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Differential hypomethylation of the repetitive *Tol2*/*Alu*-rich sequences in the genome of *Bodianus* species (Labriformes, Labridae)

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Abstract

Representatives of the order Labriformes show karyotypes of extreme conservatism together with others with high chromosomal diversification. However, the cytological characterization of epigenetic modifications remains unknown for the majority of the species. In the family Labridae, the most abundant fishes on tropical reefs, the genomes of the genus *Bodianus* Bloch, 1790 have been characterized by the occurrence of a peculiar chromosomal region, here denominated BOD. This region is exceptionally decondensed, heterochromatic, argentophilic, GC-neutral and, in contrast to classical secondary constrictions, shows no signals of hybridization with 18S rDNA probes. In order to characterize the BOD region, the

methylation pattern, the distribution of *Alu* and *Tol*2 retrotransposons and of 18S and 5S rDNA sites, respectively, were analyzed by Fluorescence *In Situ* Hybridization (FISH) on metaphase chromosomes of two *Bodianus* species, *B. insularis* Gomon & Lubbock, 1980 and *B. pulchellus* (Poey, 1860). Immunolocalization of the 5-methylcytosine revealed hypermethylated chromosomal regions, dispersed along the entire length of the chromosomes of both species, while the BOD regions exhibited a hypomethylated pattern. Hypomethylation of the BOD region is associated with the precise co-location of *Tol*2 and *Alu* elements, suggesting their active participation in the regulatory epigenetic process. This evidence underscores a probable differential methylation action during the cell cycle, as well as the role of *Tol*2/*Alu* elements in functional processes of fish genomes.

Keywords
Fish cytogenetics, Methylation, BOD region, pseudo-NORs, Mobile elements, Repetitive DNA

Introduction

Genomes of some representatives of Labriformes families carry preferential chromosomal rearrangements (Sena and Molina 2007; Molina et al. 2014; Almeida et al. 2017), and singular regional DNA organization (Molina et al. 2012; Amorim et al. 2016). Labridae, the fifth largest marine fish family, with approximately 600 species, displays remarkable ecological and evolutionary diversification (Parenti and Randall 2000). Its phylogeny, where the relationships of the highest categories have been better recognized, is a long-standing and widely discussed problem (Westneat and Alfaro 2005). Particular evolutionary trends in karyotype differentiation, such as pericentric inversions and centric fusions, occur among tribes of this family (Molina and Galetti 2004, Sena and Molina 2007, Molina et al. 2014, Almeida et al. 2017). Indeed, while some groups show karyotype conservatism (Sena and Molina 2007), others possess karyotypes modeled by pericentric inversions, e.g. in the tribe Hypsigenyini and, particularly, the species of the genus *Bodianus* Bloch, 1790 (Molina et al. 2012).

The representatives of the tribe Hypsigenyini exhibit relatively symmetrical karyotypes, with 2n = 48 and high fundamental number (NF) values as compared to other ones (Arai 2011). Some Atlantic species, such as *Bodianus rufus* (Linnaeus, 1758), *B. pulchellus* (Poey, 1860) and *B. insularis* Gomon & Lubbock, 1980, have been analyzed in detail, and phylogenetically shared particular chromosomal regions have been identified. These regions, located at the p arms of the second subtelocentric chromosome pair, were characterized as exceptionally decondensed, heterochromatic and argentophilic, suggesting the presence of rDNA sites. However, these regions are neither GC-rich, nor do they display hybridization signals with 18S rDNA probes, indicating the presence of distinct repetitive sequences with unusual organization (Molina et al. 2012).

Molecular analyses have significantly widened the knowledge of the genomic organization and epigenetic modeling of the chromatin, particularly with respect to his-

tone modifications of the euchromatin and heterochromatin (Fuchs et al. 2006). DNA methylation is catalyzed by a conserved class of DNA methyltransferases (Dnmt's) broadly present in protists, fungi, plants and animal genomes (Craig and Brickmore 1994; Dyachenko et al. 2010). Islets of CpG dinucleotides (C-phosphate-G, on the fifth carbon) are correlated with 5-methylcytosine content (5 mC) (Vanyushin et al. 1973), which indicates hyper- and hypomethylation patterns in the chromatin related to gene regulation (Baylin et al. 1991; Almeida et al. 1993; Feinberg 1993; Barbin et al. 1994).

Although the knowledge of the methylation patterns is growing among vertebrates, it is still restricted in fishes, especially in relation to repetitive DNA regions (transcriptional and non-transcriptional), which are apparently limited to the heterochromatic regions and sex chromosomes (Schmid et al. 2016). Repetitive sequences have been the target of intense investigation in several fish groups (Vicari et al. 2008; Cioffi et al. 2010b; Costa et al. 2014, 2016; Barbosa et al. 2015), showing extreme complexity in some species (Costa et al. 2015). In this context, probable synergic or antagonistic interactions between collocated distinct sequences still need to be clarified.

In this study, we analyzed the DNA methylation pattern in the metaphase chromosomes of *B. pulchellus* and *B. insularis*, phylogenetically very close species (Gomon 2006), especially in the exclusive decondensed region (Ag+/CMA0/C+), here referred as BOD, in allusion to genus *Bodianus*. The data were compared with the structural patterns of the chromosomes, identified by the 18S and 5S rDNAs and the transposable elements *Tol2* and *Alu* mapping using FISH.

Methods

Individuals, collection sites, chromosome preparation and bandings

Individuals of *Bodianus pulchellus* (n = 6, all immature individuals) from Bahia State (12°58'20"S, 38°31'05"W), on the northeastern Brazilian coast, and *B. insularis* (n = 5, 2 males and 3 immature individuals) from São Pedro and Paulo Archipelago (0°55'19"N, 29°21'44"W), were used in cytogenetic analyses. The individuals were collected under authorization provided by the Chico Mendes Institute of Biodiversity Conservation (ICMBIO/SISBIO) (license #02001.001902/06-82) and all experimental procedures followed the rules of the Animal Ethics Committee of the Federal University of Rio Grande do Norte (protocol 044/2015).

Mitosis stimulation followed the protocols developed by Molina (2001) and Molina et al. (2010). Mitotic chromosomes were obtained by means of the *in vitro* interruption of the cell cycle (Gold et al. 1990). An amount of 150µl of cell suspension was dropped onto a wet slide covered by a film of distilled water, heated to 60 °C and dried at room temperature. The Ag-NOR (Nucleolus Organizer Regions) sites and the extra nuclear argentophilic regions were identified according to Howell and Black (1980).

FISH and immunostaining of methylated DNA

FISH was performed according to Pinkel et al. (1986). The 5S and 18S rDNA sequences were detected by double-color FISH analyses. Both ribosomal sequences were isolated from the *Hoplias malabaricus* (Bloch, 1794) (Teleostei, Characiformes) genome. The 5S rDNA included 120 base pairs (bp) of the 5S rRNA gene and 200bp from the non-transcribed spacer (NTS) (Martins et al. 2006). The 18S rDNA probes corresponded to a 1400bp segment from the 18S rRNA gene, obtained through PCR of the nuclear DNA (Cioffi et al. 2010a). The 5S rDNA probes were labeled with biotin-14-dATP by nick translation according to the manufacturer's recommendations (BioNick Labeling System; Invitrogen, San Diego, CA, USA). The 18S rDNA was labeled by nick translation with Digoxigenin-11-dUTP, in line with the manufacturer's recommendations (Roche, Mannheim, Germany). The *Tol*2 transposon probes were obtained by PCR of the nuclear DNA of *Rachycentron canadum* using the primers Tol2-5F 5'-CTG TCA CTC TGA TGA AAC AG-3' and Tol2-5R 5'-CTT TGA CCT TAG GTT TGG GC-3' (Kawakami and Shima 1999). The probes were labeled with Digoxigenin-11-dUTP by nick translation following the manufacturer's recommendations (Roche, Mannheim, Germany). The (TTAGGG)n sequences were mapped by FISH using Telomere PNA FISH Kit/FITC according to manufacturer's instructions (Dako Citomation). The *Alu* transposon probes were obtained by PCR of the genomic DNA of *Rachycentron canadum* (Linnaeus, 1766) using the primers Alu CL1 5'-TCC CAA AGT GCT GGG ATT ACA G-3' and Alu CL2 5'-CTG CAC TC AGC CTG GG-3' (Lengauer et al. 1992), and were labeled with Digoxigenin-11-dUTP by nick translation following the manufacturer's recommendations (Roche, Mannheim, Germany). The chromosomes were counter-stained with Vectashield/DAPI (1.5mg/ml) (Vector) and photographed with an Olympus BX50 epifluorescence microscope coupled to an Olympus DP73 digital camera, using CELLSENS software (Olympus).

