Original Article

Cytogenetic and Genome Research

Cytogenet Genome Res 2009;126:281–304
DOI: 10.1159/000251965

Accepted after revision: June 26, 2009
by J. Smith

Cross-Species Chromosome Painting Corroborates Microchromosome Fusion during Karyotype Evolution of Birds

T. Hansmann[a] I. Nanda[a] V. Volobouev[c] F. Yang[d] M. Schartl[b] T. Haaf[a]
M. Schmid[a]

Departments of [a]Human Genetics, and [b]Physiological Chemistry I, University of Würzburg, Würzburg, Germany;
[c]Muséum National d'Histoire Naturelle, UMR 5202 'Origine, Structure et Evolution de la Biodiversité', Paris, France;
[d]Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Cambridge, UK

Key Words
Aves · Chromosome painting · Karyotype evolution · Microchromosomes · Stone curlew · Zoo-FISH

Abstract
The stone curlew, also known as thick-knee (*Burhinus oedicnemus,* BOE), represents a phylogenetically young species of the shorebirds (Charadriiformes) that exhibits one of the most atypical genome organizations known within the class of Aves, due to an extremely low diploid number (2n = 42) and only 6 pairs of microchromosomes in its complement. This distinct deviation from the 'typical' avian karyotype is attributed to repeated fusions of ancestral microchromosomes. In order to compare different species with this atypical avian karyotype and to investigate the chromosome rearrangement patterns, chromosome-specific painting probes representing the whole genome of the stone curlew were used to delineate chromosome homology between BOE and 5 species belonging to 5 different avian orders: herring gull (Charadriiformes), cockatiel (Psittaciformes), rock pigeon (Columbiformes), great gray owl (Strigiformes) and Eurasian coot (Gruiformes). Paints derived from the 20 BOE autosomes delimited 28 to 33 evolutionarily conserved segments in the karyotypes of the 5 species, similar to the number recognized by BOE paints in such a basal lineage as the chicken (28 conserved segments). This suggests a high degree of conservation in genome organization in birds. BOE paints also revealed some species-specific rearrangements. In particular, chromosomes BOE1–4 and 14, as well as to a large extent BOE5 and 6, showed conserved synteny with macrochromosomes, whereas homologous regions for BOE7–13 are found to be largely distributed on microchromosomes in the species investigated. Interestingly, the 6 pairs of BOE microchromosomes 15–20 appear to have undergone very few rearrangements in the 5 lineages investigated. Although the arrangements of BOE homologous segments on some chromosomes can be explained by complex fusions and inversions, the occurrence of homologous regions at multiple sites may point to fission of ancestral chromosomes in the karyotypes of the species investigated. However, the present results demonstrate that the ancestral microchromosomes most likely experienced fusion in the stone curlew lineage forming the medium-sized BOE chromosomes, while they have been conserved as microchromosomes in the other neoavian lineages.

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1424–8581/09/1263–0281$26.00/0

Accessible online at:
www.karger.com/cgr

Michael Schmid
Department of Human Genetics, University of Würzburg, Biocenter, Am Hubland
DE–97074 Würzburg (Germany)
Tel. +49 931 31 84077, Fax +49 931 888 4058
E-Mail m.schmid@biozentrum.uni-wuerzburg.de

The class Aves constitutes more than 10,000 species [Chiappe and Dyke, 2006], which are highly divergent in their morphology, behavior, breeding system and adaptation to different habitats. Many of these morphological features are the targets of both natural and sexual selection. Consequently, genetic changes resulting in variation of morphological traits may play an important role in avian speciation. However, despite their diverse features, the overall genomic organization of birds is rather uniform as based on their small genome sizes [Burt et al., 1999] and their relatively similar diploid chromosome number. In about 63% of the extant species the diploid chromosome number ranges from 74 to 86 [Christidis, 1990], whereas 24% of the karyotypes consist of 66 to 74 chromosomes. The low variation of the diploid chromosome number in birds is particularly striking when considering the rapid chromosomal changes in mammals that are associated with a distinct variation in chromosome number [Yang et al., 1997; Wienberg, 2004]. The typical hallmark of avian cytogenetics is characterized by the presence of 7–10 pairs of large and medium-sized macrochromosomes in combination with numerous indistinguishable microchromosomes. The latter are thought to be an ancestral feature, as they are also described for some representatives of lower vertebrates, but they became a universal feature of bird karyotypes. The avian microchromosomes are gene-rich [Smith et al., 2000] as well as enriched for CpG islands [McQueen et al., 1996], but their numbers show remarkable interspecific variation between karyotypes. How these variable numbers of microchromosomes are sustained in different species is poorly elucidated. Although the typical avian karyotype is retained among the majority of birds, the karyotypes of some birds are atypical. Especially the karyotypes of the diurnal birds of prey (Accipitridae; Falconiformes) display a moderate diploid chromosome number [de Boer, 1976; Nanda et al., 2006], distinguished by a large number of medium-sized macrochromosomes and a low number of microchromosomes, which can even be reduced to 1 single pair [Bed´Hom et al., 2003]. More striking is the situation in the stone curlew, *Burhinus oedicnemus* (Charadriiformes), in which the karyotype comprises the lowest known diploid chromosome number, 2n = 42 [Nie et al., 2009]. In all these atypical karyotypes the chromosome rearrangements favor an increase of the number of macrochromosomes, associated with a decline of the number of microchromosomes. The general assumption is that microchromosomes have originated through fission of ancestral chromosomes [Takagi and Sasaki, 1974], but then these microchromosomes have evolved further by undergoing fusions in some lineages, or even splittings by further fissions [Burt, 2002].

Although both nuclear and mitochondrial DNA sequences are extensively analyzed to understand the evolutionary relationships among major avian groups, the molecular phylogeny in birds remains difficult, partially due to their rapid divergence in early evolutionary history [Ericson et al., 2006; Hackett et al., 2008]. However, there are 2 basal divergences in the tree of living birds that are consistently supported by both morphological and molecular phylogenetic studies. The first divergence split the Aves leading to the lineages palaeognaths (ratites and tinamous) and neognaths during the middle Cretaceous about 100–120 million years ago. The second split, separating the neognaths into Galloanserae (chickens, ducks, etc.) and Neoaves, occurred approximately 100 million years ago, only a short time after the first split [van Tuinen and Hedges, 2001]. The patterns of diversification and the precise divergence times during evolution of the Neoaves, which include nearly 95% of all bird species, are still poorly inferred [Ericson et al., 2006; Hackett et al., 2008].

Although the broad picture of phylogeny of neoavian species remains largely unresolved, karyotype-based phylogenetic relationships of the extant bird species are increasingly elucidated by comparative genome studies. Especially cross-species chromosome painting (Zoo-FISH) is a powerful approach that allows the recognition of homologous chromosome segments between closely and distantly related species, as well as between taxa that are karyotypically divergent, thus revealing detailed pictures of genome rearrangements during speciation. Cross-species chromosome painting has already been used successfully in mammals [Chowdhary and Raudsepp, 2001; Yang et al., 2003; Ferguson-Smith and Trifonov, 2007; Graphodatsky et al., 2008] and currently, avian karyotype evolution is also increasingly studied and resolved by this method. Within the class Aves, the chicken (*Gallus gallus*, GGA) undoubtedly serves as a model organism and reference bird since it represents the best studied avian species [Schmid et al., 2005]. The GGA karyotype is considered to be closely related to the putative ancestral bird karyotype [Schmid et al., 2000; Shibusawa et al., 2004a; Griffin et al., 2007], and its genome is the only one in the avian class that has undergone whole-genome sequencing [Hillier et al., 2004]. Up to date about 40 bird species belonging to 10 orders have been analyzed by Zoo-FISH with chicken paints derived from flow-sorted chromosomes [Shetty et al., 1999; Schmid et al., 2000; Raudsepp et al., 2002; Guttenbach et al., 2003; Kasai et al.,

2003; Derjusheva et al., 2004; Shibusawa et al., 2004a, b; de Oliveira et al., 2005, 2008; Nanda et al., 2006, 2007; Nishida-Umehara et al., 2007; Nishida et al., 2008]. Comparative analyses with chicken paints in different birds have successfully contributed to trace evolutionary relationships among diverse species, particularly providing insights into the chromosomal reorganization of the ancestral bird karyotype. With only a few exceptions [de Oliveira et al., 2005; Nanda et al., 2006], the chicken chromosome paints revealed a high degree of conservation in avian genomes with a remarkably small number of variations and changes over millions of years [Griffin et al., 2007; Nanda et al., 2007].

Comparative chromosome painting across avian species is mostly based on the paints derived from the chicken macrochromosomes 1–10 [Shetty et al., 1999; Raudsepp et al., 2002; Guttenbach et al., 2003; Shibusawa et al., 2004a, b]. Therefore, a genome-wide interspecific comparison will not be feasible without carefully assessing the rearrangements involving the microchromosomes. Comparative karyotype studies using the Zoo-FISH technique with the objective of unraveling the microchromosomal evolution require chromosome-specific probes of single microchromosomes. Numerous attempts using flow-sorting and microdissection to generate such microchromosome-specific paints have been of limited success [Grutzner et al., 2001; Masabanda et al., 2004], and only few data from cross-species FISH studies using multiple sets of microchromosome probes are available [Derjusheva et al., 2004; de Oliveira et al., 2005; Griffin et al., 2008]. In this context, birds showing an atypical karyotype with a low number of microchromosomes will be ideal to generate chromosome-specific paints to perform genome-wide comparative studies. It is anticipated that in the case of a previous large-scale exchange between micro- and macrochromosomes, chromosome-specific painting probes of such an atypical karyotype promise to reveal a high amount of rearrangements when hybridized on metaphase preparations of species with a typical avian genome organization.

Recently, a complete set of painting probes covering the whole genome of the stone curlew (*B. oedicnemus*, BOE, 2n = 42), a neoavian species (Charadriiformes) exhibiting the lowest diploid chromosome number known in birds, has been generated [Nie et al., 2009]. The stone curlew belongs to a distinct monophyletic clade of the shorebirds, the Charadrii [Fain and Houde, 2007]. It is also a relatively young species, as the genus *Burhinus* split from its sister genus *Esacus* only in the late Eocene period [Baker et al., 2007]. The chromosome-specific BOE painting probes have been generated through flow-sorting of individual chromosomes and amplification by DOP-PCR to establish a genome-wide comparative map between the chicken and the stone curlew by reciprocal chromosome painting [Nie et al., 2009]. In the present study, the chromosome-specific painting probes of the stone curlew were applied to perform a whole-genome comparison with 5 neoavian species belonging to different orders by Zoo-FISH. This study provides new insights into the microchromosome evolution and contributes to the delineation of the interordinal relationships of chromosomes in the Neoaves.

Materials and Methods

Animals and Chromosome Preparation

Five representative species belonging to different orders were analyzed in the present study: *Nymphicus hollandicus* (2n = 72), NHO (Cacatuidae, Psittaciformes); *Larus argentatus* (2n = 70), LAR (Laridae, Charadriiformes); *Columba livia* (2n = 80), CLI (Columbidae, Columbiformes); *Strix nebulosa* (2n = 82), SNE (Strigidae, Strigiformes) and *Fulica atra* (2n = 92), FAT (Rallidae, Gruiformes). Mitotic chromosomes were prepared from fibroblast cell cultures following the standard procedures [Schmid et al., 1989].

Painting Probes and Fluorescence in situ Hybridization

The stone curlew chromosome-specific probes were generated from flow-sorted chromosomes and amplified by DOP-PCR [Nie et al., 2009]. Since chromosomes 15 and 16 and 17–20, respectively, were co-sorted as 1 peak each, these paints containing multiple chromosomes were hybridized together. The individual chromosome paints were labeled with biotin-16-dUTP or digoxigenin-11-dUTP (Roche Diagnostics, Mannheim, Germany) via DOP-PCR using the 6-MW primer [Telenius et al., 1992]. Hybridization of the probes and their detection followed the procedure described in Guttenbach et al. [2003]. The slides were mounted in Vectashield supplemented with DAPI (Vector Laboratories, Burlingame, Calif., USA) and examined by digital fluorescence microscopy using the Applied Spectral Imaging software (Neckarhausen, Germany). For 2-color chromosome painting, 2 BOE chromosome paints were separately labeled with biotin-16-dUTP and digoxigenin-11-dUTP, respectively, and hybridized together onto the same metaphase preparation. The hybridization sites were detected as previously described [Nanda et al., 2007].

Results

Chromosome homology between the stone curlew (BOE) and 5 different Neoaves species belonging to 5 different orders was established by hybridization of the whole set of BOE chromosome-specific painting probes onto metaphases of *L. argentatus* (LAR), *N. hollandicus*

(NHO), *C. livia* (CLI), *S. nebulosa* (SNE) and *F. atra* (FAT). The diploid number in these species ranges from 70 to 92 and 10 pairs of macrochromosomes can be outlined apart from the sex chromosomes. According to the recent analysis on the stone curlew karyotype, 6 of the 20 autosomal pairs are microchromosomes which are too small to be distinguished from one another [Nie et al., 2009]. All the BOE paints revealed clear and consistent signals on the chromosomes of the species investigated. Metaphases displaying distinct hybridization signals were karyotyped to identify those chromosomes that are homologous to the corresponding BOE chromosomes. The results are displayed in figures 1–5 for each species. In order to delineate associations between different conserved segments in the rearranged chromosomes dual-color FISH was performed (fig. 6). Furthermore, a schematic comparative summary of the hybridization patterns is given to provide an overview on the arrangement of corresponding BOE-conserved segments in the different karyotypes (fig. 7).

