Comparison of Karyotypes in Two Hybridizing Passerine Species: Conserved Chromosomal Structure but Divergence in Centromeric Repeats

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Changes in chromosomal structure involving chromosomal rearrangements or copy number variation of specific sequences can play an important role in speciation. Here, we explored the chromosomal structure of two hybridizing passerine species; the common nightingale (Luscinia megarhynchos) and the thrush nightingale (Luscinia luscinia), using conventional cytogenetic approaches, immunostaining of meiotic chromosomes, fluorescence in situ hybridization as well as comparative genomic hybridization (CGH). We found that the two nightingale species show conserved karyotypes with the same diploid chromosome number of 2n = 84. In addition to standard chromosomes, both species possessed a small germline restricted chromosome of similar size as a microchromosome. Just a few subtle changes in chromosome morphology were observed between the species, suggesting that only a limited number of chromosomal rearrangements occurred after the species divergence. The interspecific CGH experiment suggested that the two nightingale species might have diverged in centromeric repetitive sequences in most macro- and microchromosomes. In addition, some chromosomes showed changes in copy number of centromeric repeats between the species. The observation of very similar karyotypes in the two nightingale species is consistent with a generally slow rate of karyotype evolution in birds. The divergence of centromeric sequences between the two species could theoretically cause meiotic drive or reduced fertility in interspecific hybrids. Nevertheless, further studies are needed to evaluate the potential role of chromosomal structural variations in nightingale speciation.

Keywords: chromosomal structure, karyotype evolution, comparative genomic hybridization, rDNA, centromere, GRC, birds, Luscinia
INTRODUCTION

Despite an increasing number of sequenced avian genomes (Jarvis et al., 2014; Zhang et al., 2014; Feng et al., 2020), we still know relatively little about the organization of the genomes into chromosomes and to what degree the chromosomal structure (i.e., number, size and collinearity of chromosomes) varies among species. It has been proposed that changes in chromosomal structure, including chromosomal translocations, inversions and copy number variations, may play an important role in the origin of reproductive isolation between species (White, 1978; Rieseberg, 2001; Wellenreuther et al., 2019; Zhang et al., 2021). For example, chromosomal translocations may cause problems with chromosome pairing, recombination and segregation during meiosis, which can lead to hybrid sterility (White, 1978; King, 1993; Homolka et al., 2007). Structural changes, such as inversions, may facilitate speciation by reducing the recombination rate within the structural variant, which may help to maintain the species-specific traits in the face of gene flow (Rieseberg, 2001; Ortíz-Barrientos et al., 2002; Butlin, 2005; Hoffmann and Rieseberg, 2008). Finally, copy number variations may serve as a source of adaptive phenotypic variation (Perry et al., 2007; Zhou et al., 2011; Iskow et al., 2020a). In addition to size, macrochromosomes and microchromosomes differ in their GC content, gene density, rearrangements between species (Bi and Bogart, 2006; Bi et al., 2018; Degrandi et al., 2020a). In addition, immunostaining of the synapsed chromosomes during meiosis provides a useful approach for detection of the GRC and comparing the chromosomal structure among species (Hale et al., 1988; Torgasheva et al., 2019).

Based on the FISH technique Kallioniemi et al. (1992) developed a new fine scale molecular cytogenetic method called comparative genomic hybridization (CGH). This method allows for the detection of unbalanced chromosomal rearrangements (i.e., duplications, deletions, and copy number variation) between two sources of DNA. Originally the method was designed to detect chromosomal changes in tumor cells compared to normal cells (Kallioniemi et al., 1992). Later, it was used for sex chromosome detection using male and female DNA (e.g., Koubová et al., 2014; Pokorná et al., 2014). Finally, an interspecific design was developed to detect chromosomal rearrangements between species (Bi and Bogart, 2006; Symonová et al., 2015; de Oliveira et al., 2019). In most CGH studies done in birds, the chicken genome has been used as a reference with a microarray-based CGH platform (array-CGH) (Skinner et al., 2009, 2014; Völker et al., 2010). To our knowledge, no interspecific CGH comparisons have been performed to detect copy number variation between closely related bird species.