The DNA methylation patterns in the metaphase chromosomes were detected through binding analysis of the monoclonal antibody to 5-methylcytosine. Indirect immunodetection of the methylated DNA was conducted according to Marques et al. (2011). The slides were treated with 20 mg/ml RNAse (Invitrogen) diluted 1:200 in 2XSSC for one hour, followed by exposure to 1mg/ml pepsin (1:100) in 0.01 N HCl (100µl per slide) for 20 minutes. They were then denatured in 70% formamide for 3 min at 75 °C and blocked with 3 % BSA diluted in 1X PBS with 0.1 % Tween 20, for 30 minutes at 37 °C and incubated with the mouse-anti-5-methylcytosine primary antibody (Eurogentec) in 1 % BSA/1X PBS (1:100) overnight at 4 °C. The 5 mC was detected using anti-mouse-FITC diluted (1:200) in 1 % BSA/1X PBS for 1 hour at 37 °C. Finally, the slides were washed in 1X PBS, mounted with DAPI/Vectashield antifading (Vector Laboratories) and analyzed by fluorescence microscopy under a Leica DMBL photomicroscope (Leica Microsystems) equipped with a CCD Cohu camera (CohuHD Costar), using QFISH software. Composition of the image with the hybridization signals was done with Photoshop CS5 (Adobe) software.

The chromosomes were categorized as metacentric (m), submetacentric (sm), subtelocentric (st) and acrocentric (a), according to Levan et al. (1964) and arranged in descending order of size. A single ideogram for both species was constructed in order to highlight the repetitive sequences and the methylation patterns identified by 5 mC.

Results

Bodianus pulchellus and *B. insularis* have diploid chromosome number 2n=48 and identical karyotypes, composed of 4m+12sm+14st+18a chromosomes, NF value 78. As previously described for these species (Molina et al. 2012), the 10th subtelocentric pair exhibited an extensive decondensed terminal region that could reach up to four times the size of the largest chromosome pair (Figs 1, 2) – the BOD region.

Ag-NOR sites were located in the terminal region of the pair No. 9 in karyotypes of both species (Fig. 1a, b). These sites and the BOD region were also argentophilic (Fig. 1a, b; highlighted), as in previous descriptions (Molina et al. 2012).

Double-FISH with 5S and 18S rDNA probes revealed a non-syntenic location for these ribosomal sites. The 18S rDNA sites were exclusively located in the terminal regions on the p arms of the pair No. 9, corresponding with the Ag-NOR signals. No hybridization signals were detected in the BOD regions of both species (Fig. 1a, b). On the other hand, 5S rRNA genes were located in the terminal regions on the q arms of the pair No. 16 in both species, and an extra pericentromeric site on the p arms of the pair No. 19, only in *B. insularis* (Fig. 1a, b).

The hybridization with the transposable element *Alu* was only performed in *B. insularis*, while *Tol*2 mapping was performed in both species. These sequences exhibited a similar distribution pattern in the chromosomes preferentially located in the terminal regions of the chromosomes and particularly accumulated in the BOD one (Fig. 1c–e; highlighted).

The hybridization signals with $(TTAGGG)_n$ probes were variable, with the majority having the same size, besides some chromosomes showed no detectable signals (data not shown). Immunostaining with 5 mC revealed that most metaphase chromosomes of the two species were hypermethylated (Fig. 2b, d). By contrast, the BOD regions were distinctly hypomethylated, as well as the centromeric regions of the majority of chromosomal pairs (Fig. 2).

All results were summarized in the ideogram of the Figure 3 below.

Discussion

Structural chromosome characteristics of *Bodianus* species

The heterochromatic regions of fish genomes have been the target of intense investigation. Although displaying variations in the amount and distribution on chromosomes, hetero-

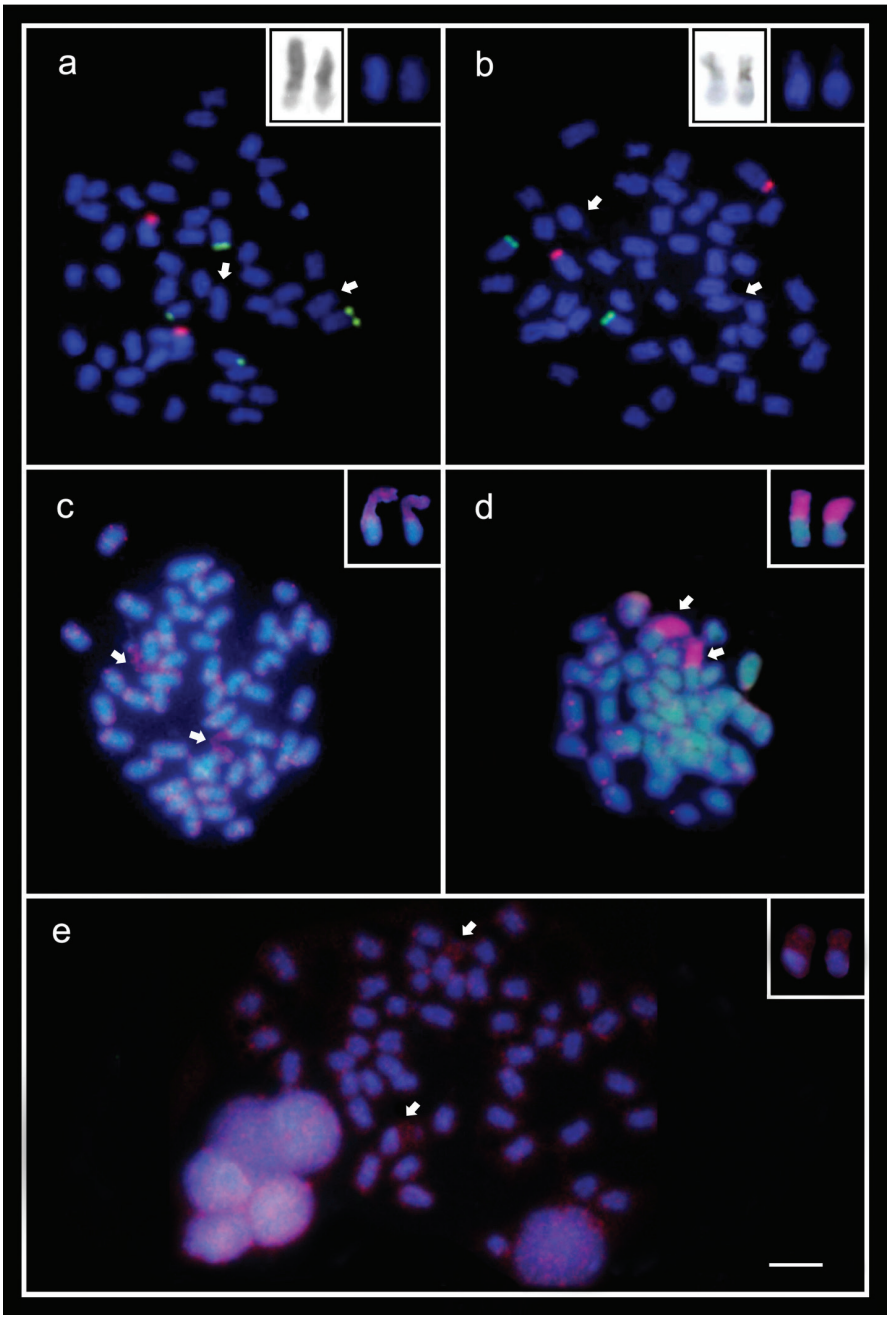

Figure 1. Metaphase chromosomes of the species *B. insularis* (**a, c, e**) and *B. pulchellus* (**b, d**); the chromosome pairs bearing the BOD region are identified with arrows and highlighted in the boxes **a, b** 18S (red signals) and 5S rDNA FISH (green signals). In the boxes, the argentophilic pattern showed in the BOD regions and the DAPI staining pattern, respectively **c, d** Distribution of the *Tol*2 element in the chromosomes. An accumulation of *Tol*2 sequences is perceptible in the BOD regions **e** - Distribution of the *Alu* transposable element on the chromosomes of *B. insularis*. Scale bar: 5μm.

