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ORIGINAL ARTICLE

A pair of gametologous genes provides further insights into avian comparative cytogenomics

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Abstract

Exploration of avian gametologous genes, i.e., homologous genes located on both the Z and W chromosomes, provides a crucial information about the underlying mechanism pertaining to the evolution of these chromosomes. The domestic chicken (*Gallus gallus* (Linnaeus 1758); GGA) traditionally serves as the primary reference subject of these comparative cytogenomic studies. Using bioinformatic, molecular (overgo BAC library scanning), and cytogenetic (BAC-based FISH) techniques, we have investigated in detail a pair of *UBE2R2*/*UBE2R2L* gametologs. By screening a gridded genomic jungle fowl BAC library, CHORI-261, with a short labeled *UBE2R2L* gene fragment called overgo probe, we detected seven specific clones. For three of them, CH261-019I23, CH261-105E16, and CH261-114G22, we identified their precise cytogenetic location on the *Gallus gallus* W chromosome (GGAW). They also co-localized with the *UBAP2L2* gene on the, as was shown previously, along with the CH261-053P09 BAC clone also containing the GGAW-specific *UBE2R2L* DNA sequence. The fine mapping of the *UBE2R2*/*UBE2R2L* homologs in the chicken genome also shed the light on comparative cytogenetic aspects in birds. Our findings provided further evidence that bird genomes moderately changed only during evolution and are suitable for successful use of interspecies hybridization using both overgo-based BAC library screen and BAC-based FISH.

Keywords Avian genome · Chromosome evolution and sex chromosomes · Pseudoautosomal region and meiotic recombination suppression · Bioinformatics tools · Overgo BAC library scanning · Fluorescence in situ hybridization

Introduction

It has been suggested that avian Z- and W-chromosomes originated from one autosome pair, but a different one from which the mammalian X- and Y-chromosomes evolved (Schmid et al. 2000). Some lines of evidence, however, lead to alternative suggestions concerning the evolution of bird and mammal chromosomes (Stiglec et al. 2007a). The W-chromosome in neognathous avian species has some features similar to the mammalian Y-chromosome, i.e., it is gene-poor and largely heterochromatic. Highly repetitive sequences belonging to *Xho*I-, *Eco*RI- and *Ssp*I-families represent about 80% of the DNA in the chicken (*Gallus gallus* (Linnaeus, 1758); GGA) W chromosome (GGAW). Only

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about 10 Mb is represented by non-repetitive DNA (Itoh and Mizuno 2002).

In all neognathous birds studied to date, the Z–W pair of sex chromosomes (gonosomes) shows strictly localized recombination in a very short pseudoautosomal region (PAR; reviewed in Pigozzi and Solari 2005). Differentiation of the Z and W chromosomes in modern birds is thought to be a result of progressive and stepwise cessation of meiotic recombination (Lawson Handley et al. 2004; Schmid et al. 2005).

A significant part of GGAW was misassembled in initial sequencing efforts, that is, it was based exclusively on repetitive sequences, which were subsequently found to be present on other chromosomes (Stiglec et al. 2007b). FISH mapping of the bacterial artificial chromosome (BAC) clones, which were putatively thought to be GGAW-specific, to its counterpart Z chromosome (GGAZ) allowed researchers to clarify the genic composition of GGAW (Stiglec et al. 2007b). Thus, several W-linked genes in this region have respective homologs on GGAZ (e.g., *CHD1*, *HINT*, *SPIN*, *UBAP2*, and *ATP5A1*), forming pairs of gametologous genes and reflecting their common origin from ancestral homologous chromosome pairs (Schmid et al. 2005).

One of these gametologous genes is *UBE2R2* encoding the ubiquitin conjugating enzyme E2 R2 that is involved in modification of proteins with ubiquitin or ubiquitin-like proteins via an E1–E2–E3 cascade, which is crucial in many signaling networks (Jin et al. 2007). Being originally unmapped in the chicken, it was presumably localized on GGAZ judging from the comparative map analysis: the human ortholog maps to the HSA9 region that corresponds to GGAZ. In the recent chicken genome assembly GRCg6a (GRCg6a 2018), it is located on GGAZ, while its W-linked counterpart, *UBE2R2L*, is mapped to GGAW within the chicken PAR (Fig. 1).

Here, we investigated the chicken *UBE2R2* genes located on GGAZ and GGAW. For this purpose, we implemented an effective genome analysis and mapping pipeline using the appropriate bioinformatic tools, molecular techniques (e.g., overgo hybridization), and cytogenetic methods such as a BAC-based fluorescence in situ hybridization (FISH). The relevant implications for, and insights into, evolutionary aspects of bird gonosome organization were inferred providing an additional information for further discussion and research in this area of avian biology.

