# Pachytene karyotypes of 17 species of birds

Anastasia Slobodchikova[1,2†], Lyubov Malinovskaya[1,2†], Ekaterina Grishko[1], Inna Pristyazhnyuk[1], Anna Torgasheva[1,2*], Pavel Borodin[1]

[1]Institute of Cytology and Genetics, Russian Academy of Sciences, Siberian Department, Novosibirsk, 630090, Russia.

[2]Novosibirsk State University, Novosibirsk 630090, Russia

Corresponding Author: Pavel Borodin. Email: borodin@bionet.nsc.ru

[†]These authors share first authorship

Anastasia Slobodchikova: slobodchikovaAY@bionet.nsc.ru

Lyubov Malinovskaya: l.malinovskaia@g.nsu.ru

Ekaterina Grishko: e.grishko@alumni.nsu.ru

Inna Pristyazhnyuk: iprist@bionet.nsc.ru

Anna Torgasheva: torgasheva@bionet.nsc.ru

Pavel Borodin: borodin@bionet.nsc.ru

**Abstract**

**Background**

To date less than 10% of bird species have been karyotyped. They are rather conservative with diploid chromosome numbers about 78-80 in most species examined. Immunostaining of meiotic chromosomes at pachytene stage enables more precise estimates of the number, morphology and

variability of macro- and microchromosomes than conventional analysis of mitotic metaphase chromosomes does. Analysis of pachytene chromosomes led to discovery of germline-restricted chromosome (GRC) that was present in germline cells and absent in somatic cells in all 16 species of passerine birds examined. GRC has not been found in any non-passerine bird.

## Results

In this study, using immunolocalization of SYCP3, the main protein of the lateral elements of the synaptonemal complex (SC) and centromere proteins we examined male pachytene karyotypes of sixteen passerine species and one outgroup species the Common cuckoo *Cuculus canorus* and provided their idiograms and precise estimates of their diploid chromosome numbers and the numbers of chromosome arms. We provided the first description of the karyotypes of three species, corrected the published data on the karyotypes of ten species and confirmed them for four species. The pachytene cells of the Gouldian finch, Brambling and Common linnet contained heteromorphic bivalents indicating heterozygosity for inversions or centromere shifts. The European pied flycatcher, Gouldian finch and Domestic canary have extended centromeres in several macro- and microchromosomes. GRCs of various sizes and shapes were detected in all passerine species examined. Their chromatin was heavily labeled by anticentromere antibodies. The lateral elements of the GRC SC varied in their size from the largest to the smallest element of the pachytene karyotype. They also varied in shape from continuous to fragmented.

## Conclusions

All songbirds examined, except the Eurasian skylark, have highly conservative karyotypes, 2n=80-82+GRC with seven pairs of macrochromosomes and 33-34 pairs of microchromosomes. The interspecies differences concern the sizes of the macrochromosomes, morphology of the microchromosomes and sizes of the centromeres. GRC is present in all songbird species examined, varying in size, morphology and SC structure even between closely related species. This indicates its fast evolution.

43 **Keywords**

44 Avian chromosomes, synaptonemal complex, GRC, SYCP3, karyotyping, germline-restricted

45 chromosome, extended centromeres, chromosome rearrangements

## Background

Birds provide an interesting model to study chromosome evolution. They have undergone rapid speciation and evolved various adaptations to a wide variety of habitats. Yet, their karyotypes (chromosome sets) are very conservative. They are bimodal, i.e. composed of macro- and microchromosomes, the feature inherited from their reptilian ancestors (Romanov et al. 2014; Damas et al. 2018). Diploid chromosome number (2n) of most karyotypes of bird species is about 78–82 (Degrandi et al. 2021). The number of chromosome arms, so called fundamental number (FN), also vary in narrow limits: 90-110. Unfortunately, less than 10% of bird species have been karyotyped. In passerines, the most speciose and diverse order of birds, the portion of karyotyped species is even smaller – 7% (Degrandi et al. 2020). Paradoxically, the karyotype is unknown even in the species whose genomes have been sequenced, annotated and studied in detail, such as the European pied flycatcher *Ficedula hypoleuca* (Kawakami et al. 2017).

Most avian karyotypes have been described in 1960-1970s with the use of conventional methods of chromosome preparation and staining available at that time (Degrandi et al. 2021). Development of the methods of chromosome analysis at the pachytene stage of meiosis provided a rather efficient karyotyping tool (Moses et al. 1977). These methods are based on visualization of synaptonemal complex (SC), the structure that mediates synapsis and recombination of homologous chromosomes. SC is composed of two lateral elements, to which the chromatin loops are attached, and the central element that holds homologous chromosomes together. At the pachytene stage of meiotic prophase, the chromosomes are less compacted than at metaphase (Pigozzi 2016). Therefore, analysis of SC provides higher resolution than the conventional analyses of metaphase chromosomes. This is especially important in the cytogenetics studies of the bimodal karyotypes, because a morphology of the microchromosomes and even their number are rather difficult to assess at the conventional chromosome spreads (Lisachov and Borodin 2016; Kichigin et al. 2018). The SC analysis is particularly efficient in detection of heterozygosity for all types of chromosome rearrangements: inversions, translocations, deletions and duplications. The heterozygotes for

71 the structural variants and heterogametic organisms produce characteristic heteromorphic SCs (Poorman
72 et al. 1981; del Priore et al. 2015; Lisachov et al. 2017).

73 Analysis of pachytene chromosomes led to discovery of germline-restricted chromosome (GRC)
74 (Pigozzi and Solari 1998). The GRC was present in germline cells and absent in somatic cells in all 16 species
75 of passerine birds examined. It has not been found in any non-passerine bird (Torgasheva et al. 2019). In
76 female germ cells GRC usually present in two copies, which synapse and recombine with each other. At the
77 meiotic prophase, the GRC bivalents are practically indistinguishable from normal autosomal bivalents.
78 They can only be revealed by a comparative subtractive analysis of pachytene and somatic karyotype. In
79 male germ cells, GRC is usually present one copy, which forms a univalent at pachytene, easily
80 distinguishable from the bivalents of the autosomes and ZZ by its thin, coiled and often fragmented SC. The
81 GRC univalents in male pachytene cells are always surrounded by dense chromatin clouds heavily labeled
82 by anticentromere antibodies. Among the Passeriformes the GRC varies in size and morphology (Pigozzi
83 and Solari 1998; Torgasheva et al. 2019, 2021a; Malinovskaya et al. 2020)

84 In this study, using immunolocalization of SYCP3, main protein of the lateral elements of
85 synaptonemal complex (SC) and centromere proteins we examined male pachytene karyotypes of sixteen
86 passerine species with special attention to the GRC and one outgroup species the Common cuckoo *Cuculus*
87 *canorus*. The source of the specimens is shown in Supplementary Table 1. In each specimen we
88 photographed, thoroughly examined and measured at least 20 well spread pachytene nuclei containing
89 complete chromosome sets. The measurements were used to generate idiograms. We compared the
90 pachytene karyotypes with the karyotypes described earlier and mostly obtained by conventional methods
91 of chromosome preparation and staining.