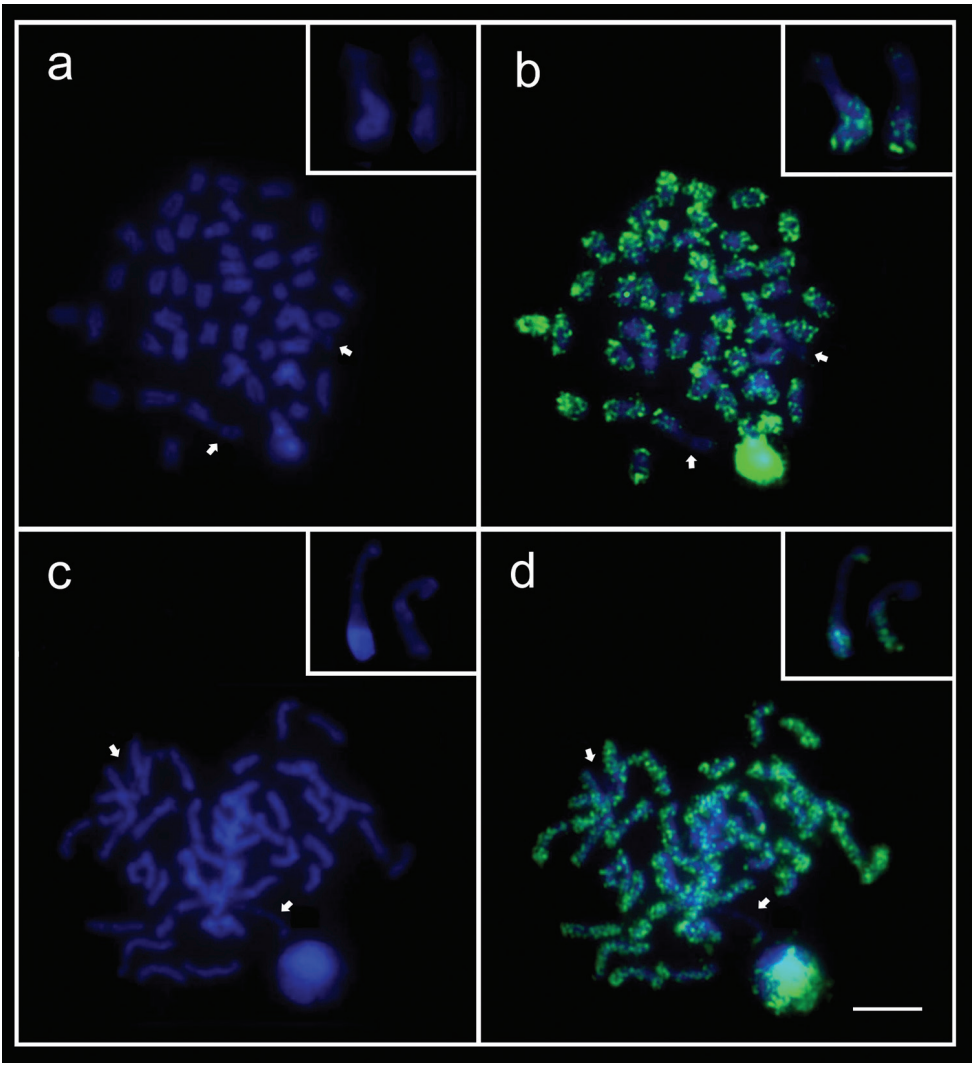

Figure 2. Metaphase chromosomes of the species *Bodianus pulchellus* (**a, b**) and *Bodianus insularis* (**c, d**) after DAPI staining (left) and sequential immunodetection of methylated sites with the monoclonal antibody 5mC (right). The chromosome pairs bearing the BOD region are denoted with arrows and highlighted in the boxes. Scale bar: 5μm.

chromatin can harbor a diversified panel of collocated sequences, whereby the effects of this interaction under gene regulation and dispersion pattern need to be better investigated (Costa et al. 2015). Functional biases could derive from the joint distribution of multigene families, or from their association with other repetitive sequences, constituting adaptive aspects and implying maintenance and dispersion in the chromosomes (Costa et al. 2016).

Argentophilic decondensed regions in vertebrates are often related to NOR sites (Árnason 1981; Schmid et al. 1982; Birstein 1984; Supanuam et al. 2012). Previ-

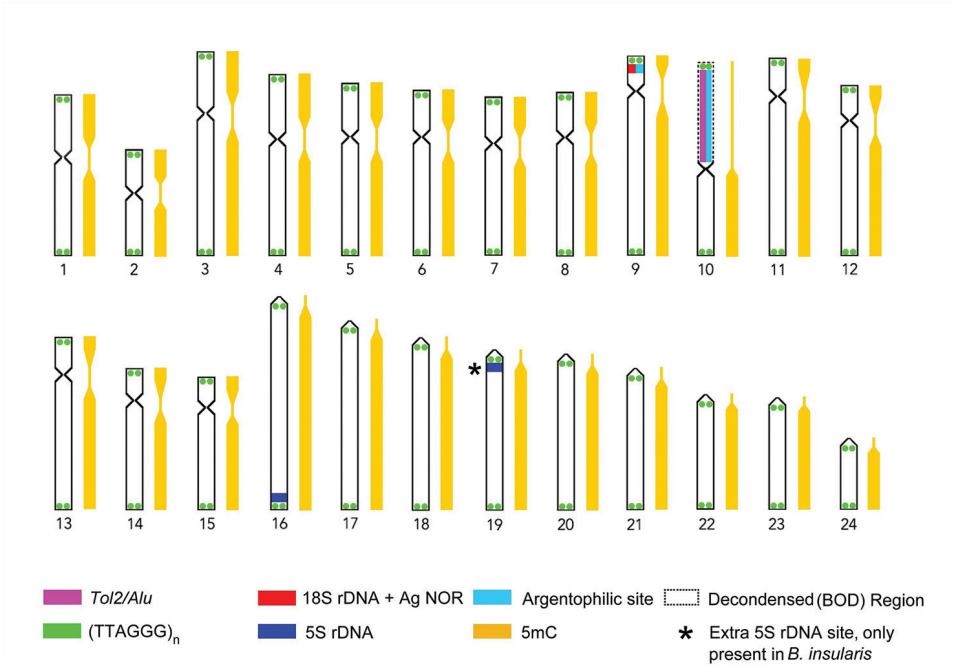

Figure 3. Ideogram showing distribution of repetitive sequences and the methylation pattern in the metaphase chromosomes of *B. insularis* and *B. pulchellus*, a dashed line highlights the decondensed BOD region.

ous cytogenetic studies identified an intriguing chromosomal region on homeologous pairs in *B. pulchellus*, *B. insularis* and *B. rufus* (Molina et al. 2012). This region, now identified as BOD one, exhibits a high decondensed structure and a heterochromatic, GC-neutral and argentophilic constitution, but that does not exhibit any hybridization signals with 18S rDNA probes. The sharing of this particular set of constitutive and functional characteristics indicates that the origin of the BOD region precedes the phyletic diversification of those species (Molina et al. 2012), representing a very favorable *sui generis* condition for the study of the complexity of repetitive DNA arrangements in fishes.