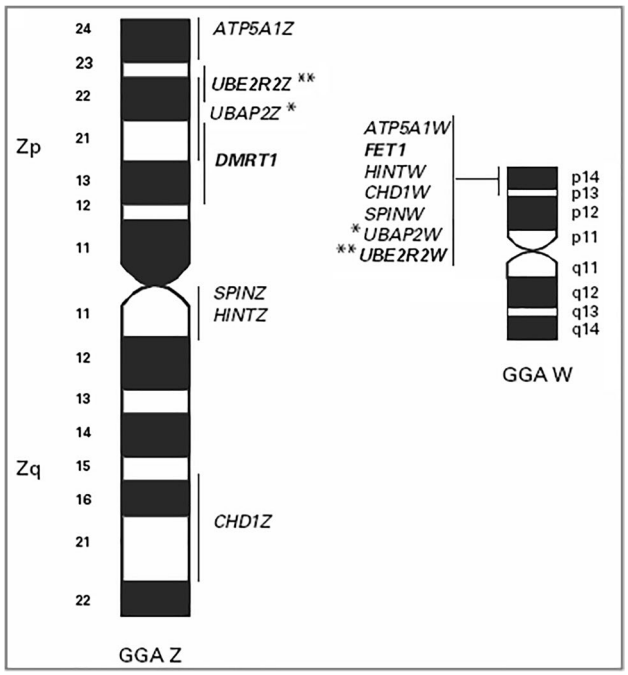

Fig. 1 G-banded ideogram of the chicken Z and W sex chromosomes showing cytological location of sex-linked genes including the gametologous genes shared between the Z and the PAR on the W (adopted from Schmid et al. 2005; Sazanov et al. 2006; **current study)

Materials and methods

Avian species

In this work, we conducted bioinformatic, molecular and cytogenetic analyses focusing on the chicken (*Gallus gallus*; order Galliformes) genome best studied among all birds. For further multifaceted comparative investigation, data obtained from our previous published and unpublished research on the following five bird species (with their respective Latin name and order in the parentheses) were also used: turkey (*Meleagris gallopavo* Linnaeus, 1758; Galliformes), Japanese quail (*Coturnix japonica* Temminck & Schlegel, 1848; Galliformes), Sunda zebra finch (*Taeniopygia guttata* (Vieillot, 1817); Passeriformes), white-throated sparrow (*Zonotrichia albicollis* (Gmelin, 1789); Passeriformes), California condor (*Gymnogyps californianus* (Shaw, 1797); Cathartiformes; alternatively, Falconiformes, Ciconiiformes or Accipitriformes), and black stork (*Ciconia nigra* (Linnaeus, 1758); Ciconiiformes).

Genome analysis and mapping pipeline

To explore the pair of *UBE2R2* genes, we developed and used an in-house genome analysis and mapping pipeline that encompassed the following components and applications: bioinformatics analyses → overgo hybridization → BAC-based FISH. The bioinformatic toolbox involved use of databases, sequence alignment tools, in silico overgo probe design, etc. Mining for UBE2R2 and related sequences was performed using NCBI- (e.g., Altschul et al. 1997; Wheeler et al. 2000; Cuff et al. 2000; Maglott et al. 2011; Sayers et al. 2021), UCSC- (Navarro Gonzalez et al. 2021) and Ensembl-based (Howe et al. 2021) genome browsers. Retrieved sequences were aligned with ClustalW (Thompson et al. 1994) and COBALT (Papadopoulos and Agarwala 2007). Contig with

BACs were detected in the Chicken FPC (ChickFPC) database (Schmid et al. 2005; Kerstens and Groenen 2004–2008). The employed molecular (overgo hybridization) and cytogenetic (FISH) procedures are outlined below (see also Online resources 1 and 2 for more detail).

BAC library scanning

The chicken genomic BAC library CHORI-261 was prepared from a red jungle fowl DNA sample (Nefedov et al. 2003; BACPAC Genomics 2020) and screened using the overgo probe hybridization approach as described in detail elsewhere (Romanov et al. 2003; Romanov and Dodgson 2006). Briefly, ~ 40-bp overgo probes comprised of two synthetic oligonucleotides with a complementary 8-bp region were designed in silico using consequently RepeatMarker (Smit et al. 1996–2010) and Overgo (Cai et al. 1998) web tools (Online resource 2). These probes were radioactively labeled with [32P]-deoxynucleotide triphosphates and hybridized at 60 °C to BAC clone array filters. The screening resulted in positively hybridized BAC clones with their IDs deposited electronically in the Michigan State University-hosted Database of BACs Assigned to Chicken Genes and Markers (Resources 2000–2013). Other avian BAC libraries were also screened using cross-species hybridization approach (Romanov and Dodgson 2006; Romanov et al. 2006, 2009, 2011).