Chromosome Homology between the Stone Curlew (BOE) and Larus argentatus *(LAR)*

Since the diploid chromosome number of *L. argentatus* has not been fully established [Christidis, 1990; 2n = 66–70], a large number of DAPI-stained metaphases were evaluated, and the diploid chromosome number of 70 determined. Paints from the 20 BOE autosomes detected 28 distinct regions in 24 different chromosomes of the herring gull (fig. 1a, b). Seven paints (BOE1–4, 6, 8 and 14) each delineated 1 homologous chromosome or chromosomal segment. Among these, BOE6 is the only probe which does not paint the complete chromosome but labels the long arm of chromosome 4 exclusively. Compared to the other 4 bird species examined, *L. argentatus* is the only species in which BOE8 hybridizes to 1 complete chromosome. Interestingly, 4 of the medium-sized LAR chromosomes (4, 6–8) display signatures of 2 different BOE probes (figs. 1a, 7a) which would signify a major rearrangement for these BOE chromosomes in the LAR lineage: BOE5 hybridizes with both the long arms of LAR7 and LAR8. The paint specific to BOE7 recognizes 3 chromosomes simultaneously in the LAR karyotype, and the hybridization signals are located on 6p, 7p and on about 30–40% of LAR6q as well as on 1 additional microchromosome. BOE9 hybridizes to the distal part of chromosome 6q and additionally to 1 microchromosome. The signals of the BOE10 paint are also located on the short arms of both chromosomes LAR4 and 8. The smaller BOE macrochromosomes 11–13 each detected 2 microchromosomes. The corresponding regions of the small microchromosomes 15–20 were found to be on microchromosomes. The 2 paints containing 2 chromosomes (15/16) and 4 smaller BOE microchromosomes (17–20), respectively, both produced signals on 3 microchromosomes.

To visualize that some of the conserved segments from different BOE chromosomes are located on the same chromosomes of the herring gull, dual-color chromosome painting was performed using 2 BOE paints (BOE6 and 10 and BOE7 and 9) that clearly substantiated the observation from the single-paint hybridization revealing the association of BOE6 and BOE10 on LAR4, as well as the association of BOE7 and BOE9 on LAR6 (fig. 6a, b).

Chromosome Homology between the Stone Curlew (BOE) and Nymphicus hollandicus *(NHO)*

In the cockatiel, the 16 BOE autosome paints recognized 30 homologous segments on 25 different chromosomes (fig. 2a, b). Compared to the other 4 species examined, the karyotype of the cockatiel exhibits the most complex hybridization pattern. The paints derived from 7 BOE chromosomes (2–4, 6, 10, 13 and 14) each hybridized to 1 entire NHO chromosome pair with the exception of BOE4 which only highlights the long arm of NHO4. All the remaining BOE macrochromosomes displayed split hybridization signals in NHO metaphase spreads. The probe from BOE1 paints 2 macrochromosomes (NHO3 and 6) entirely. Remarkably, segments from 3 different BOE chromosomes (4, 5 and 7) were found to be associated on NHO4. The long arm of NHO4 is entirely homologous to BOE4, whereas the short arm was partially labeled by BOE5 at the distal and by BOE7 at the proximal end, respectively. Furthermore, BOE7 identifies 2 additional microchromosomes. It is especially noteworthy that besides detecting the short arm of chromosome 4, BOE5 paints 2 distinct regions on the long arm of NHO5. The hybridization signal of BOE9 also revealed split signals on the same chromosome 5 as observed with BOE5. The signals for both BOE5 and BOE9 were found to be interspersed with one another on the acrocentric chromosome NHO5, revealing a complex association between the corresponding regions of these 2 BOE chromosomes. This pattern of hybridization would, therefore, indicate that NHO5 is derived from a tandem fusion of BOE5 and BOE9 followed by a paracentric inversion. These complex patterns of hybridization on NHO4 and NHO5 were further confirmed by hybridizing metaphases with BOE5 and BOE9 paints simultane-

ously (fig. 6d). Likewise, association between the conserved segments of BOE7 and BOE4 on NHO4 was demonstrated by 2-color chromosome painting (fig. 6c). Compared to the other species, NHO is the only one in which BOE10 and BOE13 labeled a single microchromosome. Aside from BOE5 and 9, BOE8, 11 and 12 paints each hybridized with 2 pairs of chromosomes, most of which appear to be microchromosomes. The hybridization pattern of the probes from microchromosomes (15/16 and 17–20) was identical to the pattern noted in LAR.

Chromosome Homology between the Stone Curlew (BOE) and Columba livia *(CLI)*

The BOE autosomes show homology with altogether 32 different chromosomes in the rock pigeon, whereupon no CLI chromosome has been detected simultaneously by 2 or more different BOE painting probes (fig. 3a, b). Probes derived from 6 BOE chromosomes (1–4, 6 and 14) each hybridized to 1 entire macro- or microchromosome pair, whereas the paints of BOE5 and BOE8–12 each mark 2 chromosomes in the CLI karyotype. BOE7 produced hybridization signals on 3 CLI chromosomes. The mixed paint containing BOE15 and BOE16 hybridizes to 3 different microchromosomes (fig. 3b). The BOE paints 13 and 17–20 showed hybridization signals on multiple tiny microchromosomes with variable hybridization intensities. Such variable patterns of signals are most likely the result of cross-hybridizations since the microchromosomes of the pigeon exhibit a high content of constitutive heterochromatin [Stefos and Arrighi, 1971]. Considering only those microchromosomes that showed strong fluorescence signals, probably deriving from specific hybridization, we spotted at least 4 hybridization signals for the BOE painting probes 13 and 17–20, while further signals could not be verified to be of homologous origin.

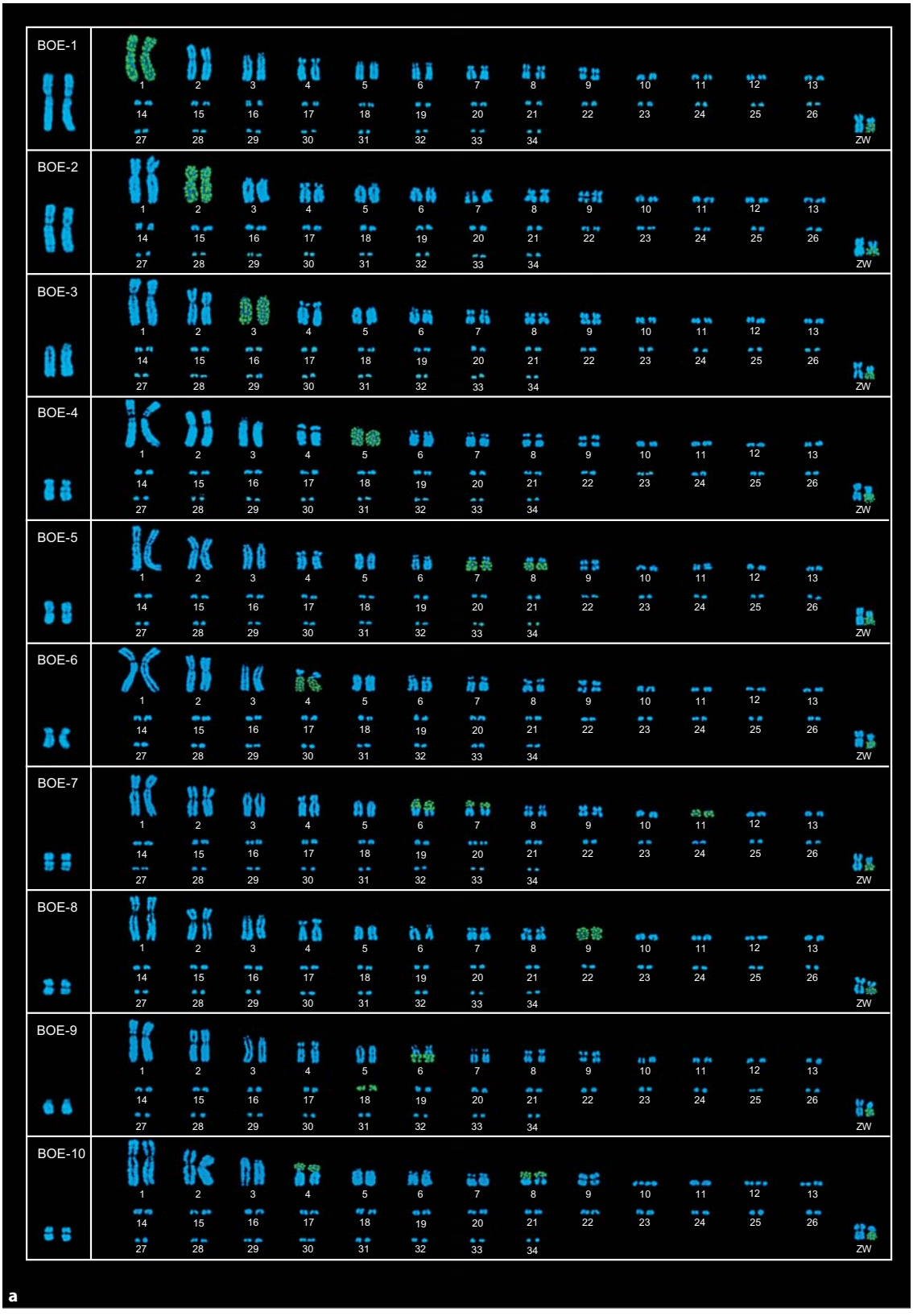
Chromosome Homology between the Stone Curlew (BOE) and Strix nebulosa *(SNE)*

The 16 BOE autosomal paints highlight 32 distinct chromosomal segments that are distributed over 31 different chromosomes in the great gray owl (fig. 4a, b). As observed for the cockatiel, BOE1 also shows homology with 2 macrochromosomes of *S. nebulosa* (SNE3 and 5). Four paints (BOE 2–4 and 14) each delineated 1 homologous chromosome or chromosomal segment. BOE4 hybridizes to the complete long arm of chromosome 2 instead of to an entire chromosome as noted for the other 3 BOE paints. The short arm of chromosome 2 is labeled by BOE6 which also paints an additional microchromosome. No double hybridization was performed since SNE2 is the largest submetacentric chromosome in the karyotype allowing unambiguous identification of the association between the corresponding regions of BOE4 and BOE6 in SNE2. As observed in the 3 aforementioned species, hybridization of BOE5 and 8–12 paints each displayed signals on 2 chromosomes whereas homology for BOE7 and BOE13 could be demonstrated on 3 different small chromosomes. The location of hybridization signals for the BOE microchromosomes (15/16 and 17–20) was comparable to that observed in the karyotype of LAR and NHO.

Chromosome Homology between the Stone Curlew (BOE) and Fulica atra *(FAT)*

In total, the 16 BOE autosomal paints revealed 33 distinct hybridization signals on 31 different chromosomes (fig. 5a, b). BOE1–3 and 14 each recognize 1 entire FAT chromosome, whereas the signals specific to BOE4, 6, and 8–12 are distributed over 2 chromosomes. Both BOE4 and BOE6 identify the same chromosome each revealing homology with one arm of the metacentric FAT4.

BOE7 detects 3 complete chromosomes which is consistent with the number of segments marked in the 4 other species. Like BOE7, also BOE5 recognizes 3 different chromosomes in *F. atra*. The hybridization signals corresponding to BOE5 are located on 1 arm of the medium-sized metacentric chromosome 5, along with additional signals on 2 small chromosomes of the Eurasian coot. The other arm of the metacentric FAT5 is labeled by BOE9 which also shows hybridization signals on an additional microchromosome pair. Since the karyotype of the Eurasian coot comprises 2 metacentric chromosomes of identical size (FAT4 and 5), the allocation of the specific BOE paints to the chromosome arms may not be accurately assigned by the single-hybridization experiments. Therefore, the specific association of BOE chromosomes on these metacentric chromosomes of the coot was outlined by co-hybridization with 2 BOE paints simultaneously, 5 and 9 and 4 and 6, respectively. Hybridization of both biotin- as well as digoxigenin-labeled paints detected by distinguishable rhodamine and fluorescein fluorescence (red and green) clearly showed the location of homologous segments corresponding to BOE5 and BOE9 on FAT5 (fig. 6e), as well as the association of BOE4 and BOE6 segments on FAT4 (not shown). BOE13 painted 3 FAT microchromosomes and the hybridization of the remaining 2 BOE paints (15/16 and 17–20) was found to be identical to the pattern detected in LAR, NHO and SNE.