In several vertebrate species, including fishes, argentophilic sites not associated with rDNA sites, known as pseudo-NORs, were already described (Ozouf-Costaz et al. 1997; Pisano et al. 2000; Caputo et al. 2002; Dobigny et al. 2002; Gromicho et al. 2005; Cabrero and Camacho 2008). Structurally, the BOD regions have similarities with pseudo-NORs that are tandem arrays of a heterologous DNA sequences. In some species, the pseudo-NORs do not exhibit promoter sequences and have high affinity for the upstream binding factor (UBF), a DNA binding protein and component of the Pol I transcription machinery which binds extensively across the rDNA repeat *in vivo* (Prieto and McStay 2008). The formation of pseudo-NORs is associated to a special class of multigene families, like histones and ribosomal genes, from both protein- and

non-protein-coding with capacity of translocation known as orphons (Childs et al. 1981, Cabrero and Camacho 2008).

Pseudo-NORs used to mimic real NORs in several aspects, as they can remain decondensed during mitosis when the transcription is inactivated and the nucleolus is broken down, forming novel silver positive secondary constrictions (Mais et al. 2005). UBF can displace histone H1 from histone octamers *in vitro* (Kermekchiev et al. 1997), thereby promoting the chromatin decompaction. Additionally, Ag-positive loci can be the result of the presence of residual acidic proteins with affinity for silver, reacting with this compound (Dobigny et al. 2002). In fact, pseudo-NORs are reactive to silver staining despite their transcriptional silence (Mais et al. 2005). The typical decondensation observed in secondary constrictions should be promoted by the action of binding argyrophilic proteins that prevent the full condensation of that region (Prieto and McStay 2008). These elements could explain the argentophilic and decondensed nature of the chromatin present in the BOD region.

Representatives of *Bodianus* display karyotypes with a larger number of biarmed chromosomes when compared to those of other Labridae genera (Sena and Molina 2007). This is a synapomorphic pattern and indicates intense structural chromosome reorganization in this clade. However, several common characteristics, such as the presence of a single 18S rDNA site and the BOD region, could indicate a lower level of diversification among the youngest branches of this group. In fact, the presence of a single chromosome pair bearing 18S rDNA sites represent the most frequent pattern found in fishes (Gornung 2013) as well as in several perciform groups (Motta-Neto et al. 2012; Costa et al. 2016). On the other hand, 5S rDNA sites show a more diversified pattern, being present on a single chromosome pair in *B. pulchellus* but on two pairs in *B. insularis*. The monitoring of ribosomal genes in chromosomes in a phylogenetic perspective makes it possible to identify the sequential patterns of change or synteny maintenance over time (Affonso et al. 2014; Fernandes et al. 2015; Costa et al. 2016), especially in conserved karyotypes, such as in *Bodianus*.

In genomes of some fish species, a high chromosome dynamism has been identified for *Tol*2 elements, which can be situated in different genomic regions (Koga and Hori 1999), or be preferentially concentrated and collocated with 18S rDNA sites (Costa et al. 2013). Therefore, the presence of structural and functional characteristics of the BOD region, typical of pseudo-NORs, may indicate that these regions were originally repositories of rDNA. Indeed, *Alu* and *Tol*2 elements exhibit a remarkable accumulation in the BOD regions. Transposable elements are transposed by a cut-and-paste mechanism, involving their excision and insertion elsewhere in the chromatin. Additionally, the spreading of transposons can be concatenated with the capacity of the orphons translocation through the genome via dispersion and magnification of minor loci consisting of a few rDNA copies (Dubcovsky and Dvorak 1995) as observed in *Aegilops speltoides* Tausch (Flaksberger 1935) (Raskina et al. 2004). If the excision process of transposons is excessive, it may affect the function of a particular gene, making it functionally unstable, requiring only that the transposon insertion occurs within or very close to the gene (Lippman et al. 2004), as observed in this study.

Alu elements concentrate huge amounts of CpG islands that are genomic regions that contain a high frequency of CpG dinucleotides, commonly representing promoters, which are usually located in GC dense regions. CpG islands tend to be hypomethylated allowing an open chromatin organization and facilitating neighboring gene expression (Jones and Baylin 2002; Gu et al. 2016). On the other hand, *Alu* sequences are punctuated by multiple CpG domains, many of which overlapping with known protein binding sites (Rowold and Herrera 2000), possibly the same aforementioned argyrophilic binding proteins which keep the chromatin decondensed and consequently opened. Thus, the marked occurrence of *Alu* and *Tol2* elements in the BOD regions could have significantly interfered in the ribosomal gene functionality, causing a pseudogenization process.

Differential methylation in *Bodianus* metaphase chromosomes

Despite the fact that a significant part of the genome of some organisms is composed of repetitive DNA sequences, their origins, dispersion and functional interaction remain largely unknown (Biémont and Vieira 2006). In this context, methylation patterns help us understand the functional aspects of the genome. DNA methylation is an important epigenetic modification in the genome of vertebrates, where only small fractions of it are hypomethylated (Nakamura et al. 2014). An overview of methylation in the vertebrate genome indicates that more basal groups such as fish and amphibians have higher methylation levels than reptiles, mammals and birds and is inversely related to body temperature (Vanyushin et al. 1973; Jabbari et al. 1997; Varriale and Bernardi 2006a, b). Despite the occurrence of chromosomal rearrangements associated with DNA methylation, this process may suppress homologous recombination, enabling genomes rich in repeats to remain relatively stable (Colot and Rossignol 1999).

The immunolocalization of 5-methylcytosine in the metaphase chromosomes of the two *Bodianus* species revealed a primarily hypermethylated pattern, despite the striking contrast observed in the BOD and the centromeric regions, both notably hypomethylated. In general, centromeric regions exhibit particular epigenetic characteristics, including DNA hypermethylation. The presence of hypomethylated regions in the centromeres of some chromosome pairs of the *Bodianus* species demonstrates an uncommon and likely functional condition of these regions, which are closely associated with the chromosome segregation process.

DNA methylation is considered a controlling mechanism of gene expression, including the ribosomal ones (Ferraro and Lavia 1983; Ferraro and Prantera 1988). Indeed, there is an inverse correlation between DNA methylation and the transcriptional activity of several eukaryotic genes (Kanungo 1994), as well as nucleolar size and the number of rDNA loci sites (Bacalini et al. 2014). In mammals, there is a strong relation between states of DNA methylation and gene silencing (Eden et al. 1994). On the other hand, in invertebrates, the origins and meaning of methylation patterns show,

in some cases, the absence of a correlation between methylation and gene expression (Tweedie et al. 1997). In more basal fish groups, GC-rich heterochromatins, which are frequently related to NOR regions (Gornung 2013), are highly methylated in the germ line, but to a lesser degree in somatic chromosomes (Covelo-Soto et al. 2014). The hypomethylation patterns of repetitive and ribosomal DNA classes can lead to chromatin decondensation (Carvalho et al. 2000; Jones and Baylin 2002), as demonstrated here for the BOD regions.

In some Perciformes species, *Tol*2 elements are distributed along the chromosomes and distinctly associated with 18S rDNA sequences (Costa et al. 2013). In *Bodianus*, both the accumulation of these transposons in the BOD regions as well their hypomethylated nature, are prominent. It has been reported that the methylation process plays a protective role against invasive DNAs or transposable elements (Yoder et al. 1997; Doerfler 1991) and is a key mechanism in gene regulation and expression (Finnegan et al. 1998; Heslop-Harrison 2000; Attwood et al. 2002). Indeed, the transposable sequences in the human genome are highly methylated (Kricker et al. 1992). Fishes have shown hypermethylated regions confined to constitutive heterochromatin, particularly in heteromorphic sex chromosomes, demonstrating that several hypermethylated regions are co-localized with repetitive elements (Schmid et al. 2016).