FISH procedure

The FISH experiments were performed following the protocol as specified elsewhere (e.g., Sazanov et al. 2006; Blagoveschensky et al. 2011; see also Online resource 3 for further detail). In short, the 96-h Brown Leghorn chick embryo cells were used to generate preparations of mitotic chromosomes using hypotonic treatment, fixation, and colchicine incubation. The double-color FISH using the respective BAC clone DNA was carried out basically in accordance with the instructions reported elsewhere (Florijn et al. 1995). A fluorescent microscopic workstation (Ista, St. Petersburg, Russia) equipped with a CCD camera and the software program VideoTest-FISH was used to record hybridization signals.

Results

Overgo design and hybridization using chicken BAC library

For a starting point of the in silico overgo probe design, we used the mRNA sequence NM_017811.4 of the human orthologous *UBE2R2* gene (located on chromosome 9p13.3) as a BLAST® search query and identified the best match amongst available chicken (Expressed Sequence Tags) EST sequences that was the chicken cDNA clone BU122359 (see for more detail Online resource 1: Fig. S1-1). In the Ensembl database conforming to an older chicken assembly, BU122359 matched one W_random and one Z_random contigs that might be indicative of two *UBE2R2* homologs that exist on both GGAZ and GGAW (see for more detail Online resource 1: Table S1).

The designed 38-mer overgo (GCAATGAGGAGTCGT GACCTGCTCTCTATGCTGTTGTA) only matched one W_random contig suggesting that it uniquely conformed to the W-linked *UBE2R2L* gene (see Online resource 1). The overgo probe was labeled and applied to screening the gridded genomic BAC library CHORI-261. As a result, we found the following seven specific clones: CH261-019I23, CH261-053P09, CH261-105E16, CH261-114G22, CH261-124O24, CH261-082I17, and CH261-095J14 (Table 1, Fig. 2).

Table 1 Summary of the chicken CHORI-261 BAC library scanning and FISH mapping results using seven BAC clones that contain the *UBE2R2L* gene

Hybridization technique	BAC clone ID (synonym)	Confirmation test[1]	Contig with BACs[2]	FISH assignment	No. of chromosomes analyzed
overgo	CH261-019I23[3]	FISH	Others	GGAW	19
overgo	CH261-053P09[4]	NT	Others	NT	–
overgo	CH261-105E16[3]	FISH	Others	GGAW	21
overgo	CH261-114G22[3]	FISH	Others	GGAW	30
overgo	CH261-124O24	NT	Others	NT	–
overgo	CH261-082I17	NT	Others	NT	–
overgo	CH261-095J14	NT	Others	NT	–

[1] Test used to confirm positive clones: FISH, fluorescence in situ hybridization; NT, not tested. [2] As detected in the Chicken FPC (ChickFPC) database (Schmid et al. 2005; Kerstens and Groenen 2004–2008). Only the clones identified in this study are listed. Others, other six clones shown in this table and included in this contig. [3] BAC clones that were also positive for, and FISH mapped to, *UBAP2L2* (or *UBAP2W*; Sazanov et al. 2006; Blagoveschensky et al. 2011). [4] BAC clone that was also positive *for UBE2R2L* and *UBAP2L2* but not tested (NT) by FISH in the previous (Sazanov et al. 2006; Blagoveschensky et al. 2011) and present studies

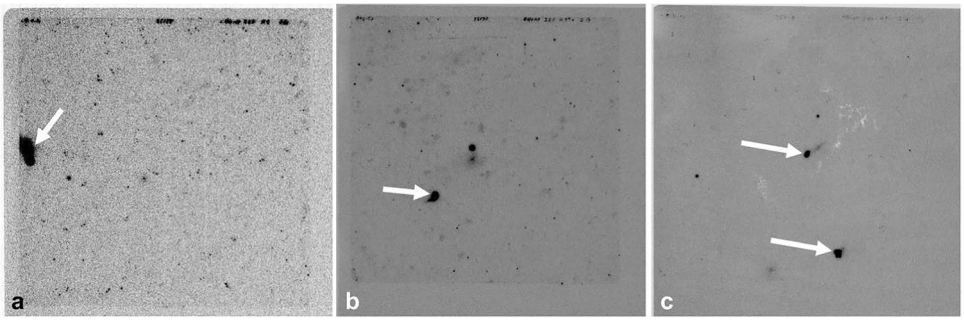

Fig. 2 Results of the CHORI-261 BAC library screen using the W-linked *UBE2R2L*-specific overgo hybridized to three BAC array filters. Arrows indicate the positive hybridization signals that corresponded to the respective BAC clones: **a** CH261-019I23; **b** CH261-053P09; and **c** CH261-114G22 (upper arrow) and CH261-105E16 (lower arrow). The same four BACs were also identified as positives for *UBAP2L2* (or *UBAP2W*)

Out of these seven BACs, there were four clones, CH261-105E16, CH261-114G22, CH261-019I23 and CH261-053P09, that also hybridized to the *UBAP2L2* (or *UBAP2W*) specific overgo probe. These appeared to overlap with both the *UBE2R2L* and *UBAP2L2* genes that can be confirmed by their close localization in the chicken genome assembly GRCg6a (Fig. 3a; Online resource 2: Fig. S2-1a). Indeed, the distance between these two genes was less than 20 Kb, with that between their Z-linked counterparts being even smaller (~ 2.5 Kb), making possible for a BAC clone to encompass the two neighboring genes.