BOE-1
BOE-2
BOE-3
BOE-4
BOE-5
BOE-6
BOE-7
BOE-8
BOE-9
BOE-10
1 2 3 4 5 6 7 8 9 10 11 12 13
14 15 16 17 18 19 20 21 22 23 24 25 26
27 28 29 30 31 32 33 34
ZW
a

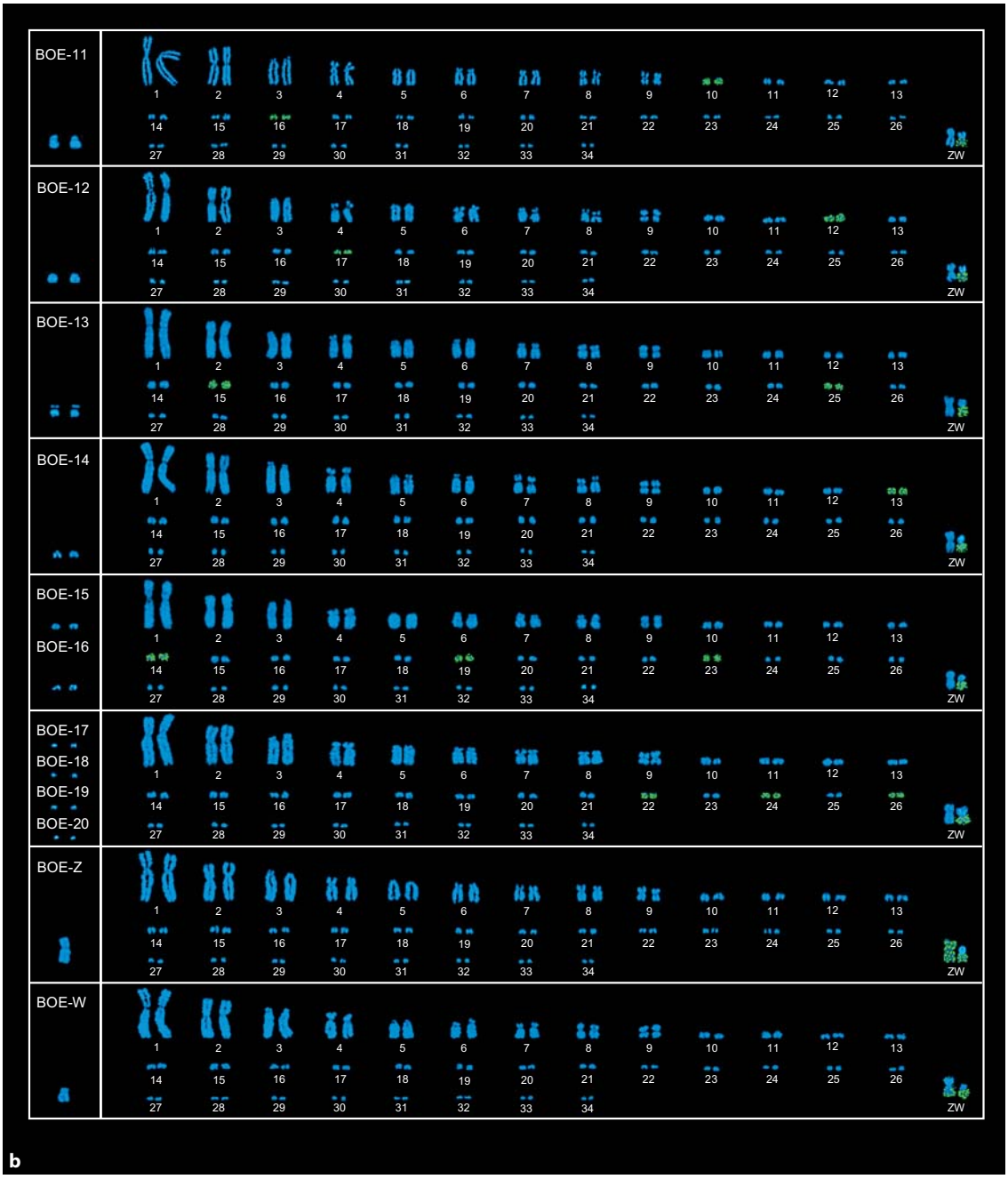
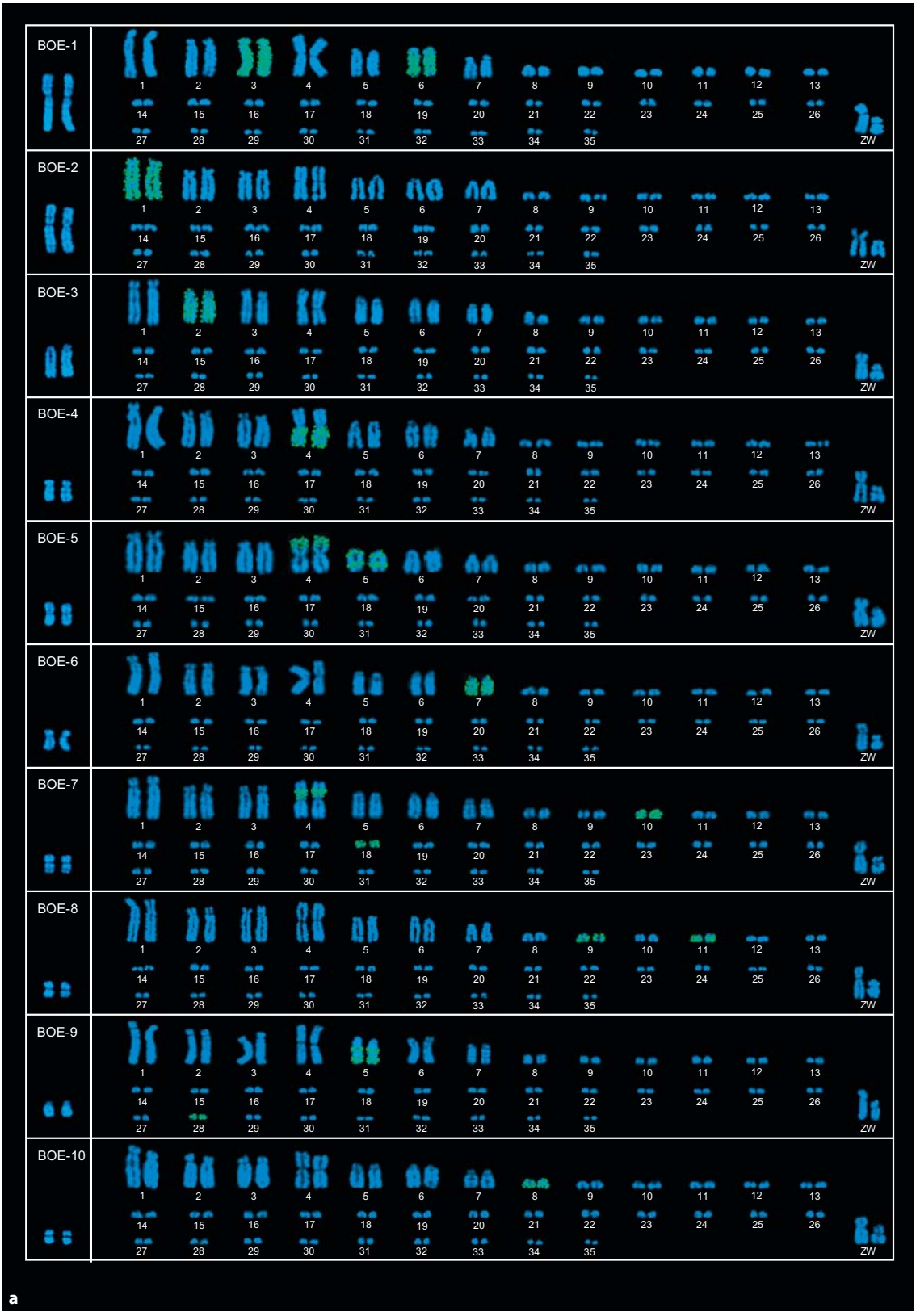


Fig. 1. FISH of the stone curlew (BOE) chromosome-specific paints on chromosomes of *L. argentatus* (LAR). The hybridization patterns (green signals) are displayed in the DAPI-counterstained female karyotype. **a** BOE chromosome-specific paints 1–10. **b** BOE chromosome-specific paints 11–20 and the BOE Z- and W-specific paints. Note that the microchromosomes 15 and 16 and 17–20 form 2 painting probes.


BOE-1
BOE-2
BOE-3
BOE-4
BOE-5
BOE-6
BOE-7
BOE-8
BOE-9
BOE-10
1 2 3 4 5 6 7 8 9 10 11 12 13
14 15 16 17 18 19 20 21 22 23 24 25 26
27 28 29 30 31 32 33 34 35
ZW
a

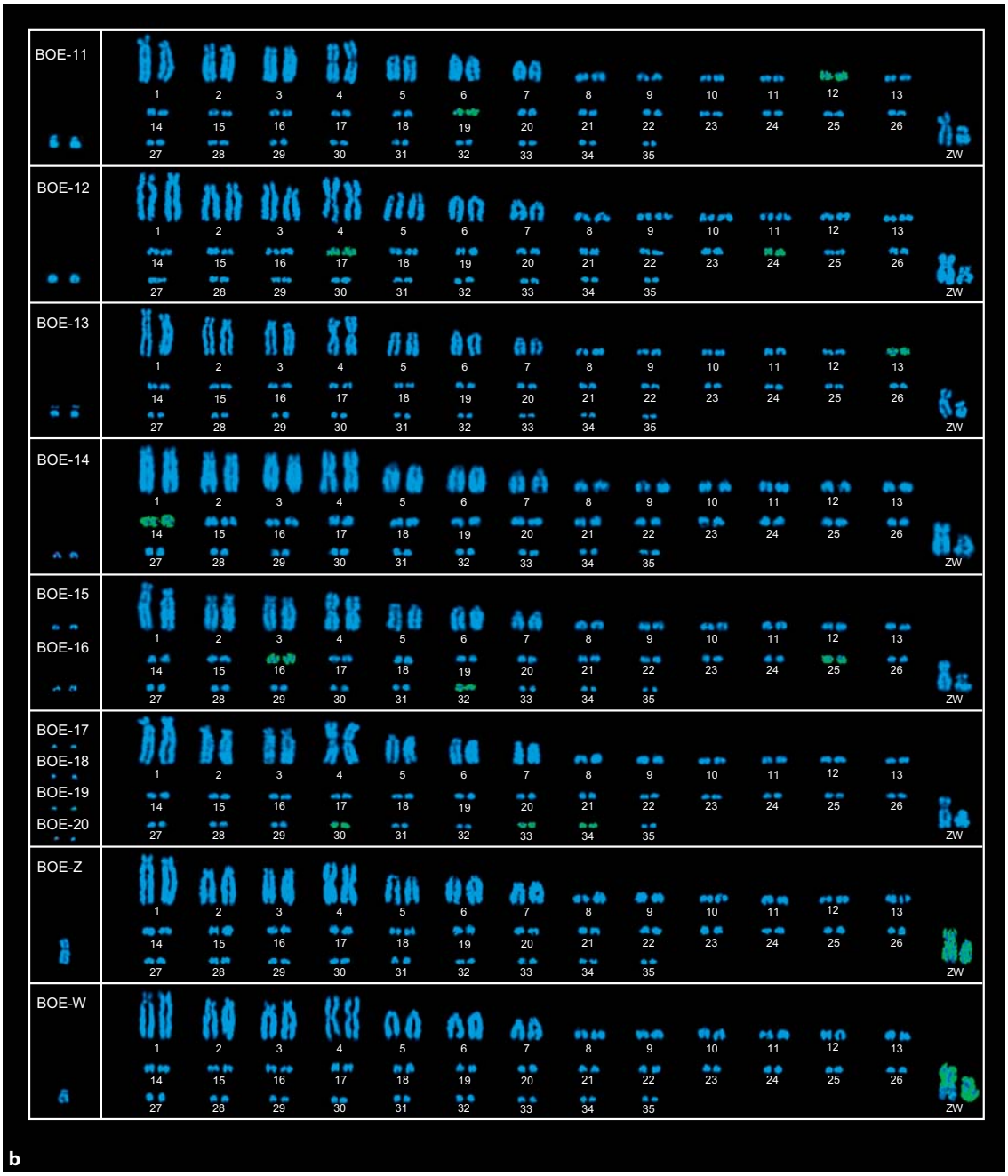
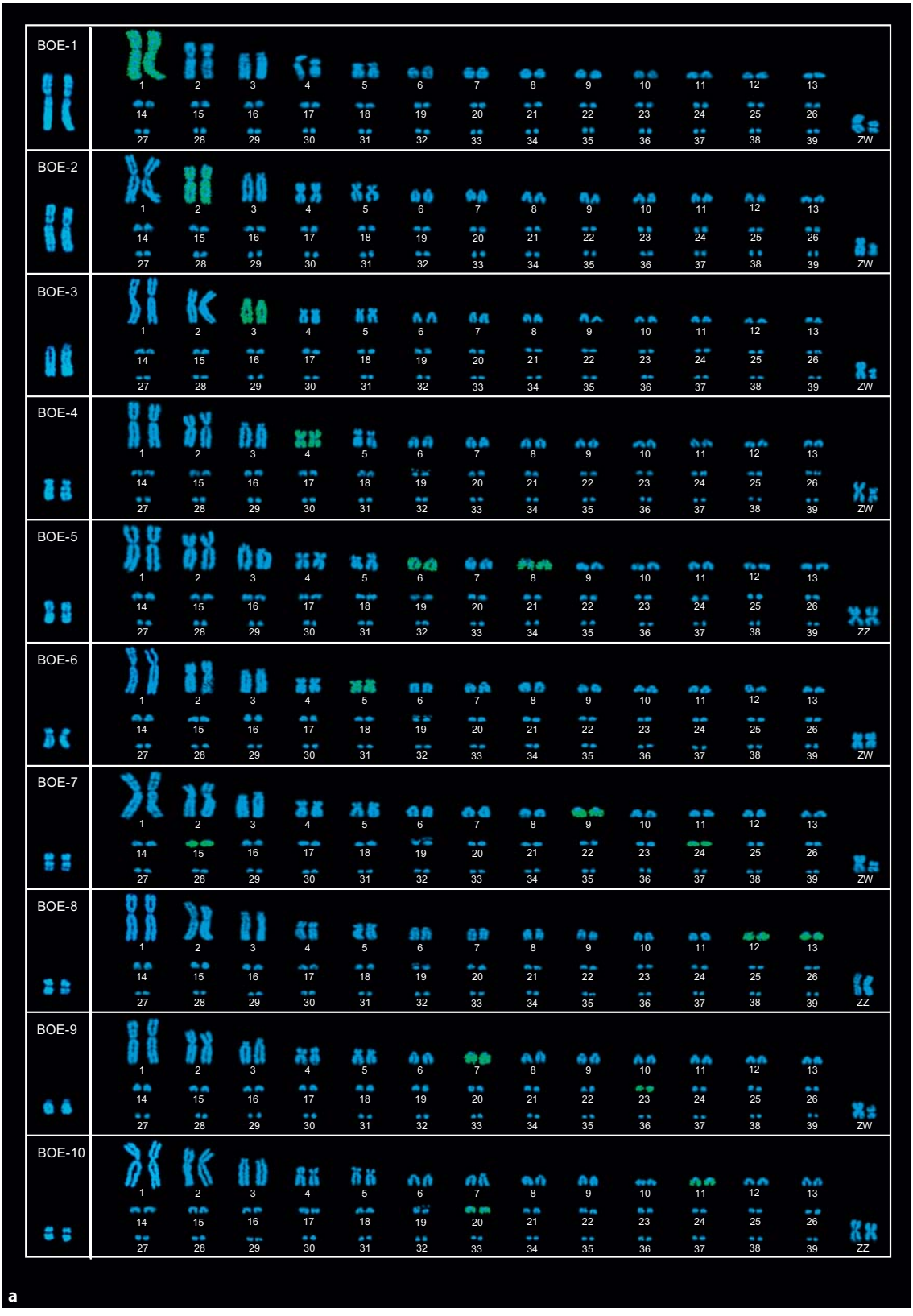