In this study, we compared the karyotypes of two closely related passerines species, the common nightingale (Luscinia megarhynchos) and the thrush nightingale (Luscinia luscinia), that diverged ∼1.8 Mya (Storchová et al., 2010) and currently hybrideize in a secondary contact zone spanning Central and Eastern Europe (Reifová et al., 2011a). These species are separated by incomplete reproductive isolation, which is mainly caused by female-limited hybrid sterility (Reifová et al., 2011b; Markovský et al., 2018) and partial ecological divergence in sympathy (Reifová et al., 2011a). This chromosome is eliminated from the somatic cells during early development; being maintained only in the germ line. In some passerines the GRC represents a big macrochromosome, while in others, a small microchromosome (Torgasheva et al., 2019). However, the size of this chromosome has only been characterized in 16 species so far and it is not clear how often it differs among closely related species (Torgasheva et al., 2019).
et al., 2018; Sottas et al., 2018, 2020). In addition, divergence in sperm morphology might contribute to postmating prezygotic isolation (Albrecht et al., 2019). Of these species, the common nightingale’s karyotype has been previously described using classical cytogenetic analysis of the somatic metaphases (Bozhko, 1971). However, the karyotype of the thrush nightingale has yet to be determined.

Here we performed a cytogenetic analysis of the nightingale karyotypes to test whether changes in chromosomal structure might be linked to reproductive isolation between the species. To do so, we applied conventional and molecular cytogenetics methods to mitotic and meiotic spreads. These methods included C-banding, immunofluorescence staining of synapsed pachytenes, and physical mapping of telomeric and 18S rDNA probes using FISH, and finally CGH.

**MATERIALS AND METHODS**

**Sampling Procedure**

The sampling of the two nightingale species was carried out in allopatric regions (where only one of the two species occurs) to avoid possible sampling of interspecific hybrids. The common nightingale was sampled in South-western Poland by the Odra river, near the town Brzeg Dolny (N 51.2602°, E 16.7440°). The thrush nightingale was sampled in North-eastern Poland by the Narew river, near the town Łomża (N 53.1621°, E 22.1246°). In total, we sampled four common nightingales (one male, three females) and two thrush nightingales (one male, one female) for mitotic spreads and two males of each species for meiotic spreads. The birds were euthanized by standard cervical dislocation and the tibiae were immediately dissected for the preparation of mitotic and meiotic chromosomal spreads, respectively. In addition, we collected a blood sample from the brachial vein from one female of each species. The blood sample was later used for DNA isolation and preparation of species-specific DNA probes for the interspecific comparative genomic hybridization (CGH) experiment. All individuals were sampled in May 2019, during the breeding season, and were captured using mist nets or collapsible traps. The work was approved by the General Directorate for Environmental Protection, Poland (permission no. DZP-WG.6401.03.123.2017.dl.3).

**Mitotic Chromosome Preparation and C-Banding**

Bone marrow from the tibias of each bird was flushed out using a syringe needle with D-MEM medium (Sigma Aldrich) and cultivated in 5 ml of D-MEM medium (Sigma Aldrich) with 75 µl of colcemid solution (Roche) for 40 min at 37°C. After that, the cells were hypotonized in pre-warmed 0.075 M KCl solution for 25 min at 37°C. Finally, cells were washed four times with fixative solution (methanol:acetic acid, 3:1) and then stored at −20°C prior to use.

Chromosomal spreading was done using the air-drying technique followed by conventional Giemsa staining (5% Giemsa in 0.07 M phosphate buffer, pH 7.4). The C-banding method was applied for visualization of constitutive heterochromatin according to Sumner (1972). More specifically, slides with chromosomal spreads were aged at 60°C for 1 h then successively soaked in 0.2 N HCl for 20 min at room temperature then in 5% Ba(OH)2 solution for 4–5 min at 45°C and subsequently in 2× SSC for 1 h at 60°C, with intermediate washes in distilled water. Finally, metaphases were mounted with 4’,6-diamidino-2-phenylindole (DAPI) in mounting medium Vectashield (Vector laboratories).