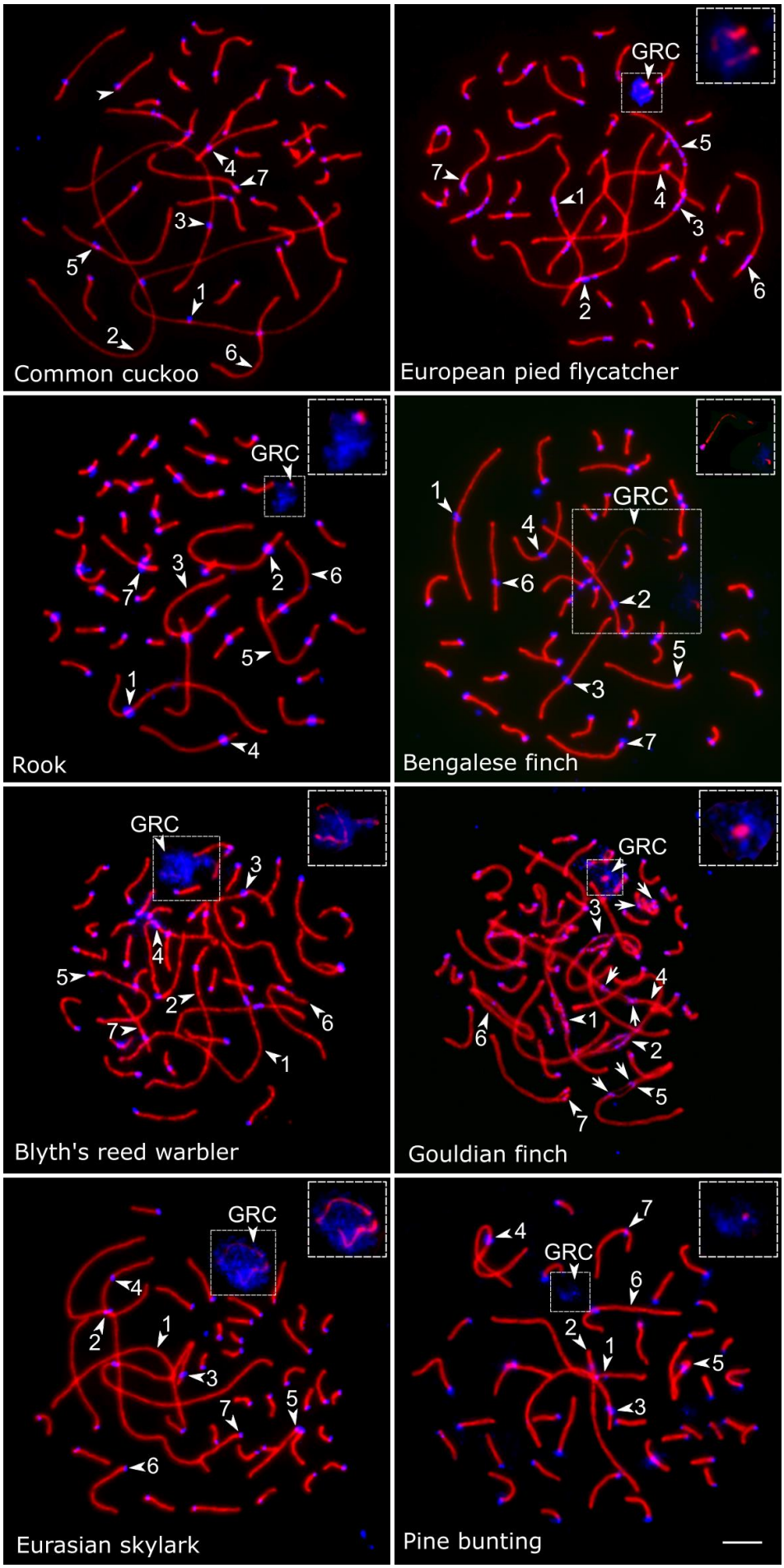
92 **Results**

93 Figure 1 shows microphotographs of the SC spreads after immunolocalization of SYCP3 (red) and
94 centromere proteins (blue) of all species examined. Figure 2 shows idiograms of pachytene karyotypes of
95 the studied species. The haploid chromosome number equal to the number of the SCs (n), the haploid

96 number of the chromosome arms equal to one for each acrocentric chromosome and to two for each
97 meta- and submetacentric chromosome (Fn), the total SC length and brief description of GRC are shown in
98 the Table 1. The descriptions of the chromosome morphologies are based on the estimates of their
99 centromeric indices. Those with the centromeric index close to 0 is scored as the acrocentrics, between 0
100 and 0.4 as the submetacentrics, and between 0.4 and 0.5 as the metacentrics. In all species, we identified
101 the seven largest SCs as macro-SC, the others – as micro-SCs. We estimated the size of GRC based on the
102 size of GRC chromatin labeled by anticentromere antibodies and the length of lateral element of GRC SC if
103 it was completely formed. We classified GRCs of size comparable to macrochromosomes of the basic set as
104 macro-GRCs, others – as micro-GRCs. In most species, we found a variation in SC appearance of macro- and
105 micro-GRCs between the cells. It could form complete, fragmented, dot-like lateral element of SC or do not
106 form it at all.

107 **Table 1.** Haploid chromosome numbers (n), haploid numbers of the chromosome arms (Fn), total
108 SC length of the basic chromosome set and characteristics of GRC of 17 bird species.

| Common name | N | Fn | SC length (µm) | GRC |
|---|---|---|---|---|
| Common cuckoo | 40 | 48 | 387±68 | Absent |
| Rook | 40 | 57 | 226±29 | Micro |
| Blyth's reed warbler | 40 | 53 | 297±51 | Macro |
| Eurasian skylark | 40 | 43 | 241±75 | Macro |
| European pied flycatcher | 40 | 55 | 309±47 | Micro |
| Bengalese finch | 40 | 46 | 208±24 | Macro |
| Gouldian finch | 40 | 46 | 238±34 | Micro |
| Pine bunting | 40 | 48 | 216±33 | Micro |
| Common chaffinch | 40 | 48 | 301±38 | Micro |
| Brambling | 40 | 50 | 237±19 | Micro |
| Eurasian bullfinch | 41 | 57 | 209±47 | Macro |
| European greenfinch | 41 | 58 | 209±17 | Micro |
| Common redpoll | 41 | 70 | 287±31 | Macro |
| Domestic canary | 41 | 56 | 302±41 | Micro |
| European goldfinch | 41 | 55 | 220±29 | Micro |
| Common linnet | 41 | 53 | 246±29 | Macro |
| Eurasian siskin | 41 | 50 | 245±66 | Macro |


Common cuckoo
European pied flycatcher
Rook
Bengalese finch
Blyth's reed warbler
Gouldian finch
Eurasian skylark
Pine bunting
GRC

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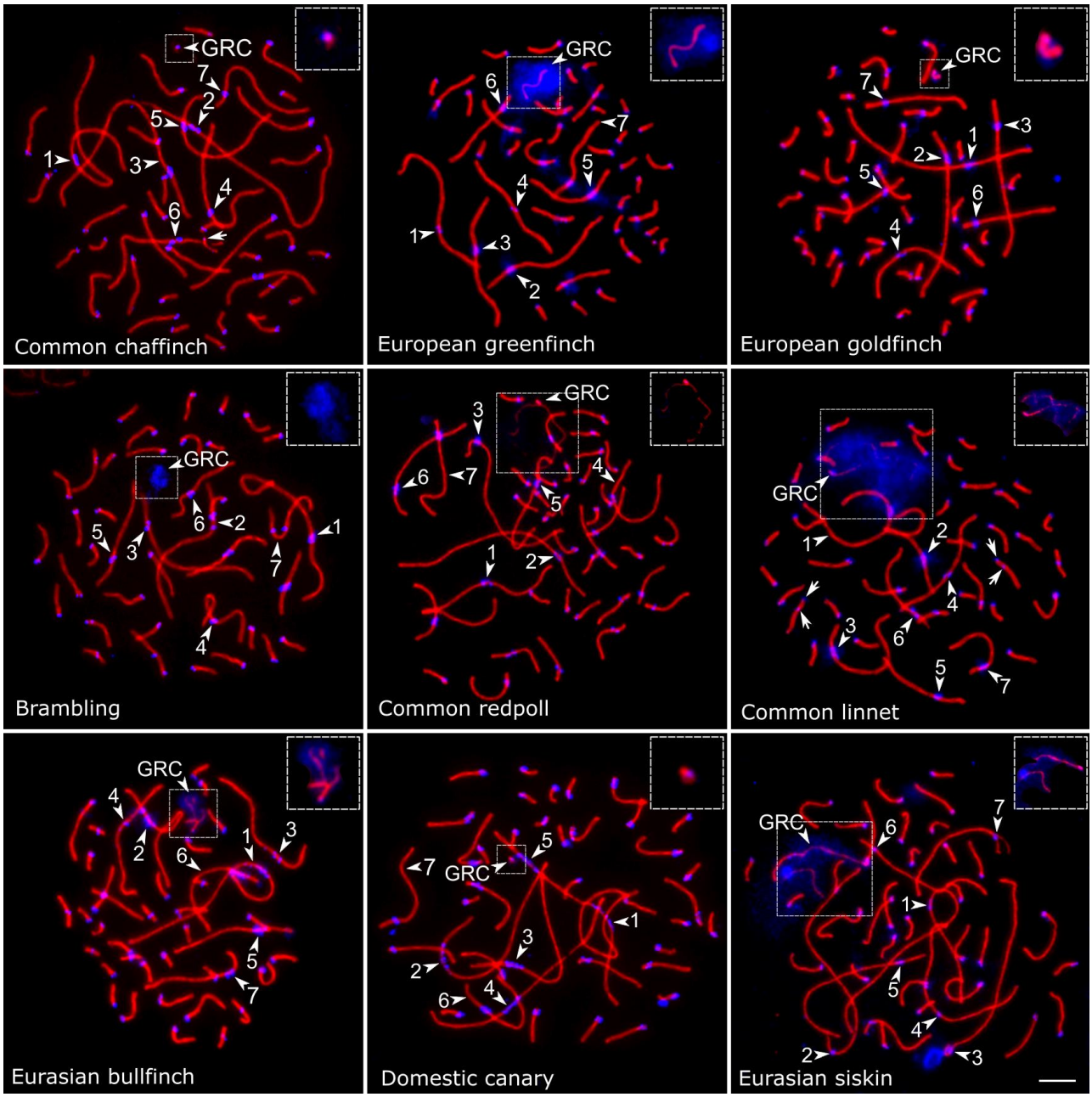
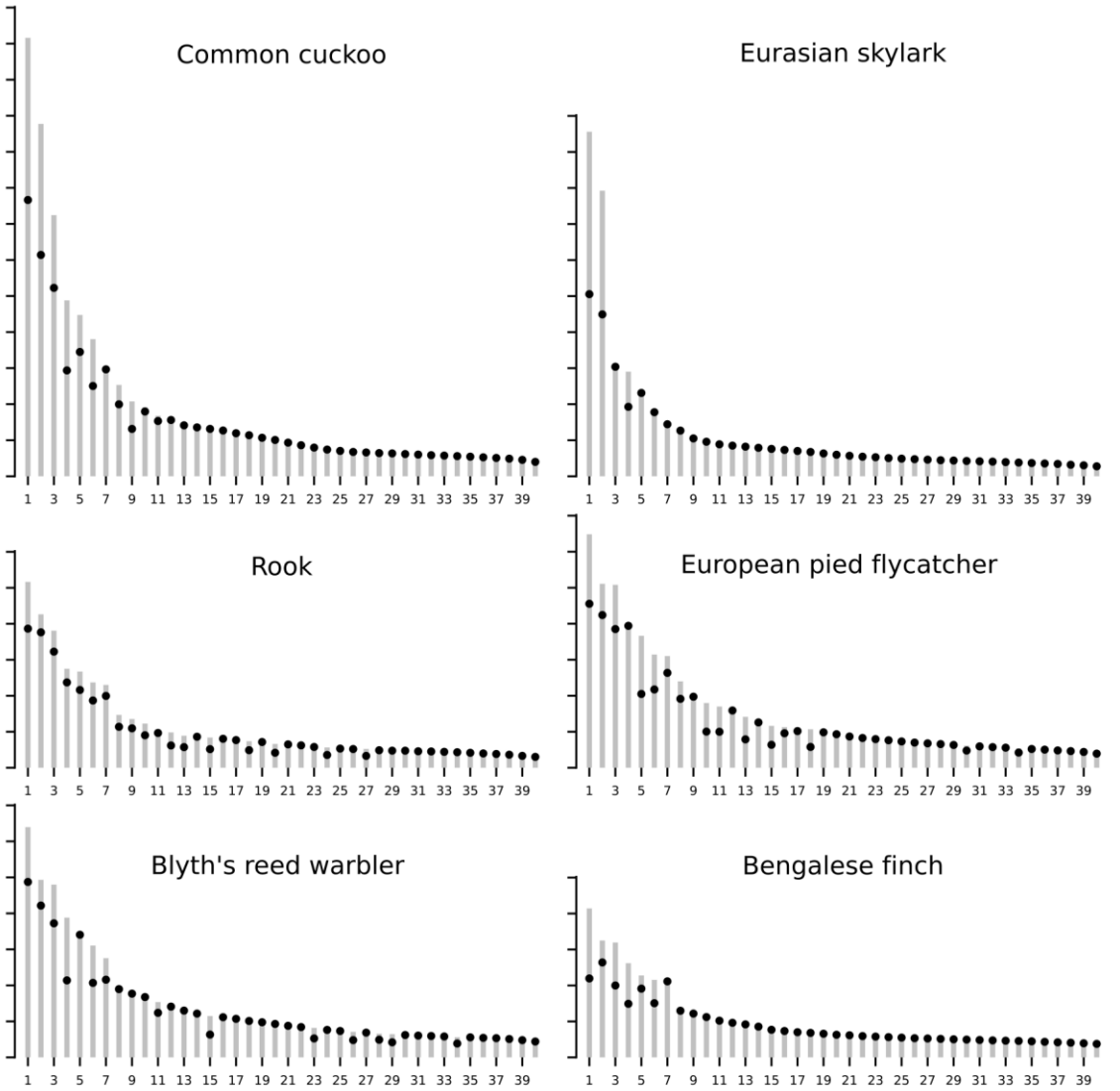