Fig. 2. FISH of the stone curlew (BOE) chromosome-specific paints on chromosomes of *N. hollandicus* (NHO). The hybridization patterns (green signals) are displayed in the DAPI-counterstained female karyotype. **a** BOE chromosome-specific paints 1–10. **b** BOE chromosome-specific paints 11–20 and the BOE Z- and W-specific paints. Note that the microchromosomes 15 and 16 and 17–20 form 2 painting probes.


BOE-1
1 2 3 4 5 6 7 8 9 10 11 12 13
14 15 16 17 18 19 20 21 22 23 24 25 26
27 28 29 30 31 32 33 34 35 36 37 38 39 ZW
BOE-2
1 2 3 4 5 6 7 8 9 10 11 12 13
14 15 16 17 18 19 20 21 22 23 24 25 26
27 28 29 30 31 32 33 34 35 36 37 38 39 ZW
BOE-3
1 2 3 4 5 6 7 8 9 10 11 12 13
14 15 16 17 18 19 20 21 22 23 24 25 26
27 28 29 30 31 32 33 34 35 36 37 38 39 ZW
BOE-4
1 2 3 4 5 6 7 8 9 10 11 12 13
14 15 16 17 18 19 20 21 22 23 24 25 26
27 28 29 30 31 32 33 34 35 36 37 38 39 ZW
BOE-5
1 2 3 4 5 6 7 8 9 10 11 12 13
14 15 16 17 18 19 20 21 22 23 24 25 26
27 28 29 30 31 32 33 34 35 36 37 38 39 ZZ
BOE-6
1 2 3 4 5 6 7 8 9 10 11 12 13
14 15 16 17 18 19 20 21 22 23 24 25 26
27 28 29 30 31 32 33 34 35 36 37 38 39 ZW
BOE-7
1 2 3 4 5 6 7 8 9 10 11 12 13
14 15 16 17 18 19 20 21 22 23 24 25 26
27 28 29 30 31 32 33 34 35 36 37 38 39 ZW
BOE-8
1 2 3 4 5 6 7 8 9 10 11 12 13
14 15 16 17 18 19 20 21 22 23 24 25 26
27 28 29 30 31 32 33 34 35 36 37 38 39 ZZ
BOE-9
1 2 3 4 5 6 7 8 9 10 11 12 13
14 15 16 17 18 19 20 21 22 23 24 25 26
27 28 29 30 31 32 33 34 35 36 37 38 39 ZW
BOE-10
1 2 3 4 5 6 7 8 9 10 11 12 13
14 15 16 17 18 19 20 21 22 23 24 25 26
27 28 29 30 31 32 33 34 35 36 37 38 39 ZZ
a

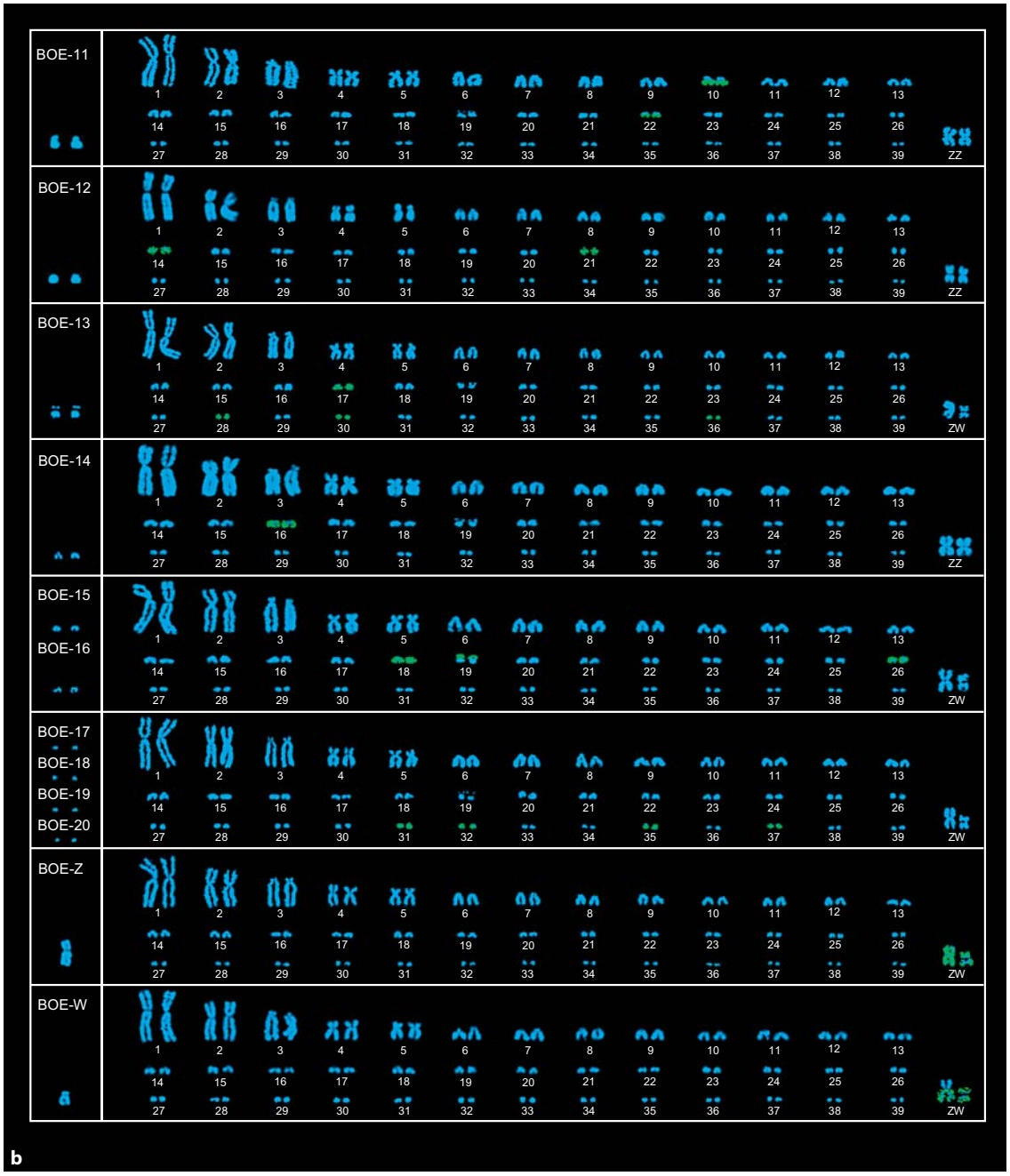
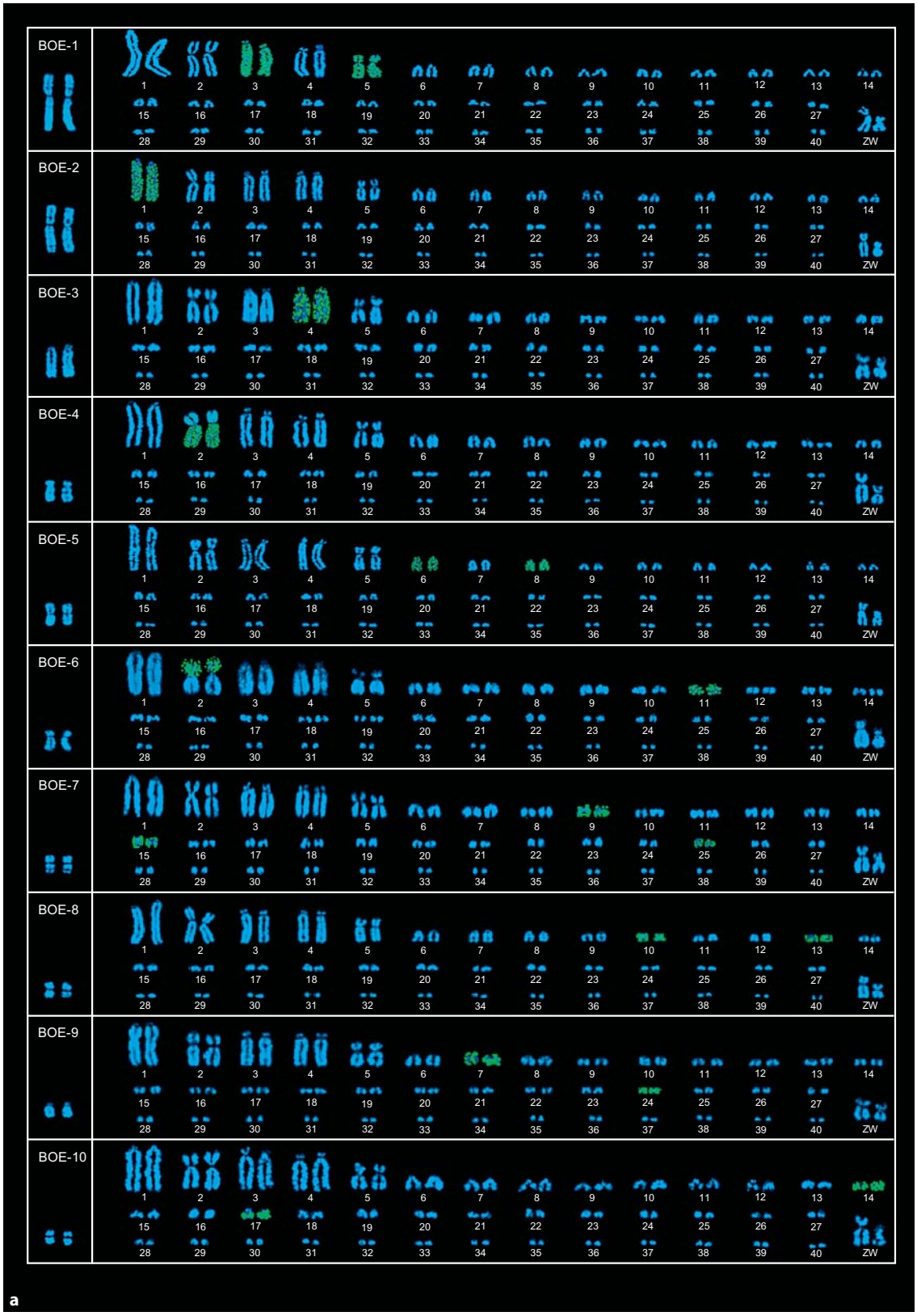


Fig. 3. FISH of the stone curlew (BOE) chromosome-specific paints on chromosomes of *C. livia* (CLI). The hybridization patterns (green signals) are displayed in the DAPI-counterstained male or female karyotypes. **a** BOE chromosome-specific paints 1–10. **b** BOE chromosome-specific paints 11–20 and the BOE Z- and W-specific paints. Note that the microchromosomes 15 and 16 and 17–20 form 2 painting probes.