**Meiotic Chromosome Preparation and Immunostaining**

Synaptosomal complex (SC) spreads were prepared from the testes of reproductively active males following Peters et al. (1997). Briefly, the left testis was cut into two pieces and placed in hypotonic solution (30 mM Tris, 50 mM sucrose, 17 mM trisodium citrate dehydrate and 5 mM EDTA; pH 8.2) for 50 min. The testis tissue was then disaggregated in 200 µl of 100 mM sucrose and the resulting cell suspension was applied in 40 µl drops and spread onto a slide previously treated with 1% PFA and 0.15% of Triton X100 (Sigma Aldrich). All slides were placed in a humid chamber for 90 min and washed for 2 min in 1× PBS. Slides were directly used for immunostaining.

Immunostaining was performed according to Moens et al. (1987) using the following primary antibodies: rabbit polyclonal anti-SYCP3 antibody (ab15093, Abcam) recognizing the lateral elements of the synaptosomal complex (dilution 1:200), human anticentromere serum (CREST, 15-234, Antibodies Incorporated) binding kinetochores (dilution 1:50). The corresponding secondary antibodies were Alexa-594-conjugated goat anti-Rabbit IgG (H + L) (A32740, Invitrogen; dilution 1:200) and Alexa-488-conjugated goat anti-Human IgG (H + L) (A-11013, Invitrogen; dilution 1:200). Primary and secondary antibodies were diluted in PBT (3% BSA and 0.05% Tween 20 in 1× PBS) and incubated in a humid chamber for 90 min. Slides were washed three times in 1× PBS and dehydrated through an ethanol row (50, 70 and 96%, 3 min each). Finally, all slides were dried and stained with DAPI in mounting medium Vectashield (Vector laboratories).

**Fluorescence In Situ Hybridization (FISH) With Telomeric and 18S rRNA Probes**

Telomeric repeat probe (TTAGGG)n was applied to meiotic and mitotic spreads using FISH. In both experiments, the telomeric repeat sequences were detected using a commercial kit probe directly labelled with Cy3 (DAKO). We followed the manufacturer’s instructions, with the hybridization extended to 1.5 h.

The distribution of 18S rDNA genes was analyzed on mitotic spreads using FISH. The 18S rDNA probe was generated by PCR amplification and nick-translation labelling according to the protocol of Cioffi et al. (2009). The template genomic DNA originated from a reptile species, slow-worm (Anguis fragilis), and the PCR product was 1,456 bp in length (sequence is provided in Supplementary Table 1). The probe showed high
sequence similarity with the rDNA of several bird species, *Gallus gallus* (97.73%), *Hirundo rustica* and *Motacilla alba* species (both 97.11%), and thus was considered similar enough to detect the distribution of 18S rDNA clusters in the nightingale species. Slides were aged for 1 h at 60°C, then treated with RNase for 1 h at 37°C and washed three times for 5 min in 2× SSC. Chromosomes were treated with pepsin for 3 min at 37°C and then fixed for 10 min in 1% formaldehyde solution. Slides were dehydrated in an ethanol row (70, 85 and 96%, 3 min each) and air-dried. The chromosomes were denatured in 75% formamide/2× SSC at 76°C for 3 min followed by dehydration in an ethanol row. Meanwhile, the probe was denatured at 80°C for 6 min and chilled on ice for 10 min prior to the hybridization. The probe-chromosome hybridization was performed overnight at 37°C. Post-hybridization washes were performed three times for 5 min in 50% formamide/2× SSC at 37°C and washed twice for 5 min in 2× SSC and finally for 5 min in 4× SSC/0.05% Tween 20 (Sigma Aldrich). Slides were first incubated for 30 min at 37°C with 4× SSC/5% blocking reagent (Roche), then the probe signal was detected by 4× SSC/5% blocking reagent mixed with fluorescein conjugated avidin (Vector laboratories) for 30 min at 37°C, followed by three washes in 4× SSC/0.05% Tween 20 for 5 min. Slides were then incubated with biotinylated anti-avidin (Vector laboratories) for 30 min at 37°C, followed by a second round of fluorescein conjugated avidin treatment for signal amplification. Slides were finally washed in 4× SSC/0.05% Tween 20 twice for 5 min, dehydrated through an ethanol row and air-dried. Chromosomes were stained with DAPI in mounting medium, Vectashield (Vector laboratories).