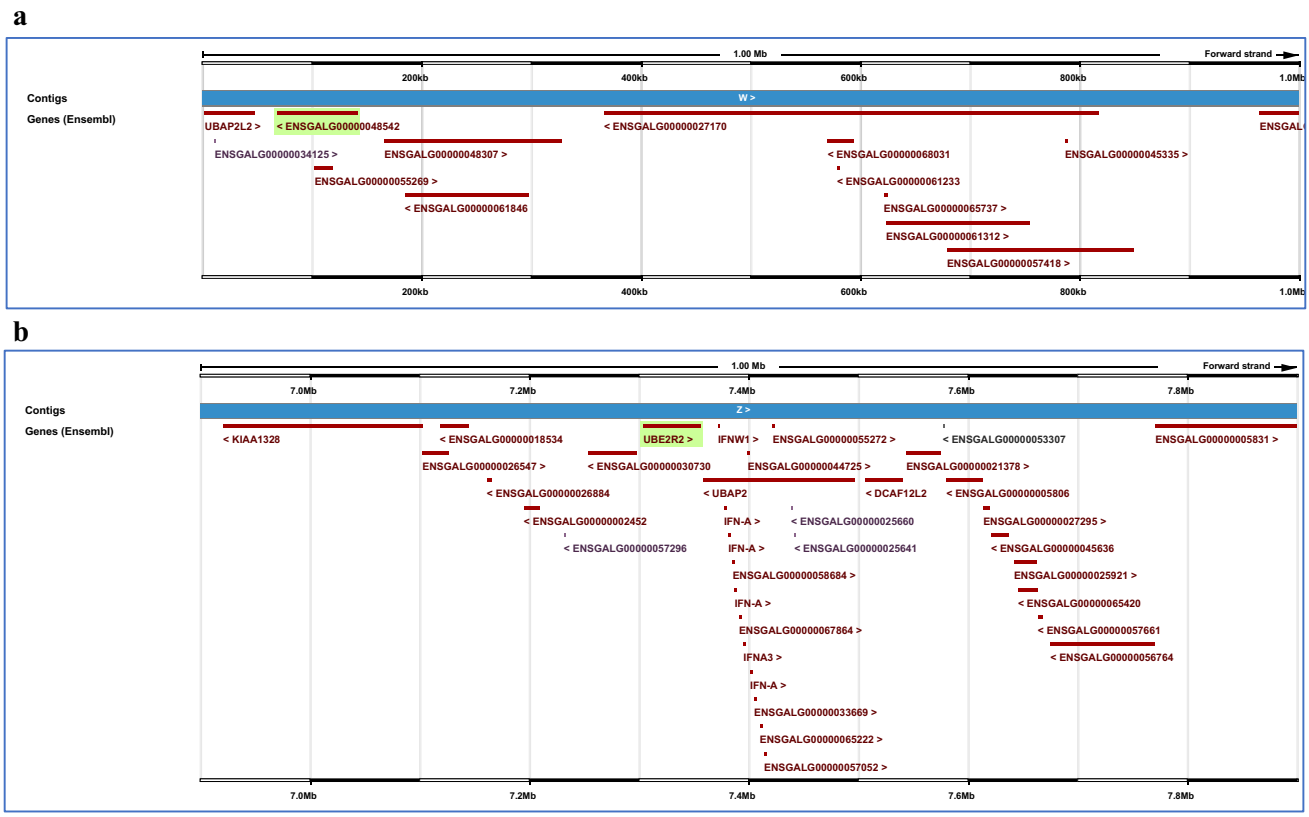

Fig. 3 Ensembl genome browser views for the GGAW (**a**) and GGAZ (**b**) genomic regions showing close localization of two gametologous gene pairs in the chicken genome assembly GRCg6a. Gene positions: **a** *UBAP2L2* at 2,472.0.48,083 bp (forward strand) vs *UBE2R2L* (or *ENSGALG00000048542*) at 67,938.0.141,552 bp (reverse strand; green highlight); **b** *UBE2R2* at 7,304,045.0.7,356,140 bp (forward strand; green highlight) vs *UBAP2* at 7,358,707.0.7,496,787 bp (reverse strand)

Overgo-based cross-species hybridization

Using the chicken *UBE2R2L*-specific overgo, we, in addition to the chicken BAC library, successfully screened four other avian libraries (Table 2). The number of positive clones ranged between 1 to 15. For instance, in the California condor BAC library CHORI-262, we identified one positive clone CH262-037I12 that was putatively mapped in silico to the W chromosome and was further used for FISH on the Z and W in this endangered species and other birds (Romanov et al. 2006, 2009; Modi et al. 2009).