It is known that DNA methylation may limit the dispersion of various transposable elements in a number of genomes (Scortecci et al. 1997; Miura et al. 2001; Iida et al. 2006). However, this condition does not occur in the BOD regions. If methylation inhibits the dispersion of transposable elements, why is the BOD region, extremely rich in *Alu* and *Tol*2 elements are not methylated? The answer may be related to the following considerations: (1) *Alu* elements appear to be preferentially located in GC-rich genomic isochores (Deininger 2006), explaining the accumulation of this transposable element in the 18S rDNA sites; (2) CpG islands, strongly present in *Alu* elements, are hypomethylated as a response to an overlapping between the CpG domains and the argyrophilic proteins binding sites, which prevent the full condensation of the heterochromatin through the displacement of the histone H1 from the histone octamers. This way, the open and decondensed heterochromatin may offer favorable conditions for the accumulation of the *Tol*2 retrotransposon in such region; (3) the epigenetic action promoted by the excessive excision of transposons inserted within or very close to the gene in the BOD region, affects its function and makes it functionally unstable. Therefore, novel NORs are formed in other chromosomal locations by the transposons spreading, associated with the translocation of orphons and the magnification of minor rDNA loci. Evidence suggests that in some fish species, such as *Oryzias latipes* (Temminck and Schlegel 1846), the *Tol*2 element, underwent a rapid expansion in the past, but acquired interactive control mechanisms (Iida et al. 2006). Therefore, in the same way compensatory evolutionary mechanisms may have been fixed in the *Bodianus* BOD region, thereby controlling the activity and dispersion of *Tol*2 and *Alu* elements. The delimitation of a preferential reservoir for these transposable elements in the BOD region would therefore constitute effective protection for genes allocated to the other chromosomes of the karyotype.

Conclusion

DNA methylation is one of the epigenetic processes that has modulated the molecular evolution of life, but its influence in karyotype evolution and interaction in the structural chromosome regions are little known, especially for fish species. The use of monoclonal antibodies in cytogenetic study of *Bodianus* species provided an overview of the methylation pattern of metaphase chromosomes, with sufficient resolution to characterize the peculiar BOD regions. The complex composition of the BOD chromatin suggests that it is a pseudo-NOR containing a relict sequence of an ancestor rDNA. The DNA organization of such region provided evidence of its functional dynamics, possibly in the transcriptional control of *Tol2* and *Alu* elements. In this sense, the methylation process, associated with the dispersion control of the transposable elements, may have played a particular active role in the evolutionary process of *Bodianus* species.

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Karyotype description and comparative analysis in Ringed Kingfisher and Green Kingfisher (Coraciiformes, Alcedinidae)

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Abstract

Kingfishers comprise about 115 species of the family Alcedinidae, and are an interesting group for cytogenetic studies, for they are among birds with most heterogeneous karyotypes. However, cytogenetics knowledge in Kingfishers is extremely limited. Thus, the aim of this study was to describe the karyotype structure of the Ringed Kingfisher (*Megaceryle torquata* Linnaeus, 1766) and Green Kingfisher (*Chloroceryle americana* Gmelin, 1788) and also compare them with related species in order to identify chromosomal rearrangements. The Ringed Kingfisher presented 2n = 84 and the Green Kingfisher had 2n = 94. The increase of the chromosome number in the Green Kingfisher possibly originated by centric fissions in macrochromosomes. In addition, karyotype comparisons in Alcedinidae show a heterogeneity in the size and morphology of macrochromosomes, and chromosome numbers ranging from 2n = 76 to 132. Thus, it is possible chromosomal fissions in macrochromosomes resulted in the increase of the diploid number, whereas chromosome fusions have originated the karyotypes with low diploid number.

Keywords

Aves, chromosome, evolution, karyotype

Introduction

Avian karyotypes are characterized by internal variation in the size of chromosomes, presenting two distinct groups, macrochromosomes and microchromosomes. About eight pairs of macrochromosomes are seen in most of birds, and the remaining are microchromosomes (Rodionov 1996). Diploid number also varies, including species with a low diploid number such as *Burhinus oedicnemus* Linnaeus, 1758 (Charadriiformes) 2n = 40 (Nie et al. 2009), and high 2n = 136-142 in *Corythaixoides concolor* Smith, 1833 (Musophagiformes) (Christidis 1990), but most of the species exhibit karyotypes with 2n = 74–86 (Tegelstrom and Ryttman 1981).

Studies of karyotype structure in birds have given valuable information about evolutionary relationships. Chromosome painting shows that, although relatively conserved, the macrochromosomes evolve through several intra and inter-chromosomal rearrangements (de Oliveira et al. 2010, Kretschmer et al. 2014). While Tandem fusions between microchromosomes and micro- with macrochromosomes have resulted in decrease of diploid number (Nishida et al. 2008, Nie et al. 2009, de Oliveira et al. 2010, 2013). Chromosome fission in recurrent breakpoints has been documented in macrochromosomes, and can result in increase of chromosome number (Skinner and Griffin 2012, Degrandi et al. 2017).

In relation to the sex chromosomes of birds, males have a homogametic ZZ pair and female have a heterogametic ZW (Schartl et al. 2015). The Z chromosome is a highly conserved macrochromosome and it comprises 7% of the haploid genome (Graves and Shetty 2001). In Piciformes, Bucerotiformes, and Coraciiformes the Z chromosome is often the largest chromosome of the complement (de Oliveira et al. 2017). Whereas the W chromosome is highly variable in size, and has been observed from homomorphic to Z in Paleognaths Ratite (Nishida-Umehara et al. 2007) to a small and heterochromatic with variable size in Neognaths birds (Graves and Shetty 2001). This size variation has been attributed to a differential accumulation and degradation of repetitive DNAs (de Oliveira et al. 2017). Also, a multiple sex chromosome system was recently described for the Adelie Penguin (*Pygoscelis adeliae* Hombron et Jacquinot, 1841/ Sphenisciformes) where males have $Z_1Z_1Z_2Z_2$ and females Z_1Z_2W (Gunski et al. 2017).

Kingfishers (Alcedinidae) comprises a diverse family of birds with approximately 115 species distributed worldwide (Gill and Donsker 2017). They are an interesting group for cytogenetic studies since they are among birds with most heterogeneous karyotypes. However, knowledge about cytogenetics in Kingfishers is extremely limited. There are records for *Dacelo novaeguineae* Hermann, 1783, 2n = 76, *Halcyon smyrnensis* Linnaeus, 1758, 2n = 76, *Halcyon pileata* Boddaert, 1783, 2n = 84, *Alcedo atthis* Linnaeus, 1758, 2n = 132, *Ceyx azureus* Latham, 1801, 2n = 122, and *Ceryle rudis* Linnaeus, 1758, 2n = 82 (De Boer and Belterman 1980, Xiaozhuang and Qingwei 1989, Christidis 1990, Youling et al. 1998, Garg and Shrivastava 2013).

The Ringed Kingfisher, *Megaceryle torquata* Linnaeus, 1766 and the Green Kingfisher, *Chloroceryle americana* Gmelin, 1788 belong to subfamily Cerylinae and their

karyotypes are unknown (Moyle 2006). In view of this, the present study aimed to describe the karyotype structure of these species. Secondly, we sought to gather karyotype information from Alcedinidae in order to compare them and to identify the chromosomal rearrangements.

Material and methods

Samples and location

The karyotype of one male and one female of *Megaceryle torquata* (Fig. 1A) collected at the Parque Ecológico El Puma in Argentina, and two males and one female of *Chloroceryle americana* (Fig. 1C) from Santa Maria/Rio Grande do Sul, Brazil were analyzed for this work. Specimens were collected according to license SISBIO 44173-1 and animal research ethics committee (CEUA 018/2014).

Cell culture

Mitotic chromosomes in *M. torquata* specimens were obtained by lymphocyte culture according to Moorhead et al. (1960). In short, blood samples were incubated in medium PBMax (Gibco) for 72 hours at 38 °C. In the last hour of incubation, 0,001 ml of colchicine solution (0.05%) was added. After these procedures, the cells were centrifuged and pellet was incubated in 10 mL of hypotonic solution (0.075 M KCl) for 20 min, followed by fixation in three washes with Methanol: Acetic acid 3:1 solution.