**Figure 1.** Microphotographs of the SC spreads after immunolocalization of SYCP3 (red) and centromere proteins (blue) of all species examined. The inserts show close-up of the GRC. Numbers indicate SCs of the macrochromosomes. Bar—5 µm.


Common cuckoo
Eurasian skylark
Rook
European pied flycatcher
Blyth's reed warbler
Bengalese finch
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39

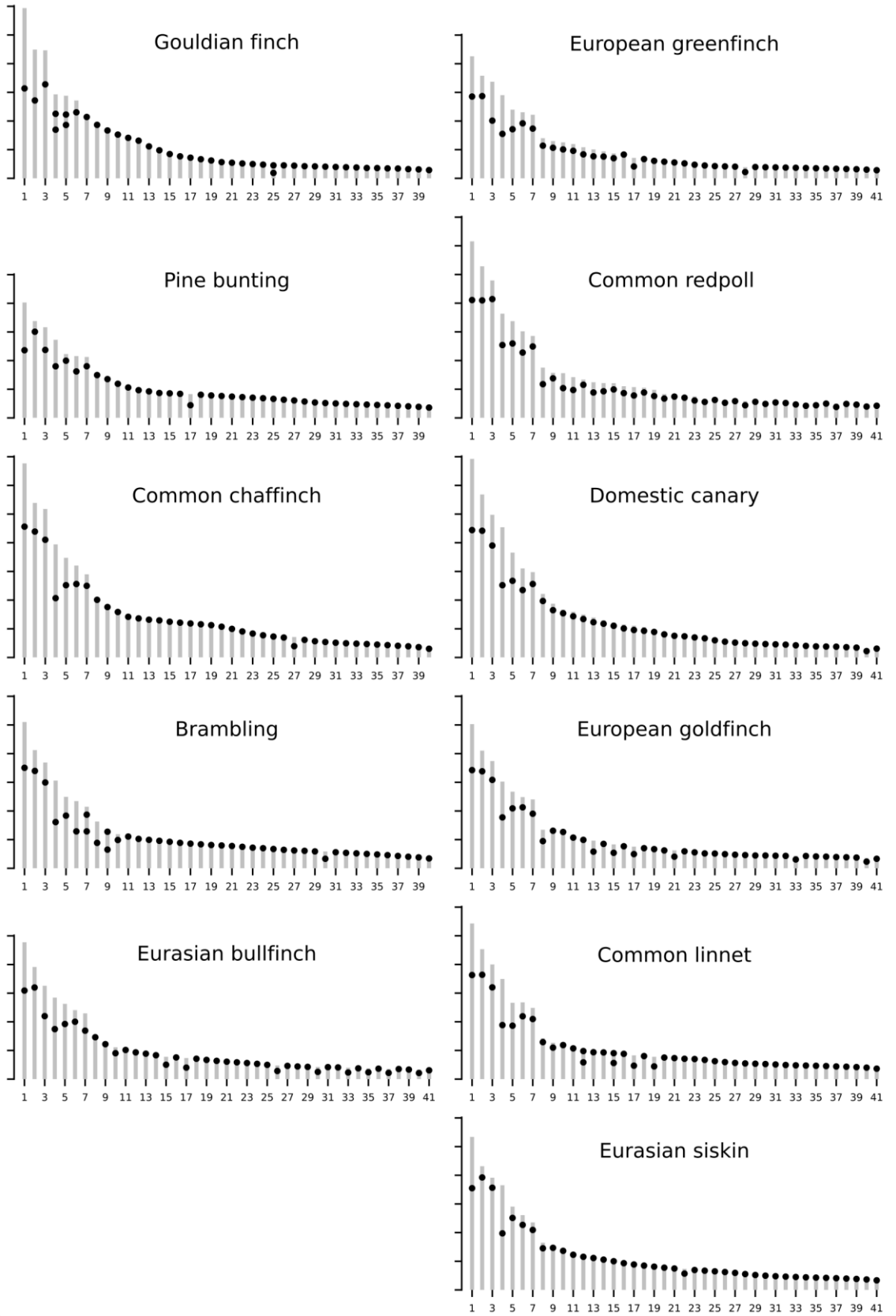



**Figure 2.** Idiograms of pachytene karyotypes without GRCs of the studied species. The *y*-axes show average SC length in µm. The x-axes SCs in decreasing size order. Black circles indicate centromeres. Arrows indicate additional centromeres in the heteromorphic bivalents

119

120 **Common cuckoo *Cuculus canorus***

121 Somatic karyotype of the Common cuckoo has been described by Bian and Li (1989). They indicated
122 2n=78. Pachytene karyotype of this species comprises 40 chromosome pairs (2n = 80). Total length of its SC
123 set is one of the largest among the bird species examined so far. The excess of the SC length is mainly due
124 to very large metacentric SCs 1 and 2 and submetacentric SC3. Macro-SC4 is metacentric. Macro-SCs 5 and
125 6 and micro-SCs 8 and 9 are submetacentrics, Macro-SC7 and all other 30 micro-SCs are acrocentrics. The
126 Common cuckoo as well as all other non-passerine birds studied so far does not have GRC in its pachytene
127 karyotype.

128 **Rook *Corvus frugilegus***

129 Somatic karyotype of the Rook has not been studied yet. The diploid number of the closely related
130 species *Corvus corax* is 80 (Roslik and Kryukov 2001). Our results confirm that basic diploid chromosome set
131 of the Rook is 80. Pachytene karyotype of Rook comprises 40 bivalents and one univalent of a GRC
132 surrounded by a small cloud of anticentromere antibodies. All of the Rook's macro-SCs are
133 submetacentrics. Among the micro-SCs, there are ten submetacentrics and twenty-three acrocentrics. In
134 most pachytene cells, GRC forms dot-like SC while in some cells SC is not formed.

135 **Blyth's reed warbler *Acrocephalus dumetorum***

136 Somatic karyotype of the Blyth's reed warbler remains unknown. The diploid number of the closely
137 related species *Acrocephalus agricola* is 78 (Bulatova 1981). Pachytene karyotype of the Blyth's reed
138 warbler comprises 40 chromosome pairs (2n = 80). Its macro-SCs 1, 2, 3, 6 and 7 are submetacentrics, SC4
139 is metacentric, SC5 is acrocentric. Most micro-SCs are acrocentrics. There are one metacentric and six
140 submetacentric micro-SCs.

141 GRC in the Blyth's reed warbler occurs as a large acrocentric univalent with fragmented SC
142 surrounded by a chromatin cloud labeled by anticentromere antibodies. The level of fragmentation varies
143 between the cells from evenly labeled SC to a dispersed series of the short fragments.