BAC-based FISH

We used three out of the seven *UBE2R2L*-specific chicken BACs, i.e., CH261-019I23, CH261-105E16 and CH261-114G22, to identify precise cytogenetic location of *UBE2R2L* on GGAW (Table 1). Previously, these three clones also produced co-localized signals for *UBAP2L2* (or *UBAP2W*) on GGAW but not for its Z-linked counterpart *UBAP2* (or *UBAP2Z*; Sazanov et al. 2006; Blagoveschensky et al. 2011). The fine FISH mapping of the three BAC clones to GGAW proved unequivocally that they contained sequences of both *UBE2R2L* and *UBAP2L2*. Examples of mapping CH261-114G22 to GGAW using double-color FISH, in combination with different *ATP5A1*-positive BACs, are presented in Fig. 4. Remarkably, the *ATP5A1*-specific clone CH261-064F22, detected cytogenetically on GGAZ (Flpter, 0.14 ± 0.033; Blagoveschensky et al. 2011), also co-localized on GGAW, along with the CH261-114G22 BAC clone containing the GGAW-specific *UBAP2L2* DNA sequence (Fig. 4e).

Discussion

The origin of sex gonosomes in birds, mammals and reptiles appear to be different. While mammals use the XX/XY system, the genetic sex determination independently evolved in birds, and utilizes the ZZ/ZW system (Matsubara et al. 2006). Effective study of the gametologous genes underlying the ZZ/ZW system, especially for cross-species comparison, requires the availability of appropriate molecular and cytogenetic tools. In this study, we demonstrated efficiency in using gene sequence-derived overgos to discover specific BACs that, in turn, are applicable to FISH map gametologs to one or both gonosomes.

Within-species use of overgos to scan BAC libraries is, as a rule, a straightforward and reliable technique. Overgo-based interspecies hybridization success rate may vary due to a number of reasons: (1) evolutionary distance from the chicken, a source for the used overgo probes, and respective sequence divergence, especially within non-coding regions (Modi et al. 2009); (2) genome coverage, i.e., genome representation in a BAC library; (3) variation in quality of BAC DNAs arrayed on the gridded filters; and (4) specific and changeable BAC filter hybridization conditions in an individual screen experiment and in a particular lab.

Comparative chromosome painting and FISH mapping of DNA clones, including BACs, have been broadly employed for studying chromosome rearrangements between chicken and other birds (e.g., Griffin et al. 1999; Shibusawa et al. 2002, 2004). By implementing BAC-based FISH and using zebra finch BACs for a number of Z chromosome genes, including *UBE2R2*, Itoh et al. (2006) were able to map them to the zebra finch Z and W chromosomes. We previously mapped pairs of the chicken gametologous genes located in the PAR: *ATP5A1Z—ATP5A1W* and *UBAP2–UBAP2L2* (Sazanov et al. 2006; Blagoveschensky et al. 2011). Here, we cytogenetically localized in the chicken genome *UBE2R2L*,

Table 2 Summarized results for screening the chicken, turkey, zebra finch, white-throated sparrow and California condor BAC libraries using the chicken *UBE2R2L*-derived overgo

Avian species	Library ID	No. of clones	Coverage	No. of positive BACs	Positive BAC IDs	Reference
Chicken	CHORI-261	73,000	11	7	See Table 1	Romanov et al. (2003)
Turkey	CHORI-260	71,000	11.1	3	067M16, 085N18, 114O20	Romanov and Dodgson (2006)
Zebra finch	TG_Ba	147,456	15.5	2	024E22, 025F15	Romanov and Dodgson (2006)
White-throated sparrow	CHORI-264	196,354	21.1	15	002G18, 009P20, 033H09, 037L14, 044L15, 072K22, 080D07, 084C20, 084I16, 098E16, 102G07, 116D17, 143B09, 184F12, 191N02	Romanov et al. (2011)
California condor	CHORI-262	89,665	14	1	037I12	Romanov et al. (2006)