In *C. americana*, mitotic cells were obtained from bone marrow according to Garnero and Gunski (2000). Initially, bone marrow was extracted from femurs and incubated in a 10 ml of RPMI 1640 medium with 0,001 ml of colchicine solution (0.05%) at 39 °C for 1 hour. Finally, cells were incubated in 10 ml of hypotonic solution (0.075 M KCl) for 20 minutes. Then cells were washed three times with Methanol: Acetic acid 3:1 solution.

Chromosomal analyses

The diploid number was determined by analyzing approximately 40 metaphases per specimen, by conventional 0,8% Giemsa staining solution. Karyotypes were organized according to chromosome size and differential staining CBG-banding (Sumner 1972) was applied to identify the W chromosome.

Morphometry of the first 15 autosomal chromosomes pairs and the ZW sex chromosomes, were performed in Alcedinidae species available. Centromeric index (CI) was estimated by ratio of short arm length by total chromosome length. Nomenclature for chromosome morphology were performed according to Guerra (1986) using CI index.

Results

The Ringed Kingfisher presented chromosome number of 2n = 84 (Figure 1B). The chromosome set is composed of ten biarmed pairs, being the submetacentric pairs (1, 3 and 4), metacentric (2, 5, 8 and 13) and acrocentric (6, 7 and 9). The remaining autosomes are telocentric. Z and W are both submetacentric macrochromosomes, with size similar to chromosome 4 and 9, respectively.

The Green Kingfisher had a diploid number of 2n = 94 (Fig.1D), consisting of only four biarmed pairs, where 1, 2 and 3 are submetacentric and 12 is metacentric. All the other chromosome pairs are telocentric. The Z chromosome is submetacentric and is the largest chromosome of the karyotype, while the W chromosome is submetacentric with size between 1 and 2.

C-banding analysis allowed correct identification of the W chromosome, since both species presented a highly heterochromatic pattern for this chromosome (Fig. 2A and B). The Z chromosome was euchromatic in both species. However, in *C. americana* a positive staining was observed near the centromere (Fig. 2 B).

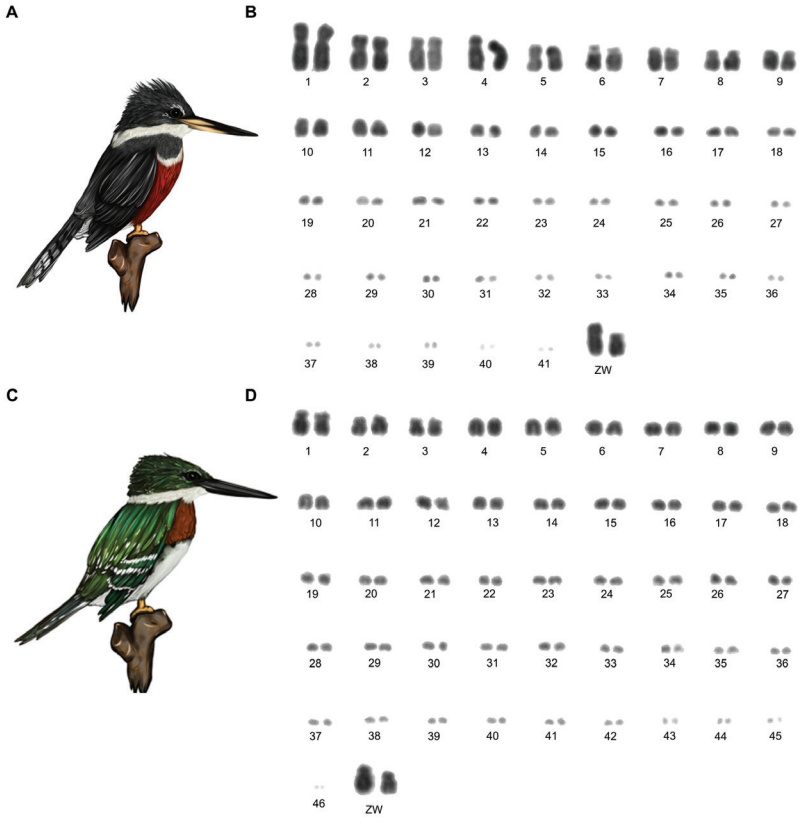

Figure 1. Ringed Kingfisher *Megaceryle torquata* (**A**), and karyotype with 2n = 84 (**B**). Green Kingfisher *Chloroceryle americana* (**C**), and karyotype with 2n = 94 (**D**).

In the literature, chromosome data were found for *C. rudis*, *H. pileata*, *A. atthis*, *H. smyrnensis*, *D. novaeguineae,* and *C. azureus* (Table 1). Unfortunately, for *H. smyrnensis*, *D. novaeguineae*, *C. azureus* only the diploid number was available. Despite this, some observations can be made: i) diploid number is highly variable; ii) number of biarmed chromosomes (metacentric, submetacentric, and acrocentric) was also variable; iii) the Z chromosome is a conserved submetacentric chromosome; and iv) the W chromosome morphology is variable among species, appearing as metacentric or submetacentric.

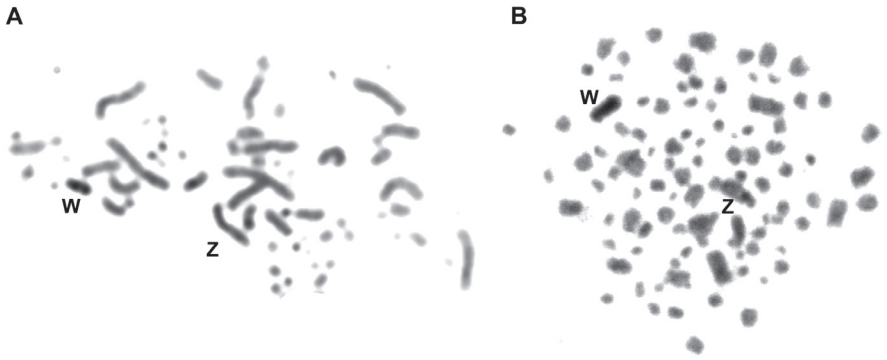

Figure 2. Comparative C-banding analysis of the Ringed Kingfisher *Megaceryle torquata* (**A**) and the Green Kingfisher *Chloroceryle americana* (**B**).

Table 1. Karyotype information's in Alcedinidae species.

Species	2n	Nº biarmed	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Z	W	Reference
Chloroceryle americana	94	4	S	S	S	T	T	T	T	T	T	T	T	M	T	T	T	S	S	Present work
Ceryle rudis	82	13	M	M	M	M	M	M	S	S	A	A	A	A	A	T	T	S	M	Garg and Shrivastava 2013.
Megaceryle torquata	84	10	S	M	S	S	M	A	A	S	A	T	T	T	M	T	T	S	S	Present work
Halcyon pileata	84	12	M	M	S	S	M	M	M	S	T	T	M	T	M	M	S	S	M	Xiaozhuang and Qingwei 1989.
Halcyon smyrnensis	76	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Youling et al. 1998.
Dacelo novaeguineae	76	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	De Boer and Belterman 1980.
Alcedo atthis	132	15	M	M	M	S	M	M	M	M	S	M	S	M	M	M	M	S	M	Xiaozhuang and Qingwei 1989.
Ceyx azureus	122	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Christidis 1990.

2n= diploid number; Nº biarmed= Number of biarmed autosomes; Chromosome morphology: (M=metacentric, S=submetacentric, A=Acrocentric and T=Telocentric); - = Not was possible to obtain the information in original work; Species names in accordance to IOC WORLD BIRD LIST (7.3) http://dx.doi.org/10.14344/IOC.ML.7.

Discussion

Unfortunately, forty years after the publication of the karyotype of *D. novaguineae* (*D. gigas* by De Boer and Beltrman 1980), information about cytogenetics of Alcedinidae species is still limited. Nevertheless, comparisons done in this work (Tab. 1) show that Kingfishers present karyotype plasticity, evidenced by variation in diploid number, number of biarmed chromosomes, and in size and morphology of macrochromosomes.