144 **Eurasian skylark *Alauda arvensis***

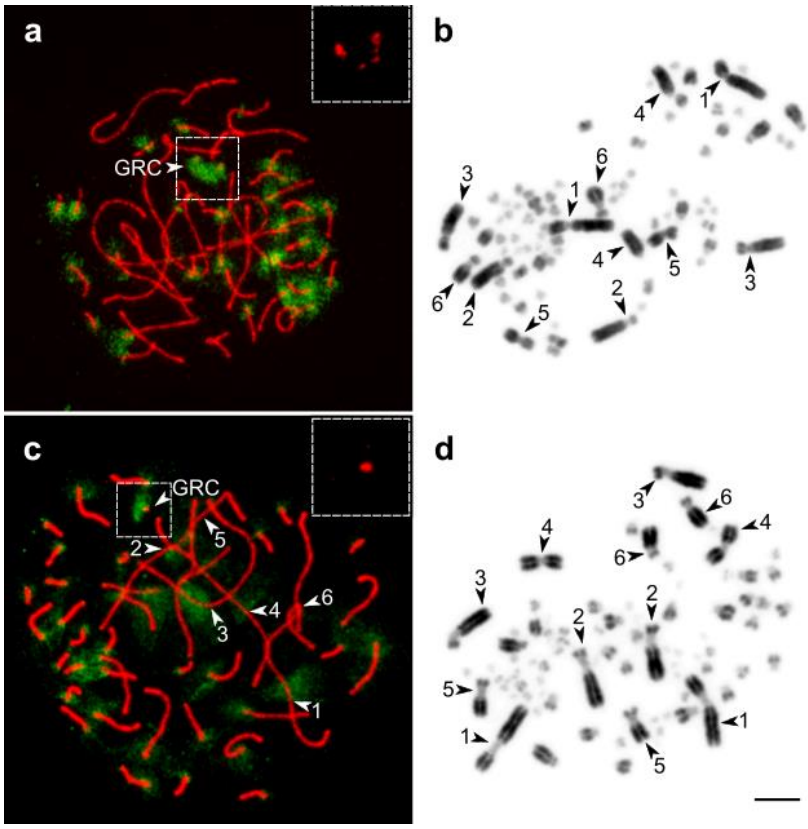
145 The first description of the Eurasian skylark somatic karyotype was provided by Udagawa (1952) on
146 the basis of histological sections of embryonic testes and ovaries. The spermatogonial 2n was estimated as
147 78, oogonial as 77. Li and Bian (1988) estimated 2n the Oriental skylark *Alauda gulgula* as 76±. We found
148 that the pachytene karyotype of the Eurasian skylark contains 40 chromosome pairs (2n =80). In terms of
149 SC sizes, it is extremely asymmetric. Metacentric SCs 1 and 2 comprise 36% of the total SC length. This is in
150 agreement with the description of the Alaudidae karyotype by Li and Bian (1988). They described the Z- and
151 W-chromosomes as the largest elements, metacentric and submetacentric respectively. The 1$^{st}$
152 macrochromosome was the second largest metacentric.

153 One of the two largest SCs in the pachytene karyotype of the Eurasian skylark is probably the neo-Z
154 chromosome. Genomic analysis indicates that Z chromosome of several skylark species resulted from
155 fusions between parts of the chromosomes Z, 3, 4A and 5. It is the largest sex chromosome found in birds
156 (about 200 Mb) (Sigeman et al. 2019, 2020). Another exceptionally large chromosome has probably also
157 evolved via several chromosome fusions. Surprisingly, the chromosome number of the Eurasian skylark is
158 the same as in most songbirds. This means that the fusions leading to the formation of the two largest
159 macrochromosomes had been preceded or followed by a series of chromosome fissions. Besides the two
160 largest macrochromosomes, the karyotype of the Eurasian skylark contains one submetacentric
161 macrochromosome. All other macrochromosomes and microchromosomes are acrocentrics.

162 GRC of the Eurasian skylark occurs as a large chromatin cloud heavily labeled with anticentromere
163 antibodies. It forms thin, pale and fragmented SC.

164 **European pied flycatcher *Ficedula hypoleuca***

165 Genome of the European pied flycatcher has been sequenced, annotated and studied in detail
166 (Kawakami et al. 2017), yet its karyotype remains unknown. Its close relative *Ficedula parva* contains 40
167 chromosome pairs (2n=80) (Bian et al. 1991). The pachytene karyotype of the European pied flycatcher
168 contains 40 bivalents (2n=80). Almost all its macro-SCs are submetacentrics, SC4 is acrocentric, SC5 is
169 metacentric. We are sure that SC4 is ZZ because analysis of pachytene oocytes of this species revealed
170 heteromorphic ZW bivalent with acrocentric Z axis (Torgasheva et al. 2021b). Among micro-SCs there are
171 five metacentrics and four submetacentrics.

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173 **Figure 3.** Extended centromeres of European pied flycatcher (a, b) and Domestic canary males (c, d).
174 (a, c) Pachytene spermatocytes immunolabeled with antibodies against SYCP3 (red) and H3K9me2/3
175 (green). (b, d) Somatic metaphase chromosomes stained with DAPI. The arrowheads indicate the six largest
176 macro-SCs and GRCs. The inserts show close-up of the GRCs. Bar—5 µm.

177

178 We observed extended pericentromeric regions in all meta- and submetacentric macro-SCs (but
179 not in the acrocentric ZZ) and in several largest micro-SCs. They appeared as beads of several dots, which
180 cover from 9% of SC1 to 41% of SC11/13 (Supplementary Table 2). The antibodies against the histone H3,
181 di- and trimethylated at lysine 9 (H3K9me2/3), marking transcriptionally inactive chromatin, produced
182 strong signals at the standard centromeres of the pachytene chromosomes, but not at the extended ones
183 (Fig. 3a). In the corresponding somatic metaphase chromosomes of this species, we observed extended
184 primary constrictions (Fig 3b).

185 GRC in the European pied flycatcher usually appears as a chromatin cloud heavily labeled with
186 anticentromere and H3K9me2/3 antibodies containing several SC fragments.

187 **Bengalese finch *Lonchura striata domestica***

188 Pachytene karyotype of the Bengalese finch comprises 40 chromosome pairs (2n=80). This
189 coincides with the description given by del Priore and Pigozzi (2014) and differs from the earlier description
190 of its somatic karyotype as 2n =78 (Takagi 1972; Ray-Chaudhuri 1976; Christidis 1986a). Its SCs 1 and 4 are
191 metacentrics, SCs 2, 3, 5 and 6 are submetacentrics. All other SCs are acrocentrics.

192 The Bengalese finch GRC has been first described by del Priore and Pigozzi (2014) and then by
193 Torgasheva *et al.* (2019). It appears as the largest acrocentric univalent in the pachytene nucleus. We found
194 a variation in appearance of GRC SC between the cells: from complete lateral element to dot-like SC.
195 Despite variation in the degree of SC polymerization, the cloud of anticentromere antibodies labeled GRC
196 was similarly large in all cells, which allowed us to classify GRC of Bengalese finch as macro-GRC in
197 accordance with previous (del Priore and Pigozzi 2014; Torgasheva et al. 2019)

198 **Gouldian finch *Erythrura gouldiae***

199 Christidis (1986a) described 2n of the Gouldian finch as 78. We found 40 chromosome pairs (2n=80)
200 in pachytene cells of this species. SCs 1 and 2 are metacentrics, SCs 3 to 6 are submetacentrics. Macro-SC7
201 and all micro-SCs are acrocentrics. SCs 1, 2 and 3 have extended centromeres, similar to those described in

202 the European pied flycatcher. They occupy around 13-20% of SC length (Supplementary Table 2). In a half
203 of cells analyzed, these regions are asynapsed whereas all other SCs are completely synapsed (Fig. 1). The
204 delayed synapsis could be caused by an unusually extended pericentromeric heterochromatin.

205 We found heteromorphic SCs 4, 5 and 25. SC4 has two centromeres in metacentric and
206 submetacentric positions. SC5 has two centromeres in different submetacentric positions. SC25 has
207 centromeres in acrocentric and metacentric positions. GRC appears as a moderately sized chromatin cloud
208 comparable to the largest microchromosomes labeled with anticentromere antibodies surrounding from
209 one to three SC fragments.

210 **Pine bunting *Emberiza leucocephalos***

211 In the Pine bunting pachytene spermatocytes we observed 40 bivalents (2n=80) while Radzhabli et
212 al. (1970) and Bulatova (1973) described diploid number of somatic karyotype of this species as 2n=78. The
213 pachytene karyotype of this species is rather similar to that of the Bengalese finch described above. There
214 are three differences. SC4 is submetacentric, SC7 is submetacentric and one of the micro-SCs is
215 metacentric. GRC of the Pine bunting is labeled by a small cloud of anticentromere antibodies. In most
216 cells, GRC does not form lateral element of SC, in some cells we detected dot-like signal of anti-SYCP3
217 antibodies.

218 **Common chaffinch *Fringilla coelebs***

219 The Common chaffinch is an important model for evolutionary genetic studies. Its high-quality
220 genome assembly has recently been published (Recuerda et al. 2021). Our estimate of the Common
221 chaffinch karyotype coincides with earlier published one (2n= 80) (Piccinni and Stella 1070). Its pachytene
222 karyotype comprises 40 chromosome pairs. Total SC length and the lengths of the macro-SCs in the
223 Common chaffinch are much longer than in the closely related Pine bunting (Table 1). However, the
224 morphology of their chromosomes is rather similar except SC1, which is submetacentric in the Common
225 chaffinch.