**Comparative Genomic Hybridization (CGH)**

The CGH experiment was performed with (i) common nightingale and (ii) thrush nightingale metaphase chromosomes. In both cases, equal concentrations of DNA probes from the common nightingale and the thrush nightingale were hybridized to the chromosomes (Figure 1) following the procedure described in Symonová et al. (2015) with slight modification. The DNA probe was labelled by biotin (detected by streptavidin-FITC, green) in the common nightingale and digoxigenin (detected by antidigoxigenine-rhodopsine, red) in the thrush nightingale. In both experimental designs, the green signal suggests a higher copy number of a particular repetitive sequence in the common nightingale, while the red signal indicates a higher copy number in the thrush nightingale. An intermediate yellow/orange signal suggests the same copy number in both species. Finally, a green signal in one design, while red in the other, indicates the presence of species-specific sequence (Figure 1).

Genomic DNA for the preparation of probes was extracted from blood samples using DNeasy Blood and Tissue Kit (Qiagen). The probes were prepared by nick translation (Abbott Laboratories) according to the manufacturer’s protocol and labelled with biotin-dUTP (Roche) and digoxigenin-dUTP (Roche). The nick translation took place at 15°C for 2 h. From each sample, 1 µg of DNA was co-precipitated overnight at −20°C with an additional 5 µl of salmon sperm DNA (10 mg/ml, Sigma Aldrich), 3 µl of 3 M sodium acetate (pH 5.2) and 2.5 volume of 96% ethanol. After precipitation, the dry pellets were resuspended in 11 µl of hybridization buffer for each slide (50% formamide in 2× SSC, 10% dextran sulfate, 10% sodium dodecyl sulfate and 1× Denhardt’s buffer, pH 7.0), denatured at 86°C for 6 min and then chilled on ice for 10 min prior to hybridization.

Metaphase slides were prepared by treatment with RNase and pepsin before being fixed with 1% formaldehyde, dehydrated through an ethanol row (70, 85 and 96%, 3 min each) and air-dried. Chromosomes were then denatured in 75% formamide/2× SSC at 76°C for 3 min and dehydrated again in an ice cold ethanol row (70, 80 and 96%, 3 min each). Finally, 11 µl of the probe mix was added to each slide and hybridized overnight at 37°C. Post-hybridization washes were performed three times for 5 min in 4× SSC at 76°C and washed twice for 5 min in 2× SSC and finally for 5 min in 0.1× SSC/0.05% Tween 20. From each sample, 1 µg of DNA was co-precipitated overnight at −20°C with 5 µl of salmon sperm DNA (10 mg/ml, Sigma Aldrich), 3 µl of 3 M sodium acetate (pH 5.2) and 2.5 volume of 96% ethanol. After precipitation, the dry pellets were resuspended in 11 µl of hybridization buffer for each slide (50% formamide in 2× SSC, 10% dextran sulfate, 10% sodium dodecyl sulfate and 1× Denhardt’s buffer, pH 7.0), denatured at 86°C for 6 min and then chilled on ice for 10 min prior to hybridization.
was applied to each slide and hybridization took place at 37°C for 48 h. The same probe mix was applied to metaphases of both nightingale species in the two CGH designs.

Post-hybridization washes were performed two and three times in 50% formamide/2× SSC and 1× SSC at 44°C, respectively. Each slide was incubated with 100 µl of 4× SSC/5% blocking reagent (Roche) at 37°C for 30 min and then with 100 µl of detection mix containing 4× SSC/5% blocking reagent, 2 µl of streptavidin-FITC (Vector Laboratories) and 1 µl of antidigoxigenin-rhodamine (Roche) at 37°C for 1 h. The slides were subsequently washed in 4× SSC/0.01% Tween 20 (Sigma Aldrich) at 44°C, dehydrated through an ethanol row (70, 85 and 96%, 3 min each) and air-dried. Finally, the chromosomes were counterstained with DAPI in mounting medium Vectashield (Vector laboratories).

Microscopy and Image Processing
Mitotic spreads were captured with an Axio Imager Z2 microscope (Zeiss) equipped with the automatic Metafer-MSearch scanning platform and a CoolCube 1 b/w digital camera (MetaSystems). Meiotic spreads were analyzed using an Olympus BX53 fluorescence microscope (Olympus) equipped with a DP30BW digital camera (Olympus). Ikaros karyotyping software (Metasystems) was used to remove the background from the metaphase images and to arrange the karyotypes. The colors of the C-banded metaphase images were inverted. All color images were captured in black and white, and later pseudocolored and superimposed using Adobe Photoshop software (version CC 2017).