According to White (1977), chromosome fusions result in the reduction of diploid number and increase of number of biarmed chromosomes, while chromosome fissions increase the diploid number and decrease the number of biarmed chromosomes. These mechanisms appear to be adequate to explain the differences in the karyotypes of Alcedinidae species.

In this work, the increasing of diploid number observed in *M. torquata* (2n = 84) to *C. americana* (2n = 94), (Fig. 1B and D) may have originated by chromosome fissions. Some characteristics support this hypothesis, such as, the number of biarmed chromosomes is reduced from 9 pairs in *M. torquata* for to 4 in *C. americana*, and Z chromosome size is similar to chromosome 4 in *M. torquata*, while in *C. americana*, the Z chromosome is the largest in the karyotype. However, experiments with chromosome painting with specific probes could confirm these hypotheses.

According to Graves and Shetty (2001) Z chromosome size is conserved in most birds. So, Z chromosome size in relation to other macrochromosomes can be considered as a marker for size and evidence of occurrence of chromosome fission or fusions. Chromosome W in *M. torquata* and *C. americana* did not present differences and shows a pattern of heterochromatinization, similar of what has been observed in other Neognaths species. However, when compared to other species of Kingfishers, it is observed that there is a variation in chromosome morphology, ranging from metacentric to submetacentric.

Conclusion

Kingfishers present interesting chromosomal characteristics. These species have a diploid number which is highly variable and probably originated by fusions and/or fissions involving macrochromosomes. Hence rearrangements in macrochromosomes result in size and morphology variations, characterizing an intra-familial karyotypic heterogeneity. Absence of G-banding pattern and chromosome painting data did not allow comparisons. Therefore, we hope that this work may encourage the development of other cytogenetic studies in Kingfishers, and that our hypothesis of fission and chromosomal fusions as mechanisms responsible for karyotypes differentiation in Kingfishers can be confirmed.

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Banding cytogenetics of the Barbary partridge *Alectoris barbara* and the Chukar partridge *Alectoris chukar* (Phasianidae): a large conservation with Domestic fowl *Gallus domesticus* revealed by high resolution chromosomes

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Abstract

The development of avian cytogenetics is significantly behind that of mammals. In fact, since the advent of cytogenetic techniques, fewer than 1500 karyotypes have been established. The Barbary partridge *Alectoris barbara* Bonnaterre, 1790 is a bird of economic interest but its genome has not been studied so far. This species is endemic to North Africa and globally declining. The Chukar partridge *Alectoris chukar* Gray, 1830 is an introduced species which shares the same habitat area as the Barbary partridge and so there could be introgressive hybridisation. A cytogenetic study has been initiated in order to contribute to the Barbary partridge and the Chukar partridge genome analyses. The GTG, RBG and RHG-banded karyotypes of these species have been described. Primary fibroblast cell lines obtained from embryos were harvested after simple and double thymidine synchronisation. The first eight autosomal pairs and Z sex chromosome have been described at high resolution and compared to those of the domestic fowl *Gallus domesticus* Linnaeus, 1758. The diploid number was established as 2n = 78 for both partridges, as well as for most species belonging to the Galliformes order, underlying the stability of chromosome number in avian karyotypes. Wide homologies were observed for macrochromosomes and gonosome except for chromosome 4, 7, 8 and Z which present differences in morphology and/or banding pattern. Neocentromere

occurrence was suggested for both partridges chromosome 4 with an assumed paracentric inversion in the Chukar partridge chromosome 4. Terminal inversion in the long arm of the Barbary partridge chromosome Z was also found. These rearrangements confirm that the avian karyotypes structure is conserved interchromosomally, but not at the intrachromosomal scale.

Keywords
Barbary partridge *Alectoris barbara*, chukar partridge *Alectoris chukar*, endemic species, banding cytogenetics, high resolution chromosomes, homologies, intrachromosomal rearrangements

Introduction

The Barbary partridge *Alectoris barbara* Bonnaterre, 1790 (Phasianidae) is the only native partridge naturally present in Algeria. This North African endemic species is found not only from Morocco to Egypt, but also in Gibraltar, Sardinia and the Canary Islands (Cramp and Simmons 1980, Madge and McGowan 2002). The Barbary partridge is a nesting sedentary bird found in different ecosystems: Mediterranean (coastal dunes and Atlas Mountains), Steppic and Saharian. This common game bird is prized for its meat; hence its overhunting leads to declining population size in some areas. Although the Barbary partridge is listed as Least Concern on the IUCN Red List (2015) (International Union of Conservation of Nature), it is nevertheless protected by several conventions. Indeed, the Barbary partridge was placed on the regulated species list protected by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES): Bird instruction 79/409 (Annex I, II / 2, III / 1). This bird is also protected by the Convention on the Conservation of European Wildlife and Natural Habitats (Bern Convention). Furthermore, the Barbary partridge has also a national scope of protection in commercialisation of some bird species on the French territory. Decline of the native population is mainly due to predation, to poaching (despite the law prohibiting hunting since 1991) and habitat degradation due to mechanisation of farming and urban proliferation (Madge and McGowan 2002). In Morocco, observations have also shown a sharp decrease in Barbary partridge populations, which could become alarming in the long term (Maghnouj 1991). Other factors such as excessive use of pesticides, hikers and stray animals could also disrupt the smooth conduct of breeding. All these factors are also responsible for the decline of partridge populations in Europe (Tejedor et al. 2007, Randi 2008).

In addition, introduction of the exotic Chukar partridge *Alectoris chukar* Gray, 1830 could also lead to introgression in the wild genome of native partridge and could give rise to infertile descendants. In fact, hybridisation may occur when isolating mechanisms break down naturally or as a result of human activity as in the *Alectoris* partridges (Barbanera et al. 2011). Several studies have recorded cases of artificial genetic pollution of *Alectoris rufa* Linnaeus, 1758 and *Alectoris graeca* Meisner, 1804 by the *Alectoris chukar* genome (Randi et al. 2003, Barbanera et al. 2005, Barilani et al. 2007, Tejedor et al. 2007). The Barbary partridge is the most phylogenetically divergent taxon in the genus *Alectoris,* while *Alectoris chukar* is the most recent gamebird (Randi 1996, Randi and Lucchini 1998, Kimball et al. 1999). *Alectoris barbara* and *chukar* lineages split

from an ancestral species about 6 million years ago, at the Miocene-Pliocene boundary (Voous 1976, Randi et al. 1992).

Preservation of this endemic species is a priority, which has led to a restocking programme with captive-reared Barbary partridge carried out by the Centre Cynégétique de Zéralda (36°42'06"N, 2°51'47"E). The goal of this project is to obtain strains able to reproduce in captivity, and formulate demographic monitoring after repopulation. Although the Barbary partridge is the main game-bird species in North Africa, scarce research has been reported and it concerns the reproduction and ecology of this species (Alaoui 1992, Akil and Boudedja 2001). However, recent genetic studies have established genetic tests aiming to identify hybrid individuals (Rodríguez-García and Galián 2014). Actually, the only classical cytogenetics data reported on *Alectoris* genus concern red-legged and Chukar partridges whose karyotypes have been described by use of conventional staining (Arruga et al. 1996, Babak et al. 2014, Ishishita et al. 2014).