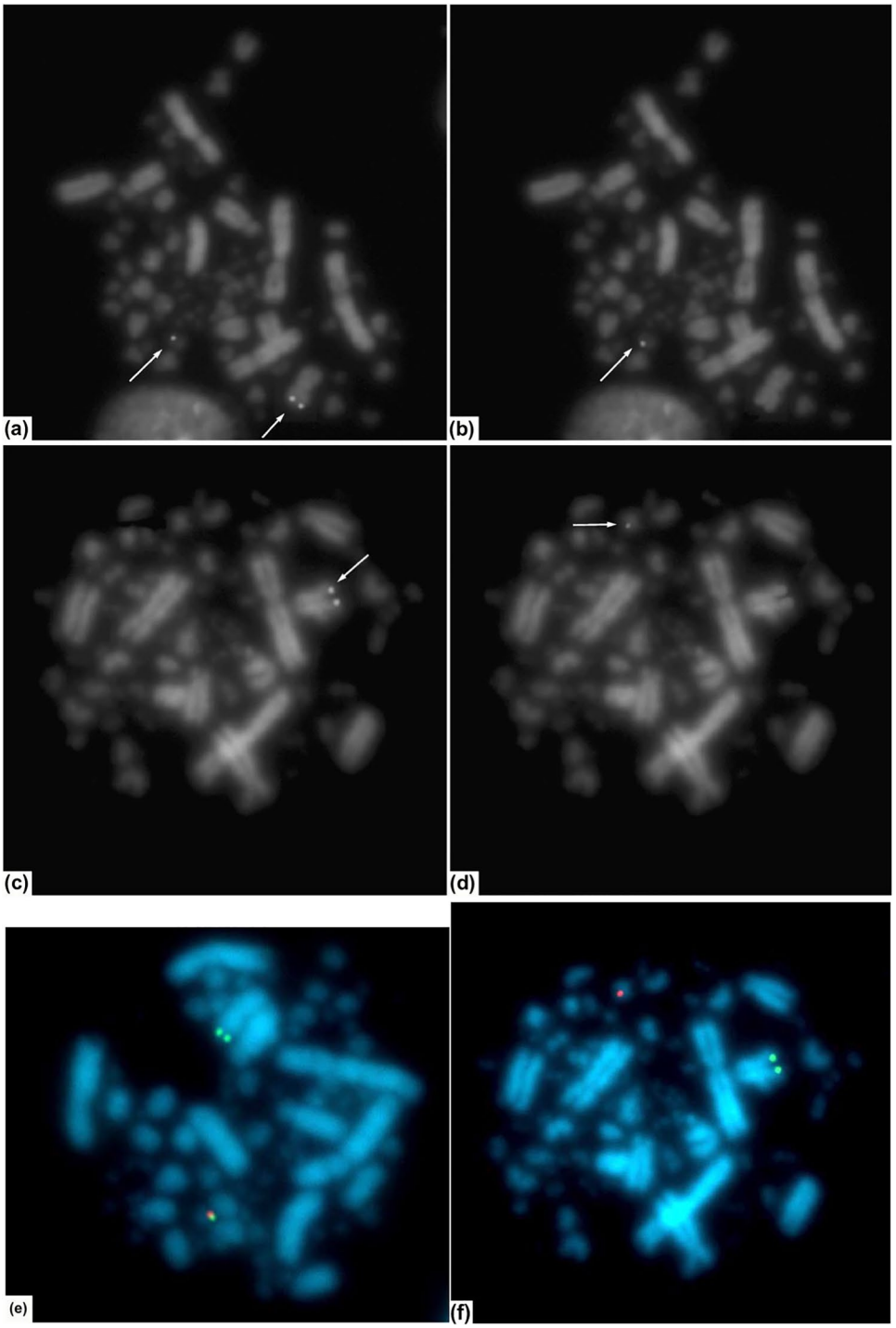

Fig. 4 In situ hybridization of chicken mitotic chromosomes with BAC clone DNA. **a**, **b** A metaphase plate showing localization for CH261-033F10 (**a**, *ATP5A1* on GGAZ and GGAW) and CH261-114G22 (**b**, *UBE2R2L* and *UBAP2L2* on GGAW). **c**, **d** A metaphase plate representing TAM31-099N01 (**c**, *ATP5A1* on GGAZ) and CH261-114G22 (**d**, *UBE2R2L* and *UBAP2L2* on GGAW). **e**, **f** Signal location for CH261-064F22 (green, *ATP5A1* on GGAZ and GGAW) and co-localized CH261-114G22 (red, *UBE2R2L* and *UBAP2L2* on GGAW); and TAM31-100C09 (**f**, green, *ATP5A1* on GGAZ) and CH261-114G22 (red, *UBE2R2L* and *UBAP2L2* on GGAW); same metaphase plate as **c**, **d**. Signals of hybridization are shown by the arrows. Photographs **a**–**d** adopted from Blagoveschensky et al. (2011)

a previously unmapped W-linked homolog of another gametologous pair, *UBE2R2–UBE2R2L*, but were unable to assign *UBE2R2L*-containing BACs to the Z gametolog. On the other hand, BAC filter hybridization using a lambda phage clone containing the W-linked turkey *AD012W* gene, also known as *LOC100303669* or *UBAP2W* (accession numbers AY188758; see for further details Harry et al. 2003; Sazanov et al. 2006; Romanov et al. 2019), resulted in discovery of five positive clones in the Texas A&M University genomic jungle fowl BAC library (code TAM31; Lee et al. 2003; Ren et al. 2003). Sazanov et al. (2006) and Blagoveschensky et al. (2011) cytogenetically assigned these BACs to Z but not to W in the chicken and Japanese quail. That could be because the turkey W-linked lambda phage clone cross-hybridized to the GGAZ-positive chicken BACs but not to GGAW-specific ones. Blagoveschensky et al.