A total of 18 and 17 metaphases from bone marrow were analyzed for the common nightingale and the thrush nightingale, respectively. The W chromosome was detected by C-banding due to its heterochromatic character. The Z chromosome was identified by comparing the female (ZW) and male (ZZ) metaphases. The size of each bivalent and its arm ratio was measured using the LEVAN plugin in the program ImageJ (Schindelin et al., 2012). Depending on the position of the centromere, we distinguished for each macrochromosome whether it was telocentric, acrocentric, submetacentric or metacentric chromosomes (Levan, 1964). For microchromosomes, the telocentric/acrocentric categories and submetacentric/metacentric categories were merged as they were difficult to distinguish clearly. The Z chromosome was identified among bivalents based on its relative size to the other macrochromosomes identified on the mitotic spreads. Chromosomes were measured in 15 pachytene cells in each species.

The metaphase chromosomes with applied CGH were analyzed using Photoshop (version CC 2017). For each CGH design, three cells were analyzed and compared. The centomeric red and green signals of the nine largest macrochromosomes and the sex chromosomes were measured using the histogram color tools. Each metaphase was measured three times to reduce the technical error associated with the signal measurement. The color ratio was calculated using the median value of both colors, after their normalization using the total red and green signal color.

RESULTS
Mitotic and Meiotic Karyotypes
Both species showed a more or less continuous decrease in chromosome size without a clear distinction between macrochromosomes and microchromosomes (Figure 2). We categorized the 10 largest chromosome pairs including the sex chromosomes to be macrochromosomes with the remaining chromosomes considered to be microchromosomes. Because the mitotic chromosomes were not always well spread, it was difficult to estimate the diploid chromosome number from metaphase spreads. This was especially true with respect to the number of microchromosomes. We thus calculated the diploid chromosome number for both species based on immunostained meiotic spreads (Hale et al., 1988; del Priore and Pigozzi, 2020). Both the common nightingale and the thrush nightingale consistently displayed 42 bivalents, establishing a diploid chromosome number of 84 for each species (Figure 3).
addition to these bivalents, both species displayed an extra univalent chromosome in male germ cells, corresponding to the GRC (Figure 3). The GRC was stained weaker by anti-SYCP3 antibody and showed a CREST signal not only in the centromere, but along the whole chromosome, as has been described previously in other passerine species (Torgasheva et al., 2019).

Staining of centromeres in meiotic chromosomes by the CREST antibody allowed us to estimate the arm ratio for each chromosome and compare chromosome morphology between species in a more precise way than was possible with mitotic chromosomes. The ten largest chromosomes had the same morphology between the species, suggesting that no chromosomal rearrangements that would have changed the position of the centromere occurred on these chromosomes. In both species, the largest chromosome, SC1, was identified as acrocentric, SC2 to SC4 as telocentric, SC5 as submetacentric, SC6 as metacentric and SC7 to SC9 as telocentric. However, SC10 was telocentric in the common nightingale, but acrocentric in the thrush nightingale (Figure 3; Supplementary Table 2), indicating that some rearrangements might have occurred on this chromosome.

Based on the comparison of male and female mitotic spreads, we identified the Z chromosome as the fourth largest chromosome in both species and in both species it was telocentric. The W chromosome was also telocentric, with a size between the 10th and 11th chromosome in the common nightingale and between the ninth and 10th chromosome in the thrush nightingale (Figure 4).

The morphology of the microchromosomes slightly differed between the two species with 17 acrocentric/telocentric and 15 submetacentric/metacentric microchromosomes in the common nightingale and 19 acrocentric/telocentric and 13 submetacentric/metacentric microchromosomes in the thrush nightingale (Figure 3; Supplementary Table 2). The GRC was present in both nightingale species, with its size corresponding to a microchromosome.