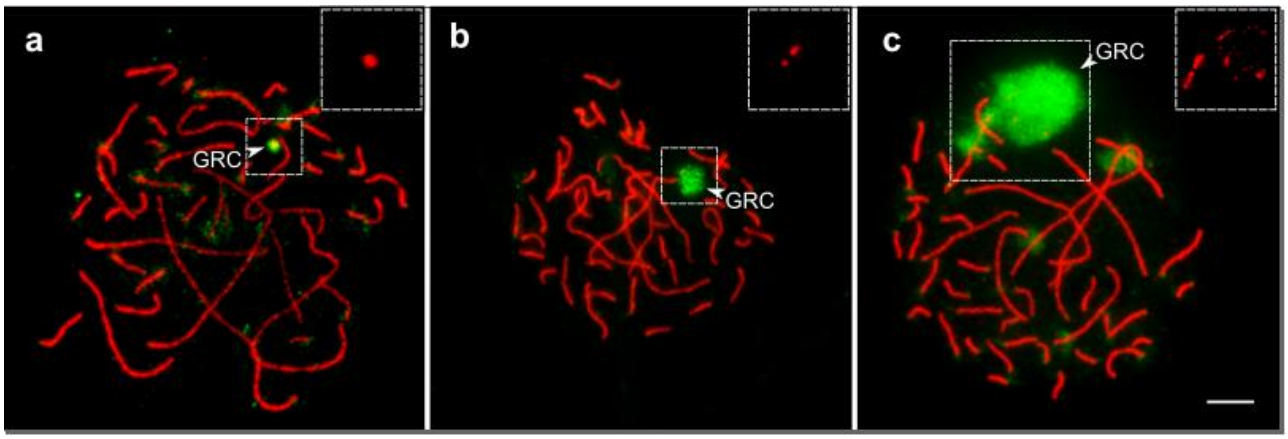


**Figure 4** Pachytene spermatocytes of the Common chaffinch (a), Brambling (b) and Common linnet (c) after immunostaining with antibodies against SYCP3 (red) and H3K9me2/3 (green). The arrowheads indicate GRCs. The inserts show close-up of the GRCs. Bar—5 µm.

GRC of the Common chaffinch forms dot-like SC labelled by a small cloud of anticentromere and H3K9me2/3 antibodies. To our surprise, in a half of pachytene spermatocytes (26 out of 49 examined) we observed a univalent of a small microchromosome. Unlike the GRC, it was not labeled either with anticentromere or with H3K9me2/3 antibodies (Fig. 4a). We suggest that this univalent was originated by a premeiotic nondisjunction of one of the microchromosomes.

**Brambling *Fringilla montifringilla***

The somatic karyotype of the Brambling has been described as 2n=78 (Li and Bian 1987). We observed 40 pairs of chromosomes (2n=80) which were morphologically similar to the pairs detected in the Common chaffinch. There are few differences. SC6 is metacentric, and two of micro-SCs are metacentric and one – is submetacentric.

The specimen examined here has heteromorphic SCs 7 and 9. Each of them contains two centromeres in all cells examined. One homolog of chromosome 7 is submetacentric as in most fringillids examined here. The other homolog of chromosome 7 is metacentric, which had probably resulted from pericentric inversion, as well as the metacentric homolog of the acrocentric chromosome 9 typical for the fringillids.

245 Similar to the GRC of the Pine bunting, the GRC of the Brambling is labeled by a small cloud of
246 anticentromere antibodies and does not form lateral element of SC in most analyzed cells. In some cells, we
247 detected dot-like signal of anti-SYCP3 antibodies.

248 **Eurasian bullfinch *Pyrrhula pyrrhula***

249 Li and Bian (1987) described the somatic karyotype of the Eurasian bullfinch as 2n=78. The
250 pachytene cells of this species contain 41 chromosome pairs (2n=82). All macro-SCs are submetacentric.
251 Among micro-SCs we observed four metacentric and five submetacentric micro-SCs.

252 GRC of this species has been described by Torgasheva *et al.* (2019). It forms long acrocentric
253 univalent evenly labelled by SYCP3 antibodies.

254 **European greenfinch *Chloris chloris***

255 Diploid chromosome number of the European greenfinch is 76 according to Hammar and Herlin
256 (1975) and 80 according to Christidis (1986b). We detected 41 bivalents (2n=82) in pachytene cells. The
257 morphology of the seven largest macro-SCs is very similar to that described for the Eurasian bullfinch
258 except the morphology of macro-SCs 3 and 4. They are metacentric in the European greenfinch. The three
259 smallest macro-SCs and eight largest micro-SCs are submetacentrics. There are also two metacentric micro-
260 SCs while all the others are acrocentrics.

261 GRC of the European greenfinch appears as partially formed lateral element of SC comparable in
262 size to the largest microchromosomes and labelled by anticentromere antibodies.

263 **Common redpoll *Acanthis flammea***

264 According to Li and Bian (1987) diploid chromosome number of the Common redpoll is 78.
265 According to our estimate, it is 82. All macro-SCs are submetacentric. Among micro-SCs, twenty-two are
266 submetacentrics and twelve are acrocentrics.

267 The pachytene karyotype of the Common redpoll also contains large acrocentric univalent of GRC
268 evenly labelled by antibodies to SYCP3. In some cells, SC of GRC was fragmented.

269 **Domestic canary *Serinus canaria* forma *domestica***

270 The karyotype of the Domestic canary has been first described by Ohno *et al.* (1964) as 2n=80± and
271 recently by Da Silva Dos Santos *et al.* (2017) and Kiazim *et al.* (2021) as 80. The Domestic canary genome
272 has been assembled and annotated but not yet to a chromosome level (Frankl-Vilches et al. 2015).

273 Pachytene cells of the Domestic canary contain 41 chromosome pairs (2n=82). Its macro-SCs are
274 larger than those in the fringillids described above, although their morphology is similar. Macro-SC4 is
275 metacentric, all other macro-SCs are submetacentric. Eight micro-SCs are submetacentrics. All other micro-
276 SCs of the Domestic canary are acrocentrics.

277 The Domestic canary shows unusually extended centromeres of macro-SCs and the largest micro-
278 SCs, similar to that detected in the European pied flycatcher and the Gouldian finch. They were about 2-3
279 µm long covering about 10% of the SCs 1, 2/3, 4, 6, and about 15% of the SCs 5 and 10/11/12 (Fig. 3c,
280 Supplementary Table 2). The antibodies against H3K9me2/3 label both the standard and extended
281 centromeres of the pachytene chromosomes (Fig. 3c). We also detected substantially extended primary
282 constrictions at the corresponding metaphase chromosomes (Fig. 3d).

283 GRC of this species has been described by Torgasheva et al. (2019). It appears as a small cloud of
284 anticentromere antibodies with dot-like lateral element of SC.

285 **European goldfinch *Carduelis carduelis***

286 Christidis (1986b) described the somatic karyotype of the European goldfinch as 2n=82. Our data
287 confirm this diploid chromosome number. We observed 41 bivalents in the pachytene cells of the European
288 goldfinch. Its seven largest macro-SCs were smaller than those of the Domestic canary, but similar in
289 morphology. We observed one metacentric and six submetacentric micro-SCs, all other micro-SCs are
290 acrocentric.

291 GRC of the European goldfinch has been described by Torgasheva et al. (2019) as micro-GRC. In
292 most cells, GRC was present as one or two dot-like lateral SC fragments labeled by a small cloud of
293 anticentromere antibodies. In some cells, GRC formed complete lateral SC element.

294 **Common linnet *Carduelis cannabina***

295 Bulatova (1973, 1981) found 82 chromosomes in bone marrow cells of the Common linnet. This is
296 in a good agreement with our observation. We found 41 bivalents. Pachytene karyotypes of the Common
297 linnet and the European goldfinch are almost the same. The difference concerns the morphology of the
298 micro-SCs. The Common linnet has only two homomorphic metacentric and two heteromorphic
299 metacentric/acrocentric micro-SCs. Micro-SC9 is submetacentric. All other micro-SCs are acrocentrics.

300 The heteromorphic SCs 12 and 15 contain two centromeres in all cells examined: one in acrocentric
301 and one in metacentric positions. The metacentric homologs had probably resulted from pericentric
302 inversions.

303 GRC of the Common linnet occurs as an acrocentric univalent with fragmented SC labeled by a large
304 cloud of anticentromere antibodies.

305 **Eurasian siskin *Spinus spinus***

306 The diploid chromosome number in the Eurasian siskin was estimated as 78 (Takagi 1972; Bulatova
307 1973; Li and Bian 1987; Christidis 1990). We found 41 chromosome pairs in the pachytene cells of this
308 species (2n=82). The morphology of the macro-SCs is rather similar to that described in two fringellid
309 species above. However, the karyotype of the Eurasian siskin contains only two submetacentric micro-SC,
310 all other micro-SCs are acrocentrics.

311 Torgasheva et al. (2019) classified its GRC as macro-GRC. In our spreads, it occurs as a large cloud of
312 anticentromere antibodies with fragmented SC.

313 **Discussion**

314 The main results of this study are:

315 1) the first description of the karyotypes of three species (the Rook, Blyth's reed warbler and
316 European pied flycatcher),
317 2) the correction of the published data on the karyotypes of ten songbird species,
318 3) the first detection of the extended centromeres in three model species (the European pied
319 flycatcher, Gouldian finch and Domestic canary),
320 4) the first detection of chromosomal polymorphism in three species (the Gouldian finch, Brambling
321 and Common linnet),
322 5) and detailed characterization of the GRCs of all songbird species examined

323 Why are we sure that our descriptions of the karyotypes are more precise and reliable than the
324 published data based on conventionally prepared and stained somatic metaphase spreads?