(2011) also mapped three other W-specific chicken clones to both Z and W in the chicken and Japanese quail. This ambiguity in applying successfully clones as FISH probes to another heterochromosome within and between species might depend on how different sequences of particular genomic regions on Z and W are, and, to some extent, how rigorous or relaxed DNA–DNA hybridization conditions are in a given experiment. In any event, it can be deduced that BAC clones cross-hybridizing to opposite gonosomes carry sequences on the Z and W chromosomes with a lesser degree of divergence, which conform to areas of the gonosomes that have recently stopped recombining and are located inside the PAR (Blagoveschensky et al. 2011).

Using the chicken *UBE2R2* mRNA sequence as a query, we additionally performed a BLAST® search as can be viewed in Fig. 5. In this BLAST® tree comprising 25 birds, a basal group was represented by three galliform species, i.e., the chicken, turkey, and rock ptarmigan (*Lagopus muta*), suggesting that their *UBE2R2* genes were most ancient by origin among the compared species. There was another gene cluster for a younger group of waterfowl including five species of geese, swans and ducks (order Anseriformes). A greater divergence relative to the chicken was characteristic of more recent evolutionary clades such as Accipitriformes, Apodiformes, Falconiformes, Psittaciformes and Passeriformes. Tinamou (Palaeognathae) got into this large group only due to availability of a partial mRNA sequence for this species that biased its divergence estimate, although in the respective protein tree (Online resource 1: Fig. S1-5b) it occupied a more basal position. Also, we noticed that some members of a younger order Passeriformes were scattered among various evolutionary tree branches (Fig. 5), which might reflect a perceptible diversification of genome organization within this clade. Similar relationships between various avian clades were also noticed based on *UBE2R2*/*UBE2R2L* gene/protein sequence alignments (Online resource 1: Figs. S1-4 and S1-5). The observed patterns of the *UBE2R2*/*UBE2R2L*-related molecular evolution was largely in agreement with the current general understanding of evolution and taxonomy in the class Aves.

In a previous study (Modi et al. 2009), the California condor clone CHORI-262 37I12 positive for the chicken *UBE2R2L* overgo was cytogenetically mapped by FISH to the condor Z-linked homolog, with no signal being determined on the W chromosome. Similarly, use of the same condor W-linked clone on the black stork chromosomes revealed its localization on Zq near telomere (though the stork W was not looked at; Modi et al. 2009; Fig. 6). In the chicken, the *UBE2R2* gene is located on Zp (Figs. 1 and 3) and demonstrates a pericentric inversion(s) in the zebra finch (Itoh et al. 2006). Similar pericentric inversion(s) of a much greater degree obviously occurred in the California condor and black stork, both species attributed to Ciconiiformes (see, however, a note on this controversial attribution for the condor in Romanov et al. 2009), with their *UBE2R2* genes being on Zq, close to the telomere. The revealed inversion patterns are indicative of specific intrachromosomal rearrangements in the Z chromosome evolution in these four avian lineages. These observations imply that the Z chromosome gene content has been conserved during the 90–100 million years since the split between the chicken and other