Distribution of Heterochromatin, 18S rDNA Genes and Telomeric Repeats in the Two Species

The distribution of the constitutive heterochromatin revealed by C-banding displayed the same pattern in the two nightingale
species. C-banding signals mainly occurred in the centromeric regions of macrochromosomes and microchromosomes, but sometimes the signal covered the entire microchromosome. The W chromosome displayed a large C-banding signal in both species, but the signal was slightly larger in the thrush nightingale than in the common nightingale (Figure 4). In both species, the Z chromosome had a small heterochromatic band in the centromeric region (Figure 4).

18S rDNA clusters were consistently located on 10 microchromosomes in both species (Figure 4). The same number and distribution of the 18S rDNA clusters suggests that no rearrangements that would include rDNA genes had occurred between the two species.

The telomeric motif (TTAGGG)$_n$ was detected at the terminal regions of all chromosomes. No interstitial telomeric signal was detected (Figure 4; Supplementary Figure 1). This can be seen in both the mitotic (Figure 4) and meiotic spreads (Supplementary Figure 1).

Divergence of Centromeric Repeats Between the Two Nightingale Species

In both interspecific CGH experimental designs (i.e., with the common nightingale and the thrush nightingale metaphases, Figure 1), the distribution pattern of the probe signal was similar to that of the heterochromatin from the C-banding experiment, meaning that the probe signal was brightest in the centromeric regions of the macrochromosomes and microchromosomes (Figures 4, 5). In some microchromosomes, the whole chromosome appeared to be generating signal, however, due to the small size of chromosomes and the signal strength of the probes, this might still only represent centromeric binding.

Interestingly, the centromeric regions of the nine largest autosomes were mostly green (common nightingale probe) in the CGH with common nightingale metaphases and red (thrush nightingale probe) in the CGH with thrush nightingale metaphases, suggesting sequence divergence of repetitive elements in the centromeric regions. The exceptions were the first and fifth chromosome pairs, which showed an increased red signal in both CGH designs, indicating a higher copy number of centromeric repetitive elements in the thrush nightingale genome. The fourth pair produced variable signals across the three metaphases, making the results difficult to interpret (Figures 5, 6; Supplementary Table 3).

The whole W chromosome displayed a higher red signal in both interspecific CGH designs, indicating a higher number of repetitive elements in the thrush nightingale genome. Contrastingly, the probe signal in the centromeric region of the Z chromosome was greener in both the common

![FIGURE 4](https://example.com/figure4.png) Distribution of heterochromatin (A,D), 18S rDNA clusters (B,E) and telomeric repeats (C,F) in the karyotype of the two nightingale species. C-banding in the common nightingale (L. megarhynchos) (A) and the thrush nightingale (L. luscinia) (D) female karyotypes. Sex chromosomes are indicated in both karyotypes. rDNA clusters (green) in the common nightingale (B) and the thrush nightingale (E). Arrowheads point to 10 microchromosomes displaying a rDNA signal. Telomeric repeat sequences (TTAGGG)$_n$ (red) in the common nightingale (C) and the thrush nightingale (F). Scale bar = 10 µm.

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nightingale and the thrush nightingale experimental designs (Figures 5, 6; Supplementary Table 3). Thus, the Z chromosome seems to have a higher copy number of centromeric repetitive elements in the common nightingale compared to the thrush nightingale.

The centromeric regions of microchromosomes were mostly green in the CGH with the common nightingale metaphases and red in the CGH with the thrush nightingale metaphases, suggesting sequence divergence of centromeric repeats on most microchromosomes (Figure 5).

**DISCUSSION**

In this study, we compared the chromosomal structure in two closely related passerine species, the common nightingale and the thrush nightingale, that show partial reproductive isolation caused mainly by hybrid female sterility and ecological differentiation (Storchová et al., 2010; Reifová et al., 2011b; Mořkovský et al., 2018; Reif et al., 2018; Sottas et al., 2018). We found that the two species have the same diploid chromosome number 2n = 84 and both possess a micro GRC in the germ cells. However, a few subtle changes in chromosome morphology imply that some chromosomal rearrangements might have occurred between the species. Interestingly, the interspecific CGH experiment suggests that the two nightingale species might have diverged in centromeric repetitive sequences on most chromosomes. Some chromosomes showed changes in copy number of centromeric repeats between the species.