325 There are at least four reasons for this. First, pachytene chromosomes of birds are about 2-3
326 times longer than somatic metaphase chromosomes (see Fig.3). Second, each object, which we identified
327 at the microphotography as the bivalent of the basic chromosome set, was simultaneously labeled with
328 two different antibodies: against SYCP3, the main protein of the lateral elements of the SC and against the
329 centromere proteins. Univalents were distinguished from the bivalents by less intense SYCP3 labeling.
330 Therefore a misidentification of the microchromosome as cell debris and the cell debris as a
331 microchromosome was unlikely. Third, these three criteria were applied to at least 30 well spread
332 pachytene cells containing complete chromosome sets. This made our estimates statistically sound. Forth,
333 the bivalents were not just counted, they were measured. This made possible objective estimate of the
334 morphology of the macrochromosomes and, what is more important and almost impossible at the somatic
335 metaphases, the morphology of the microchromosomes.

336 A comparison between our and previously published chromosome numbers of the examined
337 species shows that the earlier researchers always undercounted one or two chromosome pairs (Table 1).

338 These errors were due to very small sizes of the smallest microchromosomes and relatively low specificity
339 of the chromosome dyes. For these reasons, it was almost impossible to estimate the morphology of the
340 small microchromosomes at the mitotic metaphase spreads. It was believed that of all them were
341 acrocentric (Christidis 1990). Our data show that this is true for the Common cuckoo, the Eurasian skylark,
342 and Bengalese finch. Other songbirds have at least one meta- or submetacentric microchromosome, and
343 most of species have many of them.

344 Using anticentromere antibodies, we revealed unusual extended centromeres in almost all
345 macrochromosomes and the largest microchromosomes of three model species (the European pied
346 flycatcher, Gouldian finch and Domestic canary). They are visible both at the pachytene and mitotic
347 metaphase spreads of these species. They are also visible at the published images of the Domestic canary
348 metaphase chromosomes and shown at the idiograms (Da Silva Dos Santos et al. 2017; Kiazim et al. 2021)
349 but did not attract much attention.

350 Such long centromeres are rare. They have been detected in few species of legumes (Neumann et
351 al. 2015, 2016), fire ants (Huang et al. 2016), Indian muntjac (Brinkley et al. 1984) and marsupial hybrids
352 (Metcalfe et al. 2007). However, they have not yet been described in birds. Robertsonian translocations
353 and centromere drive have been suggested among the possible causes of the centromere
354 extension(Brinkley et al. 1984; Huang et al. 2016). The results of H3K9me2/3 immunolocalization indicate a
355 variation in epigenetic status of the extended centromeres. In the Domestic canary, both extended and
356 standard centromeres are H3K9me2/3-positive indicating their heterochromatic state. In the European
357 pied flycatcher, the standard centromeres are H3K9me2/3-positive, while the extended centromeres are
358 H3K9me2/3-negative. Genetic composition or the extended centromeres deserve special attention.

359 One more advantage of the SC analysis is its high efficiency in detection of the structural
360 heterozygosity. In this study, we detected heteromorphic SC in the Gouldian finch, Brambling and Common
361 linnet. In all cases, the bivalents displayed two misaligned centromeres. This may indicate heterozygosity
362 either for pericentric inversions or for centromere repositions (centromere shift) (Schubert and Lysak
363 2011). Both types of chromosome rearrangements are implicated for karyotypic macroevolution of birds

364 (Zhou et al. 2014; Kiazim et al. 2021). The inversions play an especially important role in restriction of the
365 gene flow between sympatric and parapatric species (Hooper and Price 2017). However, the intraspecific
366 polymorphism for inversion is poorly studied in birds. Our findings indicate the targets for the future
367 studies.

368 Our study revealed a wide variation in GRC size and appearance. Torgasheva et al. (2019)
369 suggested to classify them as macro- and micro-GRCs to fit the criteria for macro- and microchromosomes
370 of the basic set. Indeed, all analyzed chromosomes fell into one of these categories. However, some micro-
371 GRCs were apparently much smaller than the smallest microchromosomes of the basic set that can be
372 inferred from the size of the chromatin cloud labelled by anticentromere and H3K9me2/3 antibodies. This
373 variation and the lack of phylogenetic clustering by size is in accordance with the highly dynamic nature of
374 GRC (Torgasheva et al. 2019). It was shown that GRCs of different species contain different multiply
375 repeated sequences, which probably can be accumulated and/or be lost rather quickly (Kinsella et al.
376 2019).

377 The SC of GRCs in pachytene spermatocytes of most species examined here appeared
378 fragmented, however, the degree of fragmentation varied between cells. It is unclear if this feature is
379 related to the different properties of GRCs in different species (its genetic content, the degree of
380 heterochromatinization) or to the intercellular and interindividual variation in effectiveness of cohesin
381 loading and SC polymerization since for most birds studied to date, only one sample was analyzed. Thus,
382 interindividual variation in GRC appearance was observed in pachytene spermatocytes of the Great tit
383 (Torgasheva et al. 2021a).The intense chromatin labeling with antibodies to centromere proteins and H3K9-
384 modified histone allowed us to distinguish between GRC and accidental autosomal univalents.

385 The results of our study indicate several lines of future research. In order to minimize sacrificing
386 birds we described the karyotype of most species by single specimen examined. The sample size might be
387 increased at least for the most interesting species by targeted examination of the somatic karyotypes,
388 which can be obtained from short-term fibroblast cultures derived from blood or feather pulp.

389 It seems important to estimate the frequency, geographic distribution and probable adaptive
390 significance of the inversion polymorphism detected in the Gouldian finch, Brambling and Common linnet,
391 the species with wide breeding and residence areas.

392 Another interesting species is the Eurasian skylark with two giant chromosomes. The origin of its
393 Z/W chromosomes has been resolved (Sigeman et al. 2019, 2020). The genetic content of another giant
394 chromosome of the Eurasian skylark remains unknown. FISH with universal BAC-probes (Damas et al. 2017)
395 might shed a light on its origin. The microchromosomes of this species also deserve a close attention,
396 because despite the fusions of several macrochromosomes in the neo-Z and in another giant chromosome
397 its chromosome number remains the same as in most songbirds.

398 The nature, evolution and adaptive significance of the extended centromeres of Gouldian finch,
399 European pied flycatcher, and Domestic canary are of a special interest. Recent advances in sequencing and
400 bioinformatic analysis of the repetitive DNA of birds (Weissensteiner and Suh 2019) make possible to
401 address these questions.

## 402 Methods

### 403 Specimens

404 Two adult males of the European pied flycatcher, European bullfinch and Eurasian siskin and one
405 adult males of each other species were sampled at the beginning of the breeding season (April-May). The
406 sources of the material are shown in Supplementary Table 1. The birds were handled and euthanized in
407 accordance with the approved national guidelines for the care and use of laboratory animals.

### 408 Spermatocyte spreading and immunostaining

409 Chromosome spreads were prepared by the drying down method (Peters et al. 1997). Testes were
410 dissected and placed in an extraction buffer for 30–60 min. Small pieces of testis were macerated in 40 µl
411 of 0.1M sucrose on a clean glass slide. A drop of fine suspension was dropped at the slide dipped in 1%

412 paraformaldehyde solution (Sigma-Aldrich, cat# 158127), pH 9.2. The slides were incubated for 2 h in a
413 humid chamber, washed in 0.4% Kodak Photo-Flo 200 (Kodak, cat# 742057), dried at room temperature
414 and kept in sealed containers at -20°C until use.

415 Immunostaining was performed according to Anderson et al. (Anderson et al. 1999) using rabbit
416 polyclonal anti-SYCP3 (1:500; Abcam, cat# ab15093), human anticentromere (1:100; Antibodies Inc., cat#
417 15–234) and mouse monoclonal anti-H3K9me2/3 (1:100, Cell Signaling, cat# 5327) primary antibodies. The
418 secondary antibodies used were Cy3-conjugated goat anti-rabbit (1:500; Jackson ImmunoResearch, cat#
419 111-165-144), AMCA-conjugated donkey anti-human (1:100; Jackson ImmunoResearch, cat# 709-155-149)
420 and FITC-conjugated goat anti-mouse (1:100; Jackson ImmunoResearch, cat# 115-095-003). The slides were
421 incubated overnight with primary antibodies and 1 h with secondary antibodies at 37°C in a humid
422 chamber. Slides were mounted in Vectashield antifade mounting medium (Vector Laboratories, cat# H-
423 1000-10, United States).

424 **Mitotic metaphase chromosome preparations**

425 The mitotic metaphase chromosomes were obtained from the cell culture of the gonads. The
426 primary cell cultures were established from the minced gonads, that were successively treated with
427 100ng/ml Collagenase I (Sigma, cat# SCR103) and 0.25% Trypsin-EDTA (Sigma, cat# T4174) during 20 min by
428 every component at 37 °C. The cell cultures were maintained in Dulbecco's Modified Eagle's medium
429 (Gibco, cat# 41965039) supplemented with 10% Fetal bovine serum (Gibco, cat# 10270106), 2% Chicken
430 serum (Gibco, cat# 16110082), GlutaMAX supplement (TermoFisher, cat# 35050038) and
431 penicillin/streptomycin (Sigma-Aldrich, cat# P0781). Preparation of metaphase chromosomes from the
432 bird's cell culture was performed at the 2d to 3d passage according modified protocol of chicken
433 spermatogonial and follicles cell culture (Volkova et al. 2016; Zhao et al. 2018). Briefly, the cell culture
434 was incubated in culture medium with addition of 0.1 µg/ml colchicine for 3 h and collected by trypsine
435 treatment. The cells were treated with hypotonic solution (0.56% KCl) for 30 min and fixed in 3:1 Methanol:
436 Glacial Acetic Acid. Slides were mounted in Vectashield antifade mounting medium with DAPI (Vector
437 Laboratories, cat# H-1200, United States).