The Barbary partridge *Alectoris barbara* like the domestic fowl *Gallus domesticus* Linnaeus, 1758 belongs to the ancestral order of Galliformes which includes the most avian species whose genomes have been analysed. In fact, the domestic fowl is the best described one because of its economic importance. It is considered as a reference in phylogenetics and comparative genomics and represents the only standardised bird karyotype (Ladjali-Mohammedi et al. 1999). As a typical avian genome, the karyotype of the domestic fowl has 39 pairs of chromosomes represented by 10 pairs of autosomal macrochromosomes (1–10 chromosomes), 28 pairs of microchromosomes (11–38) and one pair of sex chromosomes. The male is the homogametic sex ZZ (equivalent to human XX), whereas the female is the heterogametic sex ZW (equivalent to human XY) (Masabanda et al. 2004). Despite their small physical size, microchromosomes are characterised by high gene density, high GC content (McQueen et al. 1996) and an early replicating pattern compared to macrochromosomes (Schmid et al. 1989, Ponce de Leon et al. 1992, Burt 2002). Due to the presence of high number of near-undistinguishable microchromosomes, most bird karyotypes are partial and confined to a few macrochromosomes (Shibusawa et al. 2004). However, the use of chicken probes has allowed identification of several microchromosomes in some bird species (Fillon et al. 1998, Nie et al. 2015, Galkina et al. 2017, Kretschmer et al. 2018).

On the other hand, the chicken is the first avian genome to have been sequenced (Hillier et al. 2004), followed by the zebra finch *Taeniopygia guttata* (Warren et al. 2010) and Turkey *Meleagris gallopavo* (Dalloul et al. 2010). The chicken genome assembly Gallus_gallus-4.0 covered 1.03 Gb or 96% of the total genome size, including the sequence of the 10 macrochromosomes, 19 microchromosomes and sex chromosomes (Schmid et al. 2015). Recently, coverage was improved by a gain of 183 Mb and three microchromosomes (30, 31 and 33) in the Gallus_gallus-5.0 assembly. However, 138 Mb are not yet assigned to chromosomes (Warren et al. 2017). Rapid advances in genome assembly software and technologies as Next Generation Sequencing (NGS) allowed entire genome sequencing of more than 57 birds (Dalloul et al. 2010, Jarvis et al. 2014). Among these species, 42 were a part of the Genome 10K Project which aims to facilitate the sequencing and analysis of 10.000 vertebrate genomes

(http://genome10k.soe.ucsc.edu) (Genome 10K Community of Scientists 2009). The Avian Phylogenomics Consortium announced in 2015 a great project called B10K (web.bioinfodata.org/B10K) to generate draft genome sequences for all the 10.476 avian species within the next five years (until 2020). All these sequencing data corroborate the exceptional stability of avian karyotypes (Shibusawa et al. 2002, Derjusheva et al. 2004, Shibusawa et al. 2004). Indeed, the occurrence of interchromosomal rearrangements in birds is a relatively rare event estimated to 1.25 per million years, which is considerably lower than in mammals (Zhao and Bourque 2009, Romanov et al. 2014, Zhang et al. 2014). It is assumed that interchromosomal reshuffling could be the result of an adaptive response and a cause or consequence of speciation (King 1995, Griffin et al. 2007, Romanov et al. 2014).

Although avian high resolution mapping is well advanced, reported cytogenetic studies are nevertheless partial and fewer than those of mammals despite great contribution of this discipline. In fact, classical and banding cytogenetics highlighted important features of avian karyotype as interchromosomal stability (Tegelstrôm and Ryttman 1981, Belterman and De Boer 1984, Christidis 1990, Shibusawa et al. 2004) and intrachromosomal reshuffling in some macrochromosomes (Stock and Bunch 1982, Griffin et al. 2007, Hooper and Price 2017). Banding cytogenetics has also elucidated the process of karyotypic evolution in some orders of bird (Dobigny et al. 2004, Shibusawa et al. 2004, Nishida et al. 2008) and even in mammals (Di-Nizo et al. 2017).

The aim of the present study is to describe the chromosomes of Barbary partridge *Alectoris barbara* and Chukar partridge *Alectoris chukar* at high resolution level with morphological and dynamic banding techniques. Comparison of partridges and chicken banding patterns has been conducted in order to estimate the degree of conservation and rearrangements of these species during speciation.

Material and methods

Biological material

Barbary and Chukar partridge embryos were obtained from the Centre Cynégétique de Zéralda during the laying period (March to June). Four Barbary partridge and four Chukar partridge embryos were sampled after 5–6 days incubation at 37 °C, and kept under the same temperature and hygrometry conditions in the Laboratoire de Génétique du Développement (Faculté des Sciences Biologiques, USTHB) until at least 12 days old.

Cell cultures

Primary fibroblast cell cultures were harvested from 6 to 12 days old embryos. The embryos were cleared from their annexes and totally ground in a trypsine solution (0.05%, Sigma). Cell suspension were incubated at 41 °C with an estimate concentration of

3×10^6 cells/ml in RPMI 1640 culture medium (20 mM HEPES, GIBCO) supplemented with 10% foetal calf serum (FCS, GIBCO), 1% L-Glutamine 200 mM (Sigma), 1% Penicillin, Streptomycin and Fungizone (Sigma). Trypsinisation of cells was realised to enhance division ability (adapted from Ladjali 1994, Ladjali et al. 1995).

Synchronisation of cell cultures

In order to increase the yield of metaphases and prometaphases cells, cultures were synchronised with a simple and double thymidine block during the S phase (Dutrillaux and Couturier 1981, Hayes et al. 1993, Ladjali et al. 1995). Cells were blocked for the first time during 16–18h with thymidine (final concentration: 10mg/ml, Sigma), and rinsed 2×15 min with BSS+ (Hank's Balanced Salt Solution containing 5.6% $NaHCO_3$ and 2 mM $CaCl_2$) at 41 °C. Cells were incubated again in culture medium RPMI, and the day after, the step above was repeated for a second time to produce a double thymidine block. On the third day, when cells restarted division in RPMI with 5% FCS, an analogue of thymidine 5-Bromo-2-deoxyUridine (final concentration: 10 µg/ml, BrdU, Sigma) was incorporated into cultures. An hour after, 5-Fluoro-2-uridine (final concentration: 0.5 µg/ml, FdU, Sigma) was added to enhance BrdU incorporation. These treatments are required to prepare chromosomes for dynamic R-banding staining (Dutrillaux and Couturier 1981, Schmid et al. 1989, Hayes et al. 1993, Ladjali et al. 1995).

Cell harvest

The incorporation of BrdU into the S phase lasted 6–7 hours. Meanwhile cells were continuously observed by reversed microscope until the number of mitotic round cells peaked. Cells were trypsinysed (trypsine 0.05% + 0.02% EDTA, GIBCO) and harvested in a 15 ml tube with colchicine (final concentration: 0.05 µg/ml, Sigma). After centrifugation, hypotonic treatment was undertaken during 13 min at 37 °C with diluted newborn calf serum (1:5). Intracytoplasmic structures were prefixed with 1 ml of methanol/acetic acid (3:1) at 37 °C. Fixation was finally realised at 4 °C and after centrifugation, 1 ml was let in tubes until spreading. Slides were washed, rubbed and placed in cold water. A few drops from the cell suspension were spread at 10 cm of cold slide and left to dry until staining procedures occurred (adapted from Ladjali et al. 1995).

Banding staining

GTG-banding (G-bands obtained with Trypsin and Giemsa) was realised following the Seabright modified method (1971). Approximately; 3 to 4 days after spreading,

slides were incubated for 8–10 seconds in a trypsine solution (final concentration: 0.25%, Sigma) at room temperature. Slides were rinsed twice in PBS^- (Phosphate Buffered Solution, pH=6.8) and stained in 6% Giemsa for 8–10 minutes.

RBG-FPG banding (R-bands followed by fluorochrome-photolysis) procedure was undertaken following Ladjali et al. (1995). Slides were incubated in Hoechst 33258 solution (1 mg/ml) for 20 min. Slides were then rinsed and placed for 90 min in 2 × SSC buffer (Saline Sodium Citrate) at a distance of 15 cm from UV dark light (Mazdafluor OE TFWN 20). Slides are rinsed again and placed in Earle's buffer at 87 °C for 10 min. Slides were washed and incubated for 20 min in 6% Giemsa staining solution.

RHG-banding (R-bands obtained by Heat and Giemsa) was realised on *A. chukar* spreads. Slides were incubated in Earle's buffer (ph=5,8) at 87 °C for 20 minutes, then rinsed and stained in 6% Giemsa solution (containing phosphate buffer) (Dutrