept in 70% ethanol at 4 °C.

Preparation of the Hybridization Probe and FISH. To generate a DNA probe for the GRCs of the pale martin, zebra finch, Bengalese finch, and Eurasian siskin, testicular cells of adult males were treated with hypotonic solution (0.88% KCl) at 37 °C for 3 h and then with Carnoy's solution (methanol: glacial acetic acid, 3:1). The cell suspension was dropped onto clean, cold, wet coverslips (60 mm × 24 mm, 0.17 mm thick), dried, and stained with 0.1% Giemsa solution (Sigma) for 3–5 min at room temperature. GRCs were identified as positive round bodies located near the spermatocytes I. Microdissection of GRC and amplification of DNA isolated from this chromosome were carried out with the GenomePlex Single Cell Whole Genome Amplification Kit (WGA4) (Sigma-Aldrich) (33). Microdissected DNA probes were generated from 15 copies of GRC for each studied species. The obtained PCR products were labeled with Flu-dUTP (Genetyx) in additional PCR cycles or with biotin-11-dUTP (Sileks).

FISH experiments with DNA probes on SC spreads of the studied avian species were performed as described earlier (34) with salmon sperm DNA (Ambion) as a DNA carrier. In case of suppression FISH, Cot-1 DNA (DNA enriched for repetitive DNA sequences) was added to the DNA probe to suppress the repetitive DNA hybridization. Chromosomes were counterstained with DAPI dissolved in Vectashield antifade solution (Vector Laboratories). Zebra finch GRC at the lampbrush stage was identified by FISH using biotin-labeled zebra finch microdissected probe with a 50-fold excess of *Escherichia coli* tRNA as a carrier. FISH was performed according to the DNA/DNA+RNA hybridization protocol omitting any chromosome pretreatment, as described previously (35). To detect biotin-labeled probe, we used avidin-Alexa488 and biotinylated goat antibody against avidin (both from Thermo Fisher Scientific). Lampbrush chromosomes were counterstained with DAPI in an antifade solution, containing 50% glycerol.

Immunostaining of the Zebra Finch Lampbrush Chromosomes. Immunostaining was carried out with mouse antibodies V22 (kindly donated by U. Scheer) against the phosphorylated C-terminal domain (CTD) of RNA polymerase II. Lampbrush chromosome spreads, fixed in 2% paraformaldehyde, were blocked in 0.5% blocking reagent (Sigma-Aldrich) in PBS for 1 h at 37 °C. Then preparations were incubated with primary antibodies, diluted 1:200, overnight at room temperature. Slides were washed in PBS with 0.05% Tween-20 and incubated with Alexa-488-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Lab). After washing in PBS containing 0.05% Tween-20, slides were mounted in antifade solution containing DAPI.

Microscopic Analysis. Images of fluorescently stained metaphase chromosomes and/or SC spreads were captured using a CCD camera installed on an Axioplan 2 compound microscope (Carl Zeiss) equipped with filtercubes #49, #10, and #15 (ZEISS) using ISIS4 (METASystems GmbH) at the Center for Microscopic Analysis of Biological Objects of SB RAS (Novosibirsk, Russia). For further image analysis, we used Corel PaintShop Pro X6 (Corel). The location of each imaged immunolabeled spread was recorded so that it could be relocated on the slide after FISH. Zebra finch lampbrush chromosome preparations were examined using a Leica DM4000B fluorescence microscope installed at the "Chromas" Resource Centre, Saint Petersburg State University Scientific Park (Saint Petersburg, Russia). The microscope was equipped with a black and white DFC350FX camera and filters A and I3. LAS AF (Leica) software was used to capture and process color images; Adobe Photoshop CS5 (Adobe Systems) was used for figure assembling. The length of the SC of each chromosome arm was measured in micrometers and the positions of centromeres were recorded using MicroMeasure 3.3 (36). We identified individual SCs by their relative lengths and centromeric indexes.

Preparation of Amplified DNA and Library Construction. DNA amplification of microdissected GRC chromosomal material was performed with the GenomePlex Single Cell Whole Genome Amplification kit (WGA4) (Sigma-Aldrich) according to the manufacturer's protocol. DNA library for NGS sequencing was prepared using the microdissected GRC DNA libraries using the NEBNext Ultra DNA Library Prep kit (New England Biolabs).

High Throughput Sequencing and Error Correction. NEBNext Ultra library was sequenced on an Illumina NextSeq 550 system with single-end reads at the "Genomics" core facility of the ICG SB RAS (Novosibirsk, Russia). Read lengths were 150 bp, the total number of reads obtained were 1,730,845, 1,596,722, 2,821,862, and 1,265,105 for zebra finch, Bengalese finch, Eurasian siskin, and pale martin GRC correspondingly. DNA data were quality assessed using FastQC (37) and quality trimmed using Trimmomatic (38).

Estimating the Homology to Somatic Genome and Repeat Content. Reads from the zebra finch, Bengalese finch, Eurasian siskin, and pale martin GRC sequences were aligned to the assembly of the zebra finch genome (Taeniopygia_guttata-3.2.4) using BLAT (21). A custom python script was used to estimate the coverage of the zebra finch genome in 10-kb windows. Overlapping of regions with high coverage with zebra finch Ensembl gene predictions and nonzebra finch RefSeq (23) genes was revealed with the Ensembl genome browser. Repeat content of the GRC libraries and the zebra finch genome was assessed with RepeatMasker (25) by using the avian RepBase database (26).

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