Changes in chromosomal structure are assumed to play an important role in the origin of reproductive isolation. They can for example impair meiosis in hybrids leading to hybrid sterility, or suppress recombination linking together species-specific combinations of alleles, which may help to maintain species differentiation in the face of gene flow (Rieseberg, 2001; Ortíz-Barrientos et al., 2002; Butlin, 2005). The two nightingale species have very similar karyotypes, with 10 macrochromosomes (including the sex chromosomes) and 32 microchromosomes. This observed diploid chromosome number is consistent with the previously described chromosome number for the common nightingale (Bozhko, 1971). The diploid chromosome number in other species of the family Muscicapidae varies between 2n = 64 and 2n = 86 (Udagawa, 1955; Bulatova and Panov, 1973; Mittal and Satija, 1978; Bulatova, 1981; Degrandi et al., 2020a). Thus, although some large-scale chromosomal rearrangements occurred between more distantly related species of the Muscicapidae family, the closely related nightingale species seem to have the same chromosome number, which is consistent with the generally slow evolution of bird karyotypes (Christidis, 1990; Pichugin et al., 2001; Masabanda et al., 2004; Griffin et al., 2007; Ellegren, 2010; Nanda et al., 2011).

The distribution of constitutive heterochromatin blocks observed in the nightingale species is typical for passerine birds (e.g., Kretschmer et al., 2014; Barcellos et al., 2019). The larger heterochromatin block on the W in the thrush nightingale suggests that this species might have accumulated more repetitive sequences on this chromosome compared to the common nightingale. This was supported by the CGH experiment demonstrating that the W of the thrush nightingale show a higher copy number of repetitive sequences than the common nightingale. Together these results suggest the relatively fast evolution of the W repetitive content, which might
theoretically contribute to reproductive isolation between the species (Peona et al., 2021).

The rDNA clusters are considered as hotspots of chromosomal breakage due to their repetitive nature as well as their intense transcriptome activity (Huang et al., 2008; Cazaux et al., 2011). In birds, a large variation in the number of chromosome pairs bearing the 18S rDNA cluster is observed, ranging from one to six or seven pairs, with the majority of species displaying only one chromosome pair with rDNA cluster (Degrandi et al., 2020b). Both nightingale species showed rDNA FISH signal on five microchromosome pairs, suggesting that no rDNA associated chromosomal changes have occurred between these two species. Interestingly, five chromosome pairs bearing rDNA clusters is the highest number found in passerines so far (Degrandi et al., 2020b).

In the closest related species, where rDNA has been cytogenetically localized, *Turdus rufiventris* and *Turdus albicolis*, belonging to the Turdidae family, only three and two microchromosome pairs, respectively, bear rDNA (Kretschmer et al., 2014). Such differences in the number of rDNA clusters might result from chromosome translocations, transpositions and duplications mediated by transposable elements or ectopic recombination (Nguyen et al., 2010; Teixeira et al., 2021).

Telomeric tandem repeats (TTAGGG)$_n$ are normally found at the end of chromosomes but can sometimes be present also inside the chromosomes. Such interstitial telomere sites (ITSs) may result from chromosome translocation or fusions (Nanda and Schmid, 1994; Nanda et al., 2002), although not all chromosome fusions lead to ITSs (de Oliveira et al., 2005; Nishida et al., 2008). In birds, a high number of ITSs have been found in Ratites and Galliformes (Nanda and Schmid, 1994; Nanda et al., 2002), however, in passerines only a few or no ITSs have been identified (Nanda et al., 2002; Derjusheva et al., 2004). No ITSs were detected in either of the nightingale species, providing more evidence for a conserved chromosomal structure in the two species.
It has been shown that the GRC, an extra chromosome occurring in the germline of songbirds (Pigozzi and Solari, 1998, 2005), is highly variable in its size among species (Torgasheva et al., 2019; Malinovskaya et al., 2020). Torgasheva et al. (2019) compared the size of this chromosome in 16 passerine species belonging to nine families and showed that in 10 of them the GRC is a big macrochromosome, while in six it is a small microchromosome. Our results showed that both nightingale species had a small GRC, comparable in size to a microchromosome.