BOE-1
BOE-2
BOE-3
BOE-4
BOE-5
BOE-6
BOE-7
BOE-8
BOE-9
BOE-10
1 2 3 4 5 6 7 8 9 10 11 12 13 14
15 16 17 18 19 20 21 22 23 24 25 26 27
28 29 30 31 32 33 34 35 36 37 38 39 40 ZW
a

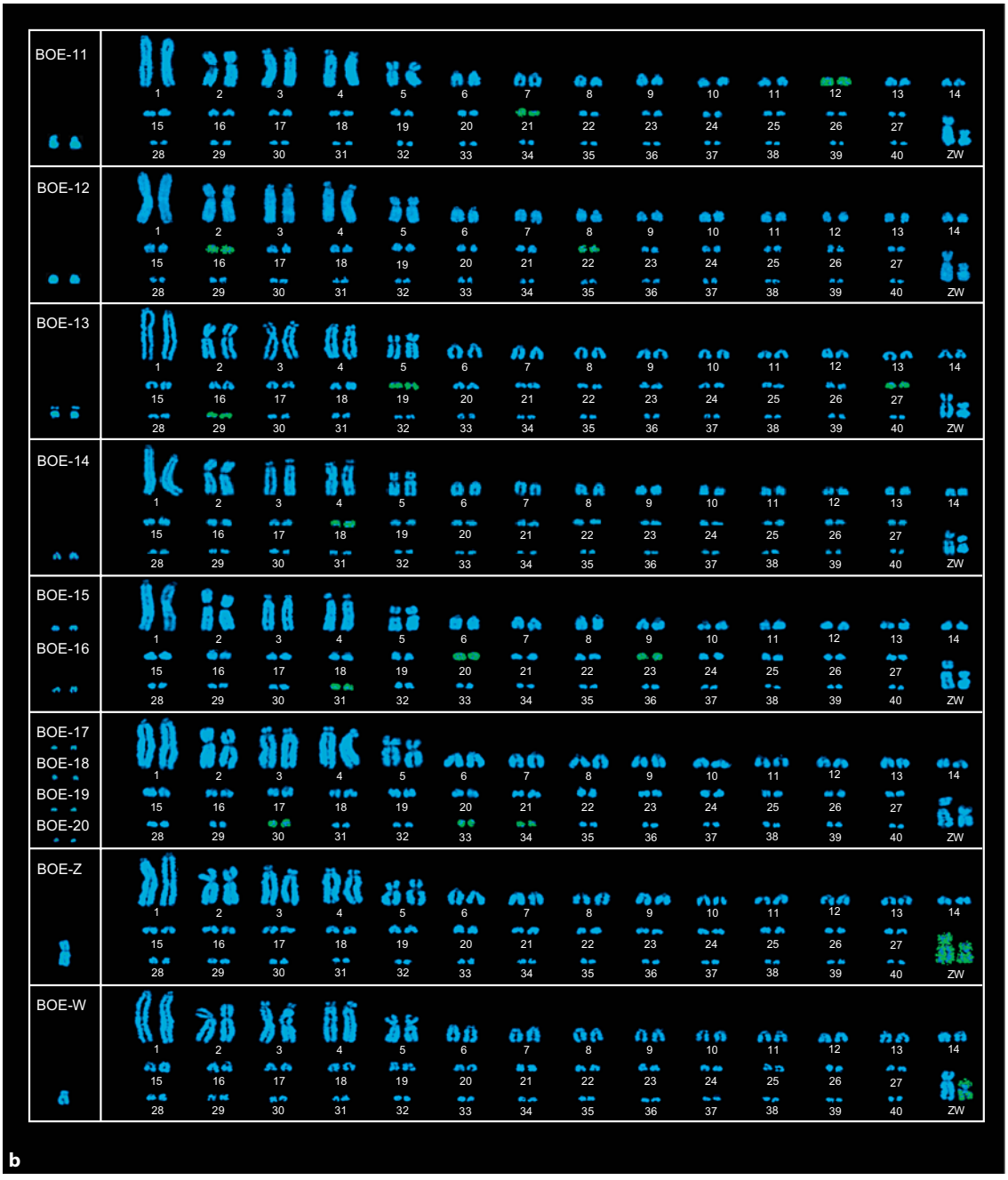
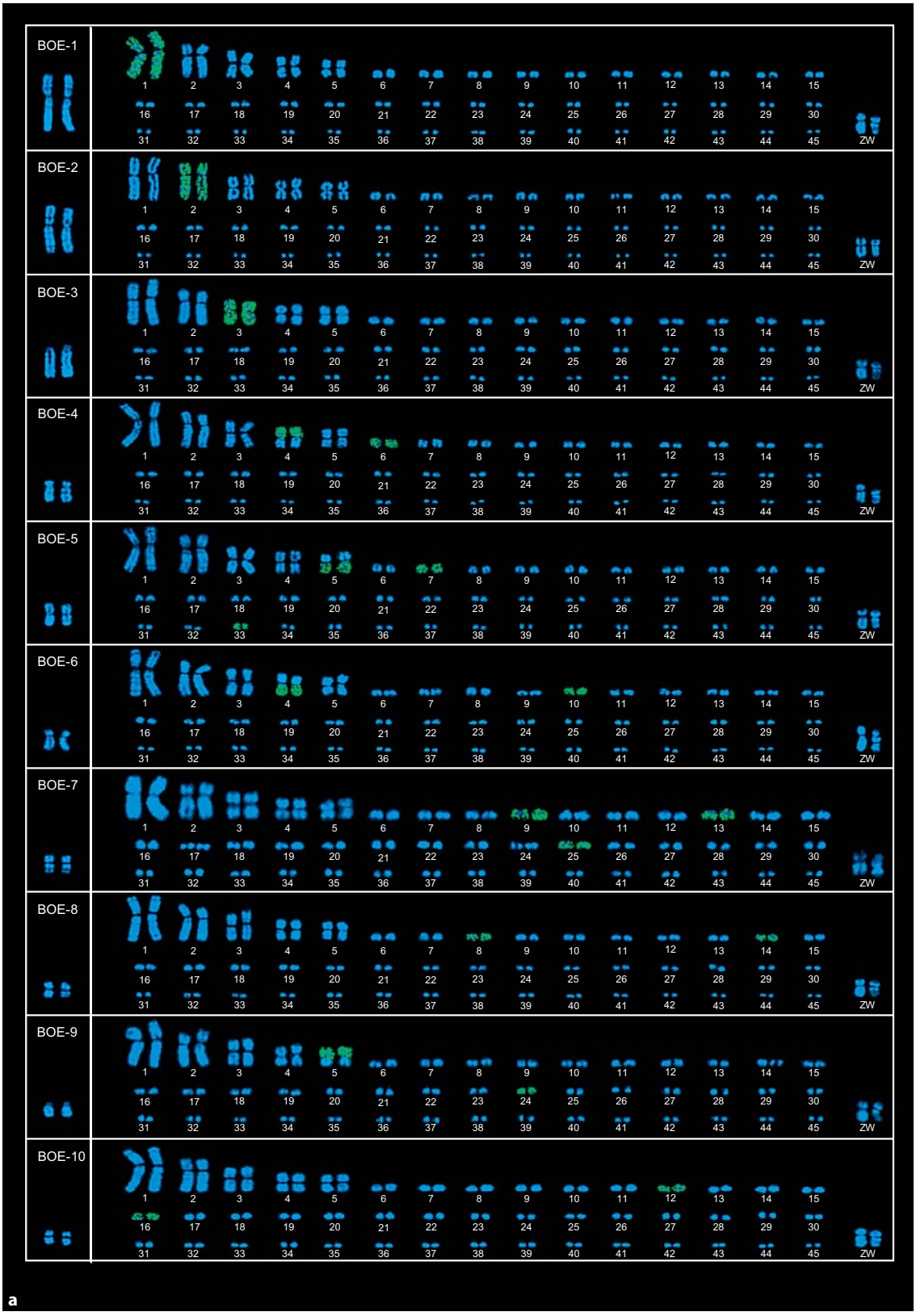


Fig. 4. FISH of the stone curlew (BOE) chromosome-specific paints on chromosomes of *S. nebulosa* (SNE). The hybridization patterns (green signals) are displayed in the DAPI-counterstained female karyotype. **a** BOE chromosome-specific paints 1–10. **b** BOE chromosome-specific paints 11–20 and the BOE Z- and W-specific paints. Note that the microchromosomes 15 and 16 and 17–20 form 2 painting probes.


BOE-1
BOE-2
BOE-3
BOE-4
BOE-5
BOE-6
BOE-7
BOE-8
BOE-9
BOE-10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
31 32 33 34 35 36 37 38 39 40 41 42 43 44 45
ZW
a

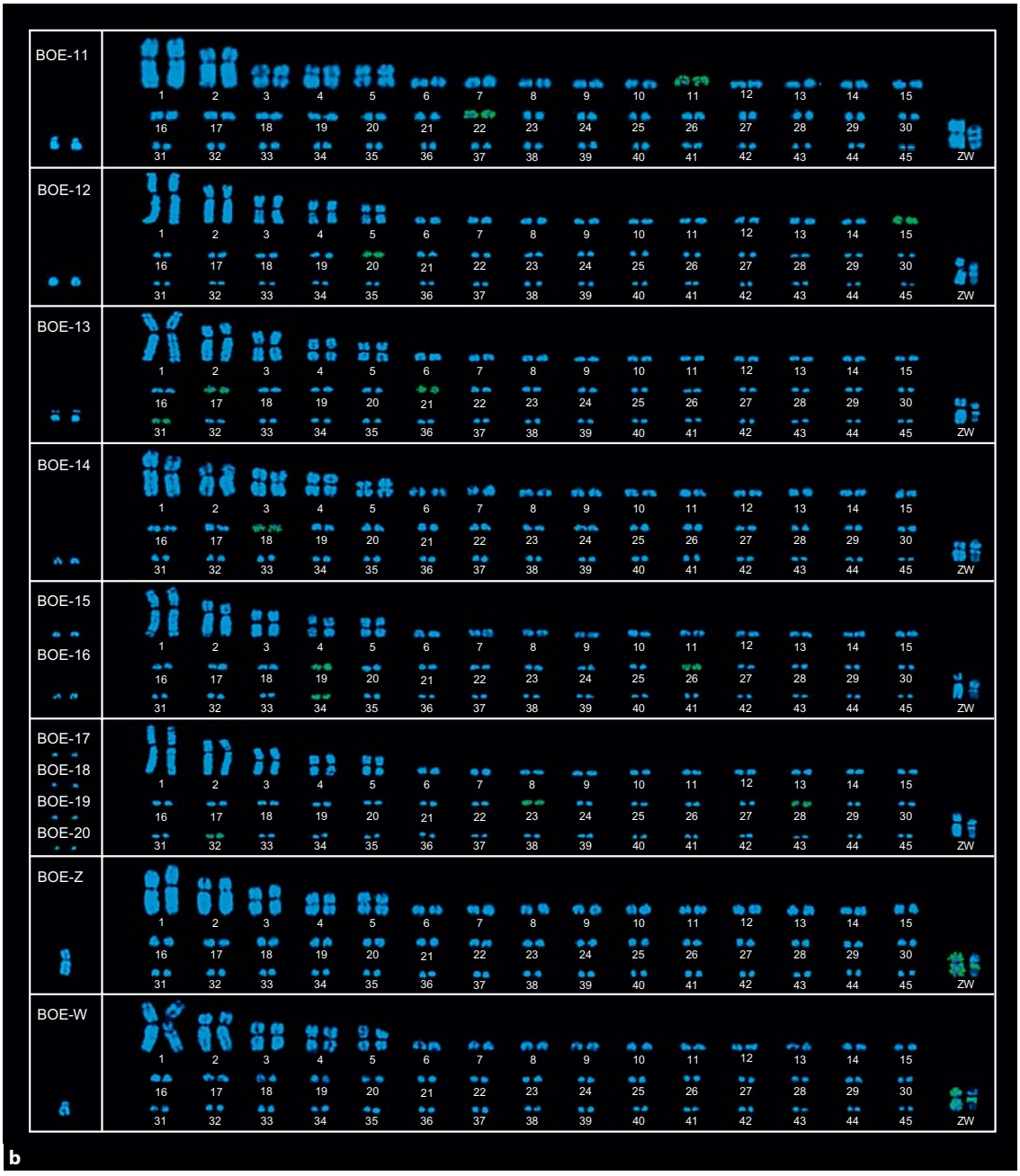


Fig. 5. FISH of the stone curlew (BOE) chromosome-specific paints on chromosomes of *F. atra* (FAT). The hybridization patterns (green signals) are displayed in the DAPI-counterstained female karyotype. **a** BOE chromosome-specific paints 1–10. **b** BOE chromosome-specific paints 11–20 and the BOE Z- and W-specific paints. Note that the microchromosomes 15 and 16 and 17–20 form 2 painting probes.

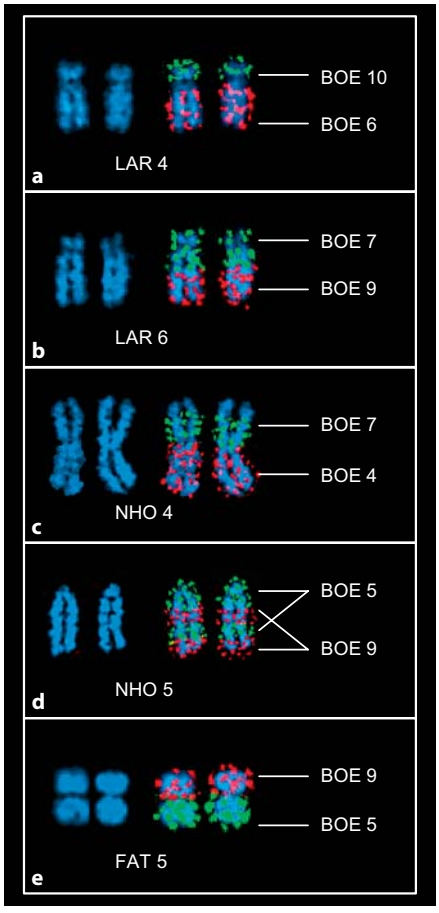


Fig. 6. Dual-color FISH of the stone curlew (BOE) chromosome-specific paints showing specific association of the corresponding homologous segments in 3 different lineages. **a** Probes BOE6 (red) and BOE10 (green) on *L. argentatus* (LAR) chromosome 4. **b** BOE7 (green) and BOE9 (red) on LAR6. **c** BOE4 (red) and BOE7 (green) on *N. hollandicus* (NHO) chromosome 4. **d** BOE5 (green) and BOE9 (red) on NHO5. **e** BOE5 (green) and BOE9 (red) on *F. atra* (FAT) chromosome 5. Chromosomes displaying hybridization signals are depicted with DAPI staining (left).