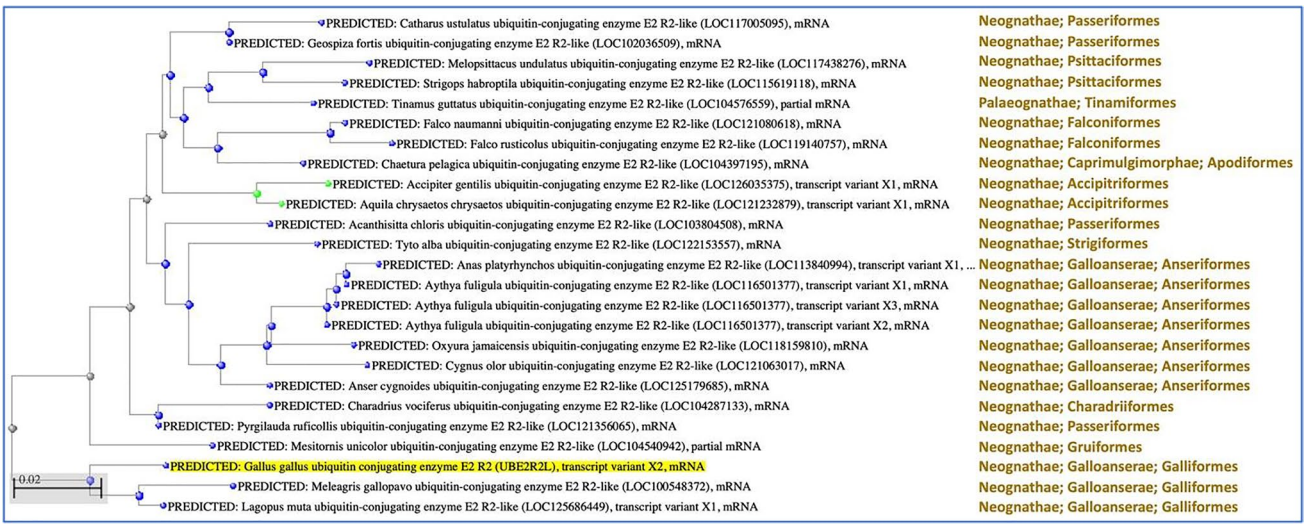

Fig. 5 A distance tree of BLAST® search results produced using BLAST® pairwise alignments between the chicken *UBE2R2L* gene sequence query (XM_025144594.3; 3185 bp) and 24 other sequences. The search was limited to records that include birds (Aves; taxid:8782). Database: nt; tree method: Fast Minimum Evolution; maximum sequence difference: 0.75. The query is highlighted in yellow; green nodes denote hawks and eagles

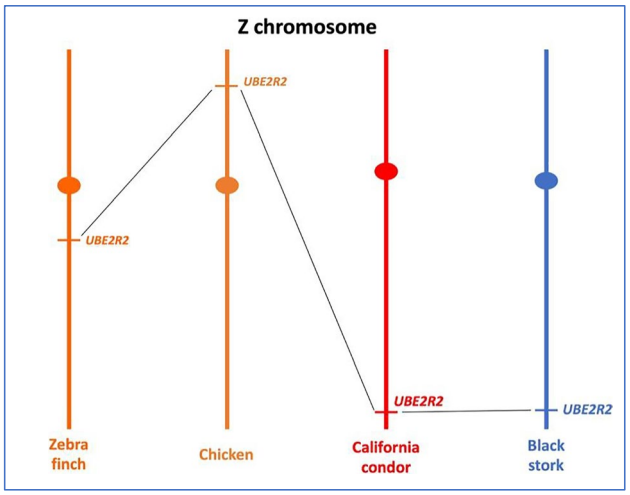

Fig. 6 Comparisons of the Z chromosomes in the zebra finch, chicken, California condor, and black stork using ideograms. Maps for the stork and condor were created using the prior FISH results (Modi et al. 2009), whereas those for the zebra finch and chicken are based on genome sequences

three lineages, while the chromosome has undergone significant reorganization (Itoh et al. 2006).

Conclusions

In the present study, we established a reliable pipeline of bioinformatic, molecular and mapping tools used for genomic analysis and mapping to explore avian sex chromosomes (gonosomes) and genes they harbor. We bioinformatically evaluated in the chicken genome a previously understudied pair of gametologous genes (i.e., Z- and W-linked homologs), *UBE2R2* and *UBE2R2L*, and, using FISH, mapped the W-linked counterpart, *UBE2R2L*. Three of the seven used BAC clones were also cytogenetically assigned to GGAW and co-localized with another sex chromosome-linked *UBAP2L2* gene. Although the chicken *UBE2R2L* (i.e. W-linked) clones were FISH mapped to GGAW, they did not cross-hybridize to GGAZ, which was in accord with other similar observations on inconsistent cross-hybridization of specific clones between the two heterochromosomes within and among species. Overall, this and other relevant investigations have established that cross-heterochromosome and interspecies hybridization can be useful for the scanning of avian BAC libraries and the FISH mapping of individual genes (including gametologs), genomic regions (like PAR), and whole chromosomes. This integrative research approach is instrumental in comprehending the evolutionary history in birds that will be further illuminated by future cytogenomic studies of various Aves clades.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11756-023-01395-6.

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Elaina M. Tuttle died before this paper was submitted to the journal.

Authors' contributions Conceptualization: A.A. Sazanov and M.N. Romanov; Methodology: M.N. Romanov, A.A. Sazanov and A.L. Sazanova; Formal analysis and investigation: M.N. Romanov; Writing – original draft preparation: A.A. Sazanov and M.N. Romanov; Writing – review and editing: D.K. Griffin and M.N. Romanov; Funding acquisition: A.A. Sazanov; Resources: M.D. Nefedov; Supervision: A.A. Sazanov and D.K. Griffin.

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Data availability All data generated or analysed during this study are included in this published article and its supplementary information files.

Declarations

Conflict of interest On behalf of all authors, the corresponding authors state that there is no conflict of interest.

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