Despite the same chromosome number in the two nightingale species, we observed a few small changes in the centromere position on one macrochromosome and several microchromosomes, suggesting that some intrachromosomal rearrangements might have occurred between these two species. More detailed analyses of nightingale karyotypes and their genomic sequence will be needed, however, to confirm the existence of structural variants between the two species and determine their size and content. We should also note that our cytogenetic approach cannot detect smaller chromosomal rearrangements, which do not change the position of the centromere, result in ITSs or change the number of rDNA clusters. Analysis of high-quality chromosome-level genome assemblies of the two species could shed more light on the possible smaller-scale structural changes between the species.

Another interesting difference in the chromosome structure of the two nightingale species was revealed by the interspecific CGH experiment. This experiment suggested that some chromosomes have different copy numbers of centromeric repeats between the two species. In addition, many macrochromosomes and microchromosomes displayed higher conspecific signals in the CGH experiment, suggesting that the two species have diverged in their centromeric repeat sequences. Our observation is consistent with other studies in birds (Ellegren et al., 2012), as well as other taxa (Haaf and Willard, 1997; Bensasson et al., 2008; Pertile et al., 2009; de Sassi et al., 2021; Oliveira et al., 2021), showing fast evolution of centromeric sequences. For example, comparison of the whole genome sequences of two closely related species of Ficedula flycatchers, which also belong to the Muscicapidae family, revealed that the centromeres were among the most differentiated regions of the genome between the species (Ellegren et al., 2012).

The rapid divergence of the centromeric sequences or their copy number, between species, is assumed to be the result of centromere-associated female meiotic drive, where some centromeric sequences can bias their transmission to the egg, leaving others to end up in the polar bodies (Henikoff, 2001; Pardo-Manuel de Villena and Sapienza, 2001). This can lead to a swift fixation of particular centromeric sequences in the population and a fast divergence of centromeric repeats, or their copy number, between the species. However, distorting the transmission ratio sometimes be harmful to the organism, for example, if it is linked to the sex chromosomes and leads to a sex ratio distortion. In such cases, it is often associated with the evolution of drive suppressors (McLaughlin and Malik, 2017). Interestingly, while most large autosomes showed species-specific sequences in our CGH experiment, sex chromosomes showed differences in the copy number of centromeric repeats, but not species-specific sequences. This suggests that the evolution of centromeric repeats on the sex chromosomes might be constrained by the sex ratio effect of sex chromosome linked meiotic drive.

It has been demonstrated that divergence of centromeric sequences between species may lead to female meiotic drive in interspecific hybrids (Chmátal et al., 2014; Akera et al., 2019; Knief et al., 2020). Theoretically, the divergence of centromeres could, in an extreme case, also cause the sterility of female hybrids and thus contribute to reproductive isolation between species (Hurst and Pomiankowski, 1991; Phadnis and Orr, 2009; Zhang et al., 2015). In nightingales, consistent with Haldane’s rule (Haldane, 1922), F1 hybrid females are sterile, while F1 males are fertile (Reifová et al., 2011b; Mořkovský et al., 2018). It is thus possible that divergence in centromeric sequences between the two nightingale species could contribute to female-limited hybrid sterility. Further studies of centromere composition in the two nightingale species should be done to explore this possibility.

In conclusion, although the two nightingale species have very similar karyotypes, it is possible that a small number of chromosomal rearrangements have occurred between them and may contribute to reproductive isolation between the species. Interestingly, the two species appear to differ in their centromeric sequences. Such divergence could cause female meiotic drive or female sterility in interspecific hybrids. Further studies are, however, needed to confirm the presence of structural variants and diverged centromeric repeats in the two nightingale species and to examine their potential role in the nightingales’ speciation.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

**ETHICS STATEMENT**

The animal study was approved by the General Directorate for Environmental Protection, Poland (permission no. DZP-WG.6401.03.123.2017.dl3).

**AUTHOR CONTRIBUTIONS**

RR, MJP, and MP conceived the research, JR, TA, MP, and TO captured birds in the field, TA, RR, and MP performed the dissections, MP, MJP, MA, DD, and ZM performed the molecular cytogenetic analyses, MP, MJP, MA, and RR analyzed and interpreted the data, MP, RR, and MJP drafted the manuscript with contribution of all co-authors. All authors have read and agreed on the final version of the manuscript.

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SUPPLEMENTARY MATERIAL
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