Homology with Sex Chromosomes

Paints specific to both BOE Z and W chromosomes hybridized to the sex chromosomes of the 5 species though some minor cross-hybridizations were visible on the large macrochromosomes and some microchromosomes. BOEZ completely painted the Z chromosome in all 5 birds and additionally displayed hybridization with the W chromosome to a variable extent (fig. 7b). Compared to the complete labeling of the W in NHO and SNE, a large part of the W chromosome was found to be labeled in LAR and CLI with BOEZ. In contrast, only a small heterochromatic segment was recognized by BOEZ on the W chromosome of FAT. Like BOEZ, the BOEW paint recognized both Z and W chromosomes in 4 species. The labeling over the W chromosome was nearly identical to the pattern observed with BOEZ. However, the hybridization pattern of the BOEW paint on the Z chromosomes is strikingly variable. In 3 lineages (LAR, NHO, CLI), BOEW hybridizes partially with the Z chromosome but it does not reveal any signals over the Z chromosome of SNE. On the other hand, the BOEW paint identified the complete Z chromosome of FAT, but the hybridization intensity was more distinct on the short arm compared to that on the long arm. In total, the labeling of the W chromosome by the Z paint and vice versa mark the extent of existing homology between the Z and W chromosomes. Intriguingly, besides highlighting the corresponding homologous segments, paints from each BOE autosome displayed additional strong hybridization signals on the long arm of the LAR W chromosome (fig. 1a, b). Since C-banding analysis revealed a high content of heterochromatin on Wq (data not shown), it might imply that some interspersed sequences present in low-copy number on autosomes might be enriched on the W chromosome resulting in strong cross-reaction with autosomal paints.

Discussion

The 2n = 80 karyotype, which is commonly found in birds, is believed to represent the ancestral figure that has been conserved for about 100 million years with only few variations [de Boer, 1980]. In contrast, the stone curlew represents an extreme exception to that general observation as the ancient chromosome number is substantially reduced in BOE to 2n = 42. This reduction of the chromosome number is evident by the presence of a very small number of microchromosomes which suggests that evolution of the BOE karyotype encompassed either a loss of microchromosomes or several fusion processes. Accordingly, the stone curlew, showing an atypical karyotype, is expected to reveal a high amount of rearrangements when it is compared to the typical bird karyotypes by Zoo-FISH examinations. Unlike BOE, the chicken is supposed to have retained the ancestral karyotype organization as revealed by comparative painting of GGA paints in the karyotypes of palaeognathous birds [Shetty et al., 1999; Nishida-Umehara et al., 2007]. A recent reciprocal Zoo-FISH study between BOE and the chicken found that the medium-sized BOE chromosomes 5 and 7–14 are homologous mostly to the GGA microchromosomes.

Thus, fusion of microchromosomes appears to be the major mechanism in restructuring the BOE karyotype [Nie et al., 2009]. In contrast, the GGA macrochromosomes 1–3, 4q and 5 have remained completely conserved apart from the few intrachromosomal rearrangements involving BOE4 and 6. Therefore, application of the whole BOE set of chromosome-specific painting probes for comparative karyotyping by Zoo-FISH promises to shed light on microchromosome rearrangements in divergent lineages. In addition, the BOE karyotype represents a phylogenetically young neoavian genome which might show improved resolution in Zoo-FISH experiments on Neoaves species compared to the painting probes from the ancient GGA karyotype. Furthermore, the location of homologous segments corresponding to the BOE chromosomes in the karyotypes of higher birds may indicate the restructuring of the ancestral conserved segments during the karyotypic diversification. In comparison to the high variation of the diploid chromosome number among the 5 species studied, the number of conserved segments detected by autosomal BOE paints does not vary extensively as they range from 28 to 33. Since these conserved segment numbers do not substantially deviate from the 28 syntenic segments reported for the chicken lineage [Nie et al., 2009], it would signify that ancestral chromosome organization represented by the chicken has not been extensively rearranged in younger karyotypes during evolution. Despite the relatively low variation in the number of conserved chromosome segments in different karyotypes, the hybridization pattern of homologous regions recognizes many intraspecific rearrangements.

Although the homologous segments in the macrochromosomes are physically large, over 50% of the conserved BOE segments detected in each lineage are found on microchromosomes. In particular, in CLI and FAT showing a high diploid chromosome number, about 70% of the overall conserved BOE segments are located on microchromosomes. Since 6 BOE autosomal paints each are homologous to at least 2 tiny microchromosomes, it may be assumed that a substantial proportion of the BOE chromosomes must have been derived from fusions of ancestral microchromosomes, which are still found to be conserved in older species like CLI and FAT.

In all 5 species analyzed the large BOE macrochromosomes 1–3 have remained overall conserved during millions of years of avian evolution. With 2 exceptions in terms of 2 fission events in the NHO and SNE lineages, these findings are essentially comparable to the results reported by reciprocal cross-species painting between BOE and GGA [Nie et al., 2009]. Thus, the high conservation of the 3 large macrochromosomes in the majority of species investigated suggests that the large ancestral avian macrochromosomes have experienced only few rearrangements, apart from the intrachromosomal or rare fission events as observed in the cockatiel and the great gray owl.

BOE4 hybridized to the long arm of GGA4 which represents the original ancestral chromosome 4 [Nie et al., 2009]. It hybridizes to 1 chromosome or chromosomal segment in all species except for the Eurasian coot in which 2 chromosomal segments were detected. This fission of the corresponding homologous segment of BOE4 observed in the Eurasian coot contributed to the formation of FAT4p and an additional medium-sized chromosome (FAT6). Since the long arm of FAT4 is detected by the paint BOE6, it would imply both fission and fusion of BOE4, or more precisely of the ancestral chromosome 4 in the Eurasian coot, whereas it has solely undergone fusion in the NHO and SNE lineages. Taken together, BOE4 has remained broadly conserved in its entity despite the fission in FAT and the remarkable fusions in the karyotypes of the cockatiel and the great gray owl. BOE6 seems to have remained conserved in 3 lineages, but has undergone fusions and fissions in SNE and FAT lineages.

In contrast to the conservation of the large BOE macrochromosomes, BOE5 and 7–10 detect at least 2 chromosomes or chromosomal segments in the species studied, with the single exception of BOE10 in NHO. Since the majority of homologous segments for BOE7–10 are located on microchromosomes in all investigated species, it is quite reasonable that the observed chromosomal rearrangements have to be referred to fusion of ancestral microchromosomes in BOE rather than to fission events in higher birds leading to microchromosomes.

Except for BOE14, the homologous segments of the 9 smallest BOE autosomes (represented by 5 paints) each correspond to at least 2 microchromosomes in all 5 lineages. Interestingly, none of the BOE microchromosomes appears to have been transferred to macrochromosomes in these 5 lineages suggesting their high evolutionary conservation. This further proposes that the smallest microchromosomes might barely participate in chromosomal rearrangements. The paint BOE13 detects a quite variable number of microchromosomes in each species, ranging from 1 in NHO to at least 4 in CLI. This might be due to the fact that the pair of nucleolar organizer regions (NORs), located on chromosome BOE13 [Nie et al., 2009] is possibly cross-hybridizing with more than 1 chromosome due to the species-specific variation in the dispersal of NOR-bearing chromosomal sites.

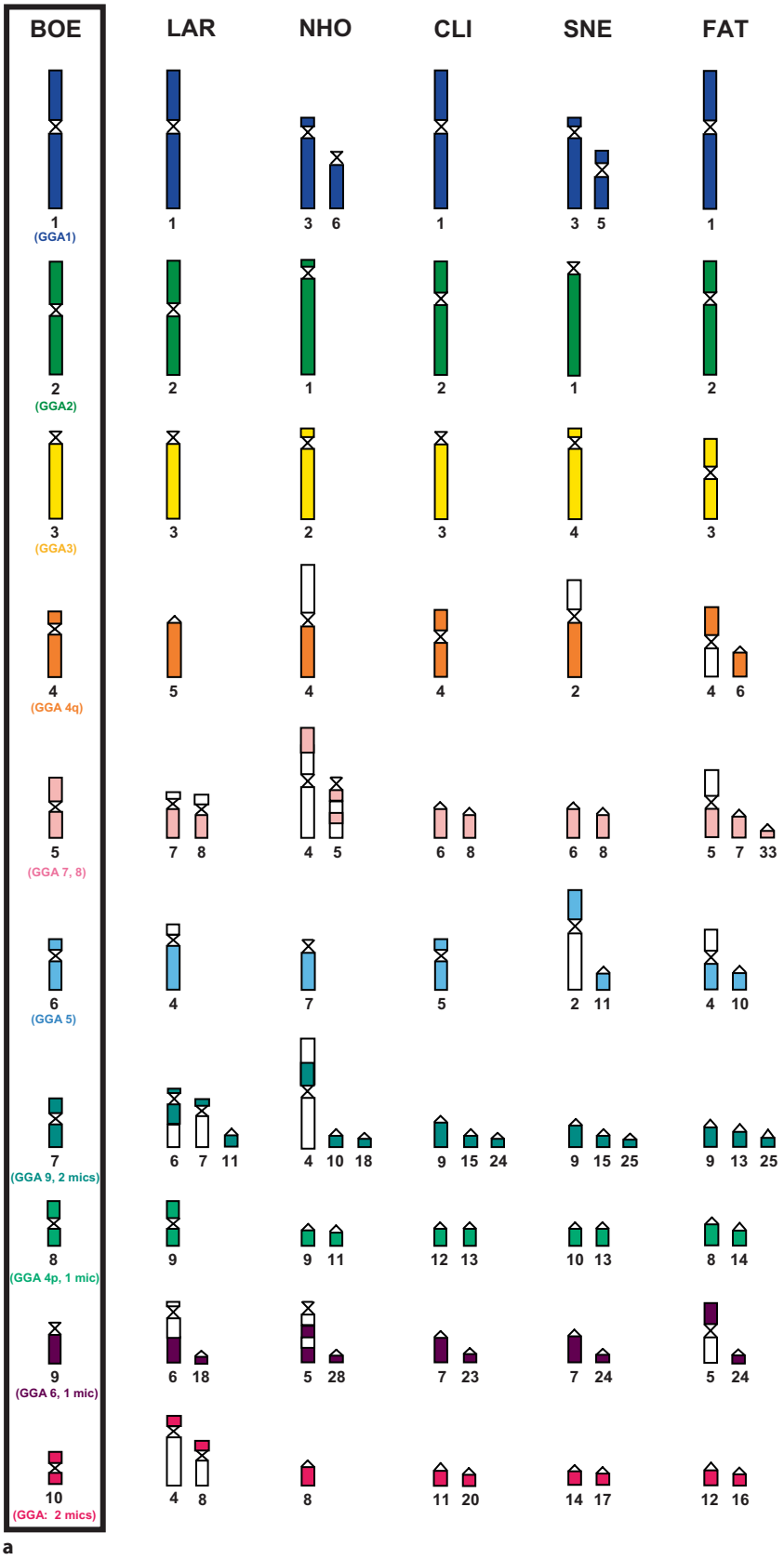
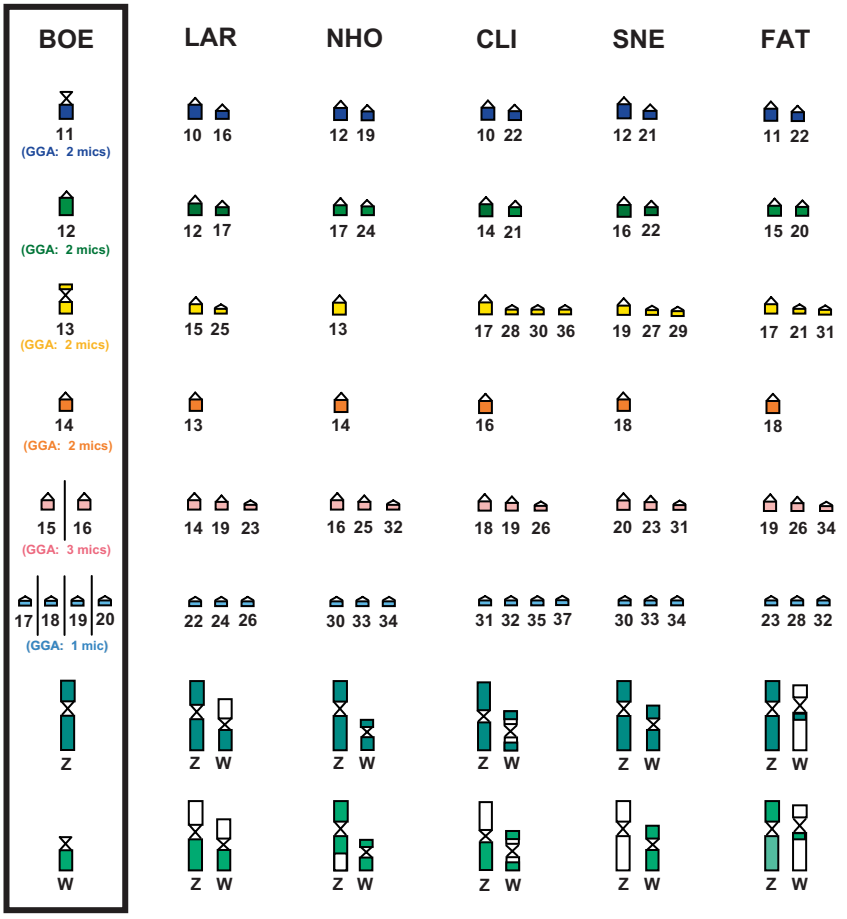


Fig. 7. Schematic representation of individual stone curlew chromosome paints compiling their hybridization patterns on the chromosomes of the 5 Neoaves species. **a** Paints 1–10. **b** Paints 11–20 and sex chromosome paints. Each BOE chromosome (left) is depicted by different colors to follow the corresponding conserved segments in the respective genomes. In addition, the corresponding chicken chromosomes [according to Nie et al., 2009] are indicated under each BOE chromosome.



b

Apart from these broad analyses of our findings, the hybridization patterns of the BOE autosomal paints also provide additional information on intraspecific chromosomal rearrangements.