438 **Microscopic image analysis.**

439 The preparations were visualized with an Axioplan 2 imaging microscope (Carl Zeiss) equipped with
440 a CCD camera (CV M300, JAI), CHROMA filter sets, and the ISIS4 image-processing package (MetaSystems
441 GmbH). In each specimen at least 30 well spread pachytene nuclei containing complete chromosome sets
442 were photographed. Brightness and contrast of all images were enhanced using Corel PaintShop Photo Pro
443 X6 (Corel Corp).

444 **Chromosome measurements and generation of idiograms.**

445 The centromeres were identified by anticentromere antibodies. The length of the SC of each
446 chromosome arm was measured in micrometers and the positions of centromeres were recorded using
447 MicroMeasure 3.3 (Reeves 2001). We plotted idiograms after measuring at least 30 cells for each species.
448 In each cell, we sorted SCs by their length in µm, identified those that can be distinguished unambiguously
449 by the length and centromere index (most macrochromosomes in most species) and measured the average
450 of these parameters across all cells. We grouped SCs that could not be unambiguously distinguished by the
451 length and/or centromere indices (most microchromosomes in most species), ranged them within the
452 group and measured the average parameters for each rank. Statistica 6.0 software package (StatSoft) was
453 used for descriptive statistics.

454 **Declarations**

455 **Ethics approval**

456 The birds were handled and euthanized in accordance with the approved national guidelines for
457 the care and use of laboratory animals. All experiments were reviewed and approved by the Animal Care
458 and Use Committee of the Institute of Cytology and Genetics SB RAS (protocol # 114 of 17 December 2021).

459 **Consent for publication**

460 Not applicable.

461 **Availability of data and materials**

462 Publicly available datasets were analyzed in this study. These data can be found here:
463 https://meiosislab.com/projects/chromosomes/17caryo.xls.

464 **Competing interests**

465 The authors declare that they have no competing interests.

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472 manuscript.

473 **Authors' contributions**

474 AT and PB provided the research idea and designed the experiments. LM, AS, EG and IP conducted
475 the experiments, collected the data, finished the data analysis and compiled the results. AT and PB
476 supervised the research and wrote the paper. All authors read and approved the final manuscript.

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## References

Anderson LK, Reeves A, Webb LM, Ashley T (1999) Distribution of crossing over on mouse synaptonemal complexes using immunofluorescent localization of MLH1 protein. Genetics 151:1569–1579

Bian X-Z, Li Q-W (1989) Studies on the karyotypes of birds. V. The 20 species of climber birds (Aves). Zool. Res. 10:309–317

Bian X-Z, Li Q-W, Ning S-X (1991) Studies on the karyotypes of birds. III. 10 species of warblers and 4 species of flycatchers (Aves). Zool Res 12:215–219

Brinkley BR, Valdivia MM, Tousson A, Brenner SL (1984) Compound kinetochores of the Indian muntjac - Evolution by linear fusion of unit kinetochores. Chromosoma 91:1–11. https://doi.org/10.1007/BF00286479

Bulatova NS (1973) A cytotaxonomic study of three related families of birds: Fringillidae, Emberizidae, Ploceidae. J Zool Syst Evol Res 11:233–239. https://doi.org/10.1111/j.1439-0469.1973.tb00144.x

Bulatova NS (1981) A comparative karyological study of Passerine birds. Acta Sci Nat Brno 15:1–44

Christidis L (1986a) Chromosomal evolution within the family Estrildidae (Aves) II. The Lonchurae. Genetica 71:99–113. https://doi.org/10.1007/BF00058692

Christidis L (1986b) Chromosomal evolution in finches and their allies (families: Ploceidae, Fringillidae, and

503 Emberizidae). Can J Genet Cytol 28:762–769. https://doi.org/10.1139/g86-107

504 Christidis L (1990) Animal cytogenetics 4: Chordata 3; B, Aves. Animal Cytogenetics. Gebrüder Borntraeger

505 Da Silva Dos Santos M, Kretschmer R, Frankl-Vilches C, et al (2017) Comparative cytogenetics between two
506 important songbird, models: The zebra finch and the canary. PLoS One 12:1–13.
507 https://doi.org/10.1371/journal.pone.0170997

508 Damas J, Kim J, Farré M, et al (2018) Reconstruction of avian ancestral karyotypes reveals differences in the
509 evolutionary history of macro- and microchromosomes. Genome Biol 19:155.
510 https://doi.org/10.1186/s13059-018-1544-8

511 Damas J, O'Connor R, Farré M, et al (2017) Upgrading short-read animal genome assemblies to
512 chromosome level using comparative genomics and a universal probe set. Genome Res 27:875–884.
513 https://doi.org/10.1101/gr.213660.116

514 Degrandi TM, Barcellos SA, Costa AL, et al (2020) Introducing the Bird Chromosome Database: An overview
515 of cytogenetic studies in birds. Cytogenet Genome Res 160:199–205.
516 https://doi.org/10.1159/000507768

517 Degrandi TM, Barcellos SA, Garnero ADV, et al (2021) Bird Chromosome Database.
518 https://sites.unipampa.edu.br/birdchromosomedatabase/

519 del Priore L, Pigozzi MI (2014) Histone modifications related to chromosome silencing and elimination
520 during male meiosis in Bengalese finch. Chromosoma 123:293–302. https://doi.org/10.1007/s00412-
521 014-0451-3

522 del Priore L, Pigozzi MI, Priore L, Pigozzi MI (2015) Heterologous synapsis and crossover suppression in
523 heterozygotes for a pericentric inversion in the Zebra Finch. Cytogenet Genome Res 147:154–160.
524 https://doi.org/10.1159/000442656

525 Frankl-Vilches C, Kuhl H, Werber M, et al (2015) Using the canary genome to decipher the evolution of
526 hormone-sensitive gene regulation in seasonal singing birds. Genome Biol 16:19.

527 https://doi.org/10.1186/s13059-014-0578-9

528 Hammar BO, Herlin M (1975) Karyotypes of four bird species of the order Passeriformes. Hereditas 80:177–
529 184. https://doi.org/10.1111/j.1601-5223.1975.tb01517.x

530 Hooper DM, Price TD (2017) Chromosomal inversion differences correlate with range overlap in passerine
531 birds. Nat Ecol Evol 1:1526–1534. https://doi.org/10.1038/s41559-017-0284-6

532 Huang Y-C, Lee C-C, Kao C-Y, et al (2016) Evolution of long centromeres in fire ants. BMC Evol Biol 16:189.
533 https://doi.org/10.1186/s12862-016-0760-7

534 Kawakami T, Mugal CF, Suh A, et al (2017) Whole-genome patterns of linkage disequilibrium across
535 flycatcher populations clarify the causes and consequences of fine-scale recombination rate variation
536 in birds. Mol Ecol 26:4158–4172. https://doi.org/10.1111/mec.14197

537 Kiazim LG, O'Connor RE, Larkin DM, et al (2021) Comparative mapping of the macrochromosomes of eight
538 avian species provides further insight into their phylogenetic relationships and avian karyotype
539 evolution. Cells 10:362. https://doi.org/10.3390/cells10020362

540 Kichigin IG, Lisachov AP, Giovannotti M, et al (2018) First report on B chromosome content in a reptilian
541 species: the case of Anolis carolinensis. Mol Genet Genomics 0:0. https://doi.org/10.1007/s00438-
542 018-1483-9

543 Kinsella CM, Ruiz-Ruano FJ, Dion-Côté A-M, et al (2019) Programmed DNA elimination of germline
544 development genes in songbirds. Nat Commun 10:5468. https://doi.org/10.1038/s41467-019-13427-4

545 Li Q, Bian X (1987) The study of bird karyotypes I: Fringillidae (Aves). Zool Res 4:387–392

546 Li X, Bian Q (1988) Studies on the karyotypes of birds Ⅱ The 19 species of 12 families of Passerine
547 birds.(Passeriformes, Aves). Zool Res 9:321–326.