Chromosomal Rearrangements between Burhinus oedicnemus *and* Larus argentatus

Like the stone curlew, the herring gull also belongs to the order Charadriiformes, which may anticipate high chromosome conservation between the 2 species. Correspondingly, conserved synteny was observed between chromosomes BOE1–4, 8, and 14 and the corresponding homologous chromosomes of LAR (LAR1–3, 5, 9 and 13). Solely a pericentric inversion could have transformed the submetacentric chromosome BOE4 to the telocentric chromosome LAR5, while no further intrachromosomal rearrangements between those chromosomes could be detected. These findings reflect a close relationship of both species and concurrently suggest that the conservation of these chromosomes dates back to an early common ancestor of BOE and LAR and might therefore represent an early charadriiform attribute. However, in contrast to the conserved syntenies, FISH of BOE probes also shows an unexpected high number of chromosome rearrangements between these 2 lineages. Probes from 7 different BOE chromosomes (5, 7, 9–13) each revealed homologous segments on at least 2 chromosomes in LAR, which is comparable to the number of segments observed in the 4 other species or in GGA [Nie et al., 2009]. However, the hybridization patterns of 4 LAR chromosomes (4, 6–8) are particularly noteworthy, as they demonstrate species-specific association of conserved segments in BOE and LAR lineages. We suppose that, like in BOE, the LAR karyotype was shaped by several fusions of ancestral chromosomes leading to the medium-sized LAR macrochromosomes. These fusions of the ancestral chromosomes occurred independently from those in BOE, leading to different associations of the conserved seg-

ments and thus forming the individual morphologies of the medium-sized macrochromosomes in the respective karyotypes of both species. We therefore assume that both karyotypes evolved in parallel except for chromosomes BOE8/LAR9, which must have emerged through fusion of 2 ancestral chromosomes in an early common ancestor of the Charadriiformes. Up to date, no FISH studies are available for charadriiform species, and current phylogenetic tree studies concentrate on comparative sequencing of mitochondrial or nuclear genes [Paton et al., 2003; Paton and Baker, 2006; Fain and Houde, 2007]. Therefore, Zoo-FISH studies using BOE chromosome-specific paints provide a highly promising tool to unravel the controversially discussed intraorder (and also interorder) relationships among the shorebirds.

Chromosomal Rearrangements between Burhinus oedicnemus *and* Nymphicus hollandicus

The cockatiel displayed the most complex hybridization pattern of all species investigated, implying extensive chromosome rearrangements during its evolution. BOE1 paint hybridized to 2 macrochromosomes (NHO3 and 6), which could support a fission event in the Psittaciformes lineage. A similar split hybridization pattern has also been observed in the great gray owl, indicating that the fission might already have occurred in a common ancestor bird of the Psittaciformes and Strigiformes. Evidence for the shared fission has been indicated through studies using the chicken chromosome 1-specific paint (homologous to BOE1) on chromosomes of the cockatiel [Nanda et al., 2007] and of *Bubo bubo* [Guttenbach et al., 2003], which, like the great gray owl, belongs to the order Strigiformes. In both studies, the GGA1 paint hybridizes to 2 macrochromosomes. In addition, Zoo-FISH studies using the GGA1 probe on representatives from Passeriformes [Guttenbach et al., 2003; Derjusheva et al., 2004] also observed a similar hybridization pattern revealing FISH signals on 2 chromosomes. Thus, fission might have already occurred in an early common ancestor of these 3 avian orders. This is in agreement with the phylogenetic tree of Hackett et al. [2008] in which Strigiformes, Psittaciformes and Passeriformes all are allocated to the group of 'land birds'. BOE2, BOE3 and BOE6 each show homology with a single NHO chromosome, but the morphology of these chromosomes differs from the corresponding BOE chromosomes, which could imply intrachromosomal rearrangements (pericentric inversions) in the NHO lineage.

In contrast to the fission event, 3 fusions are involved in the rearrangement of conserved segments in NHO. These 3 fusions, pertaining to NHO4 and 5, may explain the reduction of the ancestral chromosome number from 2n = 80 to 2n = 72 in the cockatiel. Interestingly, NHO4 has been detected by 3 different BOE painting probes (BOE4, 5 and 7) and therefore, it may be derived from fusion of 3 different homologous chromosomes. In contrast, the telocentric chromosome NHO5 is detected by both BOE5 and 9 paints, and the homologous segments are arranged interspersedly, which can be explained through a paracentric inversion subsequent to the fusion of the corresponding conserved segments of BOE5 and 9. A similar complex arrangement of homologous segments on NHO4 and NHO5 was noted with chicken paints [Nanda et al., 2007]. In the present study, the BOE conserved segments, displayed over both chromosomes NHO4 and NHO5, correspond to the GGA chromosomes which are homologous to BOE [Nie et al., 2009]. Hence, the rearrangement of conserved segments, ascertained by chicken paints in the karyotypes of other birds, can be nicely demonstrated with the paints from the highly derived chromosomes of BOE. The hybridization pattern of the remaining BOE paints is consistent with the patterns observed for the other 4 species, unequivocally supporting the fusion theory for the BOE chromosome evolution, as each smaller BOE macrochromosome is able to detect multiple chromosomes in other lineages. The only exception is the hybridization of BOE10 which, unlike in other lineages, detects just 1 NHO chromosome (NHO8), which could be due to the fact that the second chromosome might be considerably too small to be easily detected.

Chromosomal Rearrangements between Burhinus oedicnemus *and* Columba livia

The Columbiformes including the rock pigeon represent an early neoavian lineage [Hackett et al., 2008], which exhibits the ancient and largely conserved karyotype of 2n = 80. Evidence for the similarity with the assumed ancestral genome was inferred from the study of Derjusheva et al. [2004], in which GGA chromosome-specific probes 1–10 as well as 9 of the largest microchromosomes were hybridized onto metaphases of the pigeon. All chromosomes except for GGA4 showed conserved synteny with 1 entire CLI chromosome, indicating that apart from the fusion event of the ancient chromosomes 4 and 10 forming chromosome GGA4 [Schmid et al., 2000; Shibusawa et al., 2004a], no interchromosomal rearrangements have occurred between the pigeon and the chicken. Therefore, a comparative painting between BOE and CLI karyotypes is informative to compare an ancient representative of avian karyotypes (CLI) with a relatively young neoavian karyotype (BOE). The hybridization pattern is less com-

plex compared to those observed in all other species analyzed, since no distinct intrachromosomal rearrangements of conserved segments have occurred within the rock pigeon's karyotype. Instead, the hybridization pattern supports the fusion theory concerning the origin of the medium-sized BOE chromosomes: for example, BOE5 and BOE7–12 each show homology with at least 2 telocentric CLI chromosomes, which would support the notion that the respective BOE chromosomes indeed originated from fusions of ancient chromosomes. Altogether at least 11 fusions involving conserved segments must have occurred to reconstruct the exceptional BOE karyotype. Moreover, the paints from BOE seem to be more informative than chicken paints, as they were able to detect a higher number of rearrangements of conserved segments in CLI compared to the observation with the chicken paints [Derjusheva et al., 2004].

Chromosomal Rearrangements between Burhinus oedicnemus *and* Strix nebulosa

In the great gray owl 2 fissions involving chromosomes BOE1 and BOE6 as well as a fusion of chromosome BOE4 with a derived chromosome have occurred. The latter originated from the aforementioned fission of the ancestral chromosome that is homologous to BOE6 and GGA5, respectively. Since chromosome BOE6 shows conserved synteny with single chromosomes in CLI, LAR and the closely related NHO, its split into 2 chromosomes (2p and 11) in SNE must have occurred only in the Strigiformes lineage. Interestingly, GGA5, which is the corresponding chromosome of BOE6, also detects 2 chromosomes in Strigiformes species, *B. bubo* and *Pulsatrix perspicillata* [Guttenbach et al., 2003; de Oliviera et al., 2008], indicating that paints from both the neoavian and the near ancestral species are able to reveal comparable arrangement of ancestral chromosomes in the karyotype of higher birds. Moreover, if the fission of chromosome BOE6 into chromosomes SNE2p and SNE11 happened prior to the fusion of chromosome SNE2p with the conserved segment of chromosome BOE4 (SNE2q) remains to be established and might be resolved by Zoo-FISH analyses within other Strigiformes species. The fission of chromosome BOE1 in SNE forming chromosomes SNE3 and 5 is similar to a fission event found in 2 independent studies [Guttenbach et al., 2003; de Oliviera et al., 2008] using GGA probes on the Strigiformes species *B. bubo* (BBU) and *P. perspicillata* (PPE), where the GGA1 paint labeled chromosomes BBU3 and 4 and PPE1 and 4, respectively. Since chromosome BOE1 is homologous to chromosome GGA1, we conclude that the reported fission of the chromosome BOE1/GGA1 must have appeared in an early ancestor species of the Strigiformes.

Besides the interchromosomal rearrangements, involvement of pericentric inversions can be visualized by comparing the structure of chromosomes BOE2 and BOE3 with their homologous counterparts, chromosomes SNE1 and SNE4. The probes of chromosome BOE5 and 7–12 each hybridized to at least 2 chromosome pairs or chromosomal segments, which is in concordance with the number counted in the other 4 species, indicating that the respective BOE chromosomes are fusion products of ancient microchromosomes that are still separate in SNE. Altogether, at least 15 chromosomal rearrangements (including intrachromosomal rearrangements), explain the differences in the karyotype organization between BOE and SNE.

Chromosomal Rearrangements between Burhinus oedicnemus *and* Fulica atra

With 2n = 92, the Eurasian coot exhibits a considerably high number of chromosomes. Compared to the stone curlew, in which the low chromosome number is explained by fusion events of ancestral chromosomes [Nie et al., 2009], the FAT karyotype may have evolved from the ancestral one by undergoing several fissions which would be consistent with the fission-fusion theory of Burt [2002]. Correspondingly, hybridization of the BOE chromosomes 4, 5 and 6 revealed 1 additional homologous chromosome compared to the pattern in the 4 other Neoaves species, indicating fission events that at least partially explain the increased chromosome number in FAT. Further evidence comes from the study of Nie et al. [2009] that clearly showed the evolutionary conservation of BOE4 (GGA4q) and 6 (GGA5). By using GGA as the ancestral outgroup we can easily assume that the additional segments found in FAT must have emerged from fissions of the ancestral chromosomes. Likewise, BOE5, which is homologous to GGA7 and 8 [Nie et al., 2009], detects 3 instead of 2 segments in FAT, pointing to a fission event of 1 of the fused ancestral chromosomes (GGA7 or 8) in the FAT lineage. In addition, before or after those fissions have occurred, 2 centric fusions have generated the metacentric FAT chromosomes 4 and 5, whose arms show homology with parts of the conserved chromosomes BOE4 and 6 and chromosomes BOE5 and 9, respectively. Moreover, BOE9 being homologous to GGA6 and another microchromosome [Nie et al., 2009] revealed that the ancestral chromosome GGA6 underwent no further rearrangements apart from the fusion with one part of BOE5 (GGA7 or 8) in FAT.

Like in FAT5, a fusion between chromosomes BOE5 and 9 is also observed in chromosome 5 of the cockatiel, in which an additional paracentric inversion may have generated the split hybridization pattern. However, we assume that this fusion event rather happened independently than being a synapomorphic pattern of both species, since their lineages are too distantly related according to phylogenetic studies [Ericson et al., 2006; Hackett et al., 2008]. Moreover, no such fusion was found in SNE which is closely related to the cockatiel, indicating that the fusion of chromosomes BOE5 and 9 was a stochastic analogous event in both the NHO and FAT lineages. In addition to the interchromosomal rearrangements in FAT, also pericentric inversions leading to changes in the chromosome morphology can be assumed by comparing chromosomes FAT2 and FAT3 with their homologous BOE chromosomes. The hybridization patterns for the remaining BOE paints (7, 8, 10–20) are again directly comparable to those observed in CLI and SNE indicating multiple fusion events.