548 Lisachov AP, Borodin PM (2016) Microchromosome polymorphism in the sand lizard, Lacerta agilis
549 Linnaeus, 1758 (Reptilia, Squamata). Comp Cytogenet 10:387–399.
550 https://doi.org/10.3897/CompCytogen.v10i3.7655

551 Lisachov AP, Trifonov VA, Giovannotti M, et al (2017) Heteromorphism of "homomorphic sex chromosomes
552 in two anole species (Squamata, Dactyloidae) revealed by synaptonemal complex analysis. Cytogenet
553 Genome Res 151:89–95. https://doi.org/10.1159/000460829

554 Malinovskaya LP, Zadesenets KS, Karamysheva T V, et al (2020) Germline-restricted chromosome (GRC) in
555 the sand martin and the pale martin (Hirundinidae, Aves): synapsis, recombination and copy number
556 variation. Sci Rep 10:1058. https://doi.org/10.1038/s41598-020-58032-4

557 Metcalfe CJ, Bulazel K V., Ferreri GC, et al (2007) Genomic instability within centromeres of interspecific
558 marsupial hybrids. Genetics 177:2507–2517. https://doi.org/10.1534/genetics.107.082313

559 Moses MJ, Slatton GH, Gambling TM, Starmer CF (1977) Synaptonemal complex karyotyping in
560 spermatocytes of the Chinese hamster (Cricetulus griseus). III. Quantitative evaluation. Chromosoma
561 60:345–375

562 Neumann P, Pavlíková Z, Koblížková A, et al (2015) Centromeres off the hook: Massive changes in
563 centromere size and structure following duplication of cenh3 gene in fabeae species. Mol Biol Evol
564 32:1862–1879. https://doi.org/10.1093/molbev/msv070

565 Neumann P, Schubert V, Fuková I, et al (2016) Epigenetic histone marks of extended meta-polycentric
566 centromeres of lathyrus and pisum chromosomes. Front Plant Sci 7:234.
567 https://doi.org/10.3389/fpls.2016.00234

568 Ohno S, Stenius C, Christian LC, et al (1964) Chromosomal uniformity in the avian subclass Carinatae.
569 Chromosoma 15:280–288. https://doi.org/10.1007/BF00321513

570 Peters AH, Plug AW, van Vugt MJ, de Boer P (1997) A drying-down technique for the spreading of
571 mammalian meiocytes from the male and female germline. Chromosom Res 5:66–68.
572 https://doi.org/10.1023/a:1018445520117

573 Piccinni E, Stella M (1070) Some avian karyograms. Caryologia 23:189–202

574 Pigozzi MI (2016) The chromosomes of birds during meiosis. Cytogenet Genome Res 150:128–138.

575 https://doi.org/10.1159/000453541

576 Pigozzi MI, Solari AJ (1998) Germ cell restriction and regular transmission of an accessory chromosome that
577 mimics a sex body in the zebra finch, Taeniopygia guttata. Chromosom Res 6:105–113.
578 https://doi.org/10.1023/A:1009234912307

579 Poorman PA, Moses MJ, Davisson MT, Roderick TH (1981) Synaptonemal complex analysis of mouse
580 chromosomal rearrangements. III. Cytogenetic observations on two paracentric inversions.
581 Chromosoma 83:419–429

582 Radzhabli SI, Panov EN, N.S. B (1970) Comparative studies of the karyotype of two closely related species of
583 buntings (Emberiza citrinella and E. leucocephalos) hybridized in areas of overlap. Zool Zh 49:1857–
584 1863

585 Ray-Chaudhuri R (1976) Cytotaxonomy and chromosome evolution in Passeriformes (Ayes): A comparative
586 karyotype study of seventeen species. J Zool Syst Evol Res 14:299–320.
587 https://doi.org/10.1111/j.1439-0469.1976.tb00528.x

588 Recuerda M, Vizueta J, Cuevas-Caballé C, et al (2021) Chromosome-level genome assembly of the common
589 chaffinch (Aves: Fringilla coelebs): a valuable resource for evolutionary biology. Genome Biol Evol
590 13:1–6. https://doi.org/10.1093/gbe/evab034

591 Reeves A (2001) MicroMeasure: a new computer program for the collection and analysis of cytogenetic
592 data. Genome 44:439–443

593 Romanov MN, Farré M, Lithgow PE, et al (2014) Reconstruction of gross avian genome structure,
594 organization and evolution suggests that the chicken lineage most closely resembles the dinosaur
595 avian ancestor. BMC Genomics 15:1060. https://doi.org/10.1186/1471-2164-15-1060

596 Roslik G V., Kryukov AP (2001) A karyological study of some corvine birds (Corvidae, Aves). Russ J Genet
597 37:796–806. https://doi.org/10.1023/A:1016703127516

598 Schubert I, Lysak MA (2011) Interpretation of karyotype evolution should consider chromosome structural

599 constraints. Trends Genet. 27:207–216

600 Sigeman H, Ponnikas S, Chauhan P, et al (2019) Repeated sex chromosome evolution in vertebrates
601 supported by expanded avian sex chromosomes. Proc R Soc B Biol Sci 286:.
602 https://doi.org/10.1098/rspb.2019.2051

603 Sigeman H, Ponnikas S, Hansson B (2020) Whole-genome analysis across 10 songbird families within
604 Sylvioidea reveals a novel autosome–sex chromosome fusion. Biol Lett 16:20200082.
605 https://doi.org/10.1098/rsbl.2020.0082

606 Takagi N (1972) A comparative study of the chromosome replication in 6 species of birds. Japanese J Genet
607 47:115–123. https://doi.org/10.1266/jjg.47.115

608 Torgasheva AA, Malinovskaya LP, Zadesenets KS, et al (2019) Germline-restricted chromosome (GRC) is
609 widespread among songbirds. Proc Natl Acad Sci 116:11845–11650.
610 https://doi.org/10.1073/pnas.1817373116

611 Torgasheva A, Malinovskaya L, Zadesenets K, et al (2021a) Germline restricted chromosome (GRC) in
612 female and male meiosis of the great tit (Parus major, Linnaeus, 1758). Front Genet 12:2008.
613 https://doi.org/10.3389/fgene.2021.768056

614 Torgasheva A, Malinovskaya L, Zadesenets KS, et al (2021b) Highly conservative pattern of sex chromosome
615 synapsis and recombination in Neognathae Birds. Genes (Basel) 12:1358.
616 https://doi.org/10.3390/genes12091358

617 Udagawa T (1952) Karyogram Studies in Birds, I: Chromosomes of five passeres. Cytologia (Tokyo) 17:311–
618 316. https://doi.org/10.1508/cytologia.17.311

619 Volkova NA, Korzhikova S V, Kotova TO, et al (2016) Isolation and characterization of rooster (Gallus gallus)
620 spermatogonia. Sel'skokhozyaistvennaya Biol 51:450–458.
621 https://doi.org/10.15389/agrobiology.2016.4.450eng

622 Weissensteiner MH, Suh A (2019) Repetitive DNA: The dark matter of avian genomics. In: Avian Genomics

623 in Ecology and Evolution. pp 93–150

624 Zhao D, Leghari IH, Li J, et al (2018) Isolation and culture of chicken growing follicles in 2- and 3-dimensional
625 models. Theriogenology 111:43–51. https://doi.org/10.1016/j.theriogenology.2018.01.012

626 Zhou Q, Zhang J, Bachtrog D, et al (2014) Complex evolutionary trajectories of sex chromosomes across bird
627 taxa. Science (80- ) 346:1246338–1246338. https://doi.org/10.1126/science.1246338

628

629

## Supplementary materials

Supplementary Table 1. Sources of the birds examined

| Common name | Source |
|---|---|
| Common cuckoo | Provided by Bird Rehabilitation Centre after fatal accident trauma |
| Rook | Provided by Bird Rehabilitation Centre after fatal accident trauma |
| Blyth's reed warbler | Trapped in Novosibirsk |
| Eurasian skylark | Trapped in Novosibirsk |
| European pied flycatcher | Trapped in Novosibirsk |
| Bengalese finch | Purchased a commercial breeder |
| Gouldian finch | Purchased a commercial breeder |
| Pine bunting | Trapped in Novosibirsk |
| Common chaffinch | Purchased from a commercial breeder |
| Brambling | Purchased from a commercial breeder |
| Eurasian bullfinch | Provided by Bird Rehabilitation Centre after fatal accident trauma |
| European greenfinch | Purchased from a commercial breeder |
| Common redpoll | Purchased from a commercial breeder |
| Domestic canary | Purchased from a commercial breeder |
| European goldfinch | Purchased from a commercial breeder |
| Common linnet | Purchased from a commercial breeder |
| Eurasian siskin | Trapped in Novosibirsk |

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636 **Supplementary Table 2** Size of expanded centromeres

| Species | SC rank | SC length, µm | Centromere length, µm | Centromere length, % |
|---|---|---|---|---|
| Flycatcher | SC1 | 26.2±1.9 | 2.3±0.4 | 8.6 |
| | SC2 | 20.3±2.3 | 2.2±0.7 | 11.2 |
| | SC3 | 21.9±3.9 | 2.5±0.4 | 11.6 |
| | SC5 | 15.0±1.9 | 3.9±0.7 | 25.9 |
| | SC6/7 | 13.4±1.6 | 2.1±0.6 | 15.4 |
| | SC8/10 | 8.8±1.5 | 2.3±0.4 | 26.3 |
| | SC11/13 | 6.8±0.4 | 2.8±0.3 | 41.3 |
| Canary | SC1 | 34.7±5.2 | 3.5±0.8 | 10.1 |
| | SC2/3 | 27.3±5.7 | 3.2±0.8 | 11.7 |
| | SC4 | 24.0±3.7 | 2.3±0.4 | 9.4 |
| | SC5 | 19.1±4.6 | 3.0±0.8 | 15.6 |
| | SC6 | 17.1±5.5 | 1.4±0.3 | 8.2 |
| | SC10/11/12 | 9.5±1.8 | 1.7±0.3 | 17.8 |
| Gouldian finch | SC1 | 27.9±4.4 | 3.6±0.6 | 13.6 |
| | SC2 | 22.5±3.6 | 4.4±0.6 | 19.8 |
| | SC3 | 22.3±3.7 | 3.8±0.7 | 17.1 |

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