Noticeably, the whole set of BOE probes representing the whole genome of the stone curlew failed to detect several chromosomes in all 5 species. In NHO and SNE, 9 of the small chromosome pairs were not labeled by any BOE paint, whereas 10 pairs in LAR, 7 in CLI and even 14 in FAT remained unlabeled. All the unlabeled chromosomes are tiny microchromosomes representing the smallest part of the respective karyotypes, and their physical sizes are comparable to the chicken D group chromosomes. There are several explanations to account for the lack of hybridization signals in these microchromosomes. One explanation is that the resolution of chromosome painting in closely related species is restricted to 5–10 Mb [Schröck et al., 1996]. Thus, the discrepancy between the total chromosome number and the number of painted homologous chromosomes observed in each species might be due to technical constraints in resolving small conserved segments with chromosome paints. Another tentative explanation might be the loss of some microchromosomes during the evolution of the BOE karyotype, whereas the same ancestral microchromosomes may have remained conserved in the karyotypes of the other evolving Neoaves. In this scenario, these conserved microchromosomes may escape labeling with BOE chromosome paints. In fact, loss of microchromosomes in the BOE lineage can be also presumed from the reciprocal chromosome painting between BOE and GGA, as the hybridization of GGA paints from multiple microchromosomes showed relatively fewer labeling sites on BOE chromosomes compared to the number of labeled microchromosomes in GGA [Nie et al., 2009]. The undetected microchromosomes most likely could represent parts of the avian genome which contain plenty of repetitive sequences that usually react poorly with the painting probes. In this regard, it is important to emphasize that avian microchromosomes for example exhibit a higher density of telomere-specific sequences $(TTAGGG)_n$ than the macrochromosomes [Nanda et al., 2002]. Such chromosomes can experience fissions without imparting any genomic constraints, or they even might get lost. In *F. atra*, about one third of the total chromosome number has not been labeled by the BOE painting probes. Thus, it may be considered likewise that additional microchromosomes might have emerged in other lineages during evolution by fission of those microchromosomes enriched with certain repeats. Those types of microchromosomes that can undergo fissions or that even might get lost without any impairment of the genomic function are probably underrepresented in the derived BOE genome.

Conclusion

The present study for the first time establishes genome-wide chromosomal homology among the karyotypes of Neoaves using the probes from a highly derived karyotype of a Charadriiformes species. The comparative map demonstrates that the large macrochromosomes (BOE1–4) have remained generally stable undergoing only few rearrangements during evolution, while the medium-sized BOE chromosomes originated from fusions of ancestral microchromosomes which are still found separated in chicken [Nie et al., 2009] and in most of the neoavian species examined so far. Our findings in the present Zoo-FISH study support the fission-fusion model for microchromosome evolution proposed by Burt [2002] and demonstrate that BOE painting probes are an efficient tool to delineate avian chromosome evolution. Future studies with BOE paints on other bird species will complement the existing data from previous painting studies using GGA probes and might provide a better insight into microchromosome dispersal throughout avian genomes and their evolution.

Acknowledgements

We thank Dr. Barbara Klupp and Dr. Roland Riebe, Friedrich-Löffler-Institut, Federal Research Institute for Animal Health, Greifswald-Insel Riems, Germany for providing the *L. argentatus* fibroblasts.

References

Baker AJ, Pereira SL, Paton TA: Phylogenetic relationships and divergence times of Charadriiformes genera: multigene evidence for the Cretaceous origin of at least 14 clades of shorebirds. Biol Lett 3:205–209 (2007).

Bed´Hom B, Coullin P, Guillier-Gencik Z, Moulin S, Bernheim A, Volobouev V: Characterization of the atypical karyotype of the black winged kite *Elanus caeruleus* (Falconiformes: Accipitridae) by means of classical and molecular cytogenetic techniques. Chromosome Res 11:335–343 (2003).

Burt DW: Origin and evolution of avian microchromosomes. Cytogenet Genome Res 96: 97–112 (2002).

Burt DW, Bruley C, Dunn IC, Jones CT, Ramage A, et al: The dynamics of chromosome evolution in birds and mammals. Nature 402:411–413 (1999).

Chiappe LM, Dyke GJ: The early evolutionary history of birds. J Paleont Soc Korea 22:133–151 (2006).

Chowdhary BP, Raudsepp T: Chromosome painting in farm, pet and wild animal species. Methods Cell Sci 23:37–55 (2001).

Christidis L: Aves, in John B (ed): Animal Cytogenetics, Vol 4, Chordata 3 (Gebrüder Borntträger, Berlin 1990).

de Boer LEM: The somatic chromosome complements of 16 species of Falconiformes (Aves) and the karyological relationships of the order. Genetica 46:77–113 (1976).

de Boer LE: Do the chromosomes of the kiwi provide evidence for a monophyletic origin of the ratites? Nature 287:84–85 (1980).

de Oliveira EHC, Habermann FA, Lacerda O, Sbalqueiro IJ, Wienberg J, Müller S: Chromosome reshuffling in birds of prey: the karyotype of the world's largest eagle (Harpy eagle, *Harpia harpyja*) compared to that of the chicken *(Gallus gallus)*. Chromosoma 114:338–343 (2005).

de Oliveira EH, de Moura SP, dos Anjos LJ, Nagamachi CY, Pieczarka JC, et al: Comparative chromosome painting between chicken and spectacled owl *(Pulsatrix perspicillata)*: implications for chromosomal evolution in the Strigidae (Aves, Strigiformes). Cytogenet Genome Res 122:157–162 (2008).

Derjusheva S, Kurganova A, Habermann F, Gaginskaya E: High chromosome conservation detected by comparative chromosome painting in chicken, pigeon and passerine birds. Chromosome Res 12:715–723 (2004).

Ericson PG, Anderson CL, Britton T, Elzanowski A, Johansson US, et al: Diversification of Neoaves: integration of molecular sequence data and fossils. Biol Lett 2:543–547 (2006).

Fain MG, Houde P: Multilocus perspectives on the monophyly and phylogeny of the order Charadriiformes (Aves). BMC Evol Biol 7:35 (2007).

Ferguson-Smith MA, Trifonov V: Mammalian karyotype evolution. Nat Rev Genet 8:950–962 (2007).

Graphodatsky AS, Yang F, Dobigny G, Romanenko SA, Biltueva LS, et al: Tracking genome organization in rodents by Zoo-FISH. Chromosome Res 16:261–274 (2008).

Griffin DK, Robertson LBW, Tempest HG, Skinner BM: The evolution of the avian genome as revealed by comparative molecular cytogenetics. Cytogenet Genome Res 117:64–77 (2007).

Griffin DK, Robertson LB, Tempest HG, Vignal A, Fillon V, et al: Whole genome comparative studies between chicken and turkey and their implications for avian genome evolution. BMC Genomics 9:168 (2008).

Grutzner F, Zend-Ajusch E, Stout K, Munsche S, Niveleau A, et al: Chicken microchromosomes are hypermethylated and can be identified by specific painting probes. Cytogenet Cell Genet 93:265–269 (2001).

Guttenbach M, Nanda I, Feichtinger W, Masabanda JS, Griffin DK, Schmid M: Comparative chromosome painting of chicken autosomal paints 1–9 in nine different bird species. Cytogenet Genome Res 103:173–184 (2003).

Hackett SJ, Kimball RT, Reddy S, Bowie RC, Braun EL, et al: A phylogenomic study of birds reveals their evolutionary history. Science 320:1763–1768 (2008).

Hillier LW, Miller W, Birney E, Warren W, Hardison RC, et al: Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. Nature 432:695–716 (2004).

Kasai F, Garcia C, Arruga MV, Ferguson-Smith MA: Chromosome homology between chicken *(Gallus gallus domesticus)* and the red-legged partridge *(Alectoris rufa)*; evidence of the occurrence of a neocentromere during evolution. Cytogenet Genome Res 102:326–330 (2003).

Masabanda JS, Burt DW, O'Brien PC, Vignal A, Fillon V, et al: Molecular cytogenetic definition of the chicken genome: the first complete avian karyotype. Genetics 166:1367–1373 (2004).

McQueen HA, Fantes J, Cross SH, Clark VH, Archibald AL, Bird AP: CpG islands of chicken are concentrated on microchromosomes. Nat Genet 12:321–324 (1996).

Nanda I, Schrama D, Feichtinger W, Haaf T, Schartl M, Schmid M: Distribution of telomeric $(TTAGGG)_n$ sequences in avian chromosomes. Chromosoma 111:215–227 (2002).

Nanda I, Karl E, Volobouev V, Griffin DK, Schartl M, Schmid M: Extensive gross genomic rearrangements between chicken and Old World vultures (Falconiformes: Accipitridae). Cytogenet Genome Res 112:286–295 (2006).

Nanda I, Karl E, Griffin DK, Schartl M, Schmid M: Chromosome repatterning in three representative parrots (Psittaciformes) inferred from comparative chromosome painting. Cytogenet Genome Res 117:43–53 (2007).

Nie W, O'Brien PC, Ng BL, Fu B, Volobouev V, et al: Avian comparative genomics: reciprocal chromosome painting between domestic chicken *(Gallus gallus)* and the stone curlew (*Burhinus oedicnemus*, Charadriiformes) - An atypical species with low diploid number. Chromosome Res 17:99–113 (2009).

Nishida C, Ishijima J, Kosaka A, Tanabe H, Habermann FA, et al: Characterization of chromosome structures of Falconinae (Falconidae, Falconiformes, Aves) by chromosome painting and delineation of chromosome rearrangements during their differentiation. Chromosome Res 16:171–181 (2008).

Nishida-Umehara C, Tsuda Y, Ishijima J, Ando J, Fujiwara A, et al: The molecular basis of chromosome orthologies and sex chromosomal differentiation in palaeognathous birds. Chromosome Res 15:721–734 (2007).

Paton TA, Baker AJ: Sequences from 14 mitochondrial genes provide a well-supported phylogeny of the Charadriiform birds congruent with the nuclear RAG-1 tree. Mol Phylogenet Evol 39:657–667 (2006).

Paton TA, Baker AJ, Groth JG, Barrowclough GF: RAG-1 sequences resolve phylogenetic relationships within Charadriiform birds. Mol Phylogenet Evol 29:268–278 (2003).

Raudsepp T, Houck ML, O'Brien PC, Ferguson-Smith MA, Ryder OA, Chowdhary BP: Cytogenetic analysis of California condor *(Gymnogyps californianus)* chromosomes: comparison with chicken *(Gallus gallus)* macrochromosomes. Cytogenet Genome Res 98:54–60 (2002).

Schmid M, Enderle E, Schindler D, Schempp W: Chromosome banding and DNA replication patterns in bird karyotypes. Cytogenet Cell Genet 52:139–146 (1989).

Schmid M, Nanda I, Guttenbach M, Steinlein C, Hoehn M, et al: First report on chicken genes and chromosomes 2000. Cytogenet Cell Genet 90:169–218 (2000).

Schmid M, Nanda I, Hoehn H, Schartl M, Haaf T, et al: Second report on chicken genes and chromosomes 2005. Cytogenet Genome Res 109:415–479 (2005).

Schröck E, du Manoir S, Veldman T, Schoell B, Wienberg J, et al: Multicolor spectral karyotyping of human chromosomes. Science 273: 494–497 (1996).

Shetty S, Griffin DK, Graves JAM: Comparative painting reveals strong chromosome homology over 80 million years of bird evolution. Chromosome Res 7:289–295 (1999).

Shibusawa M, Nishibori M, Nishida-Umehara C, Tsudzuki M, Masabanda J, et al: Karyotypic evolution in the Galliformes: An examination of the process of karyotypic evolution by comparison of the molecular cytogenetic findings with the molecular phylogeny. Cytogenet Genome Res 106:111–119 (2004a).

Shibusawa M, Nishida-Umehara C, Tsudzuki M, Masabanda J, Griffin DK, Matsuda Y: A comparative karyological study of the bluebreasted quail (*Coturnix chinensis*, Phasianidae) and California quail (*Callipepla californica*, Odontophoridae). Cytogenet Genome Res 106:82–90 (2004b).

Smith J, Bruley CK, Paton IR, Dunn I, Jones CT, et al: Differences in gene density on chicken macrochromosomes and microchromosomes. Anim Genet 31:96–103 (2000).

Stefos K, Arrighi FE: Heterochromatic nature of W chromosomes in bird karyotypes. Chromosoma 46:91–120 (1971).

Takagi N, Sasaki M: A phylogenetic study of bird karyotypes. Chromosoma 46:91–120 (1974).

Telenius H, Pelmear AH, Tunnacliffe A, Carter NP, Behmel A, et al: Cytogenetic analysis by chromosome painting using DOP-PCR amplified flow-sorted chromosomes. Genes Chromosomes Cancer 4:257–263 (1992).

van Tuinen M, Hedges SB: Calibration of avian molecular clocks. Mol Biol Evol 18:206–213 (2001).

Wienberg J: The evolution of eutherian chromosomes. Curr Opin Genet Dev 14:657–666 (2004).

Yang F, Müller S, Just R, Ferguson-Smith MA, Wienberg J: Comparative chromosome painting in mammals: human and the Indian muntjac *(Muntiacus muntjak vaginalis)*. Genomics 39:396–401 (1997).

Yang F, Alkalaeva EZ, Perelman PL, Pardini AT, Harrison WR, et al: Reciprocal chromosome painting among human, aardvark, and elephant (superorder Afrotheria) reveals the likely eutherian ancestral karyotype. Proc Natl Acad Sci USA 100:1062–1066 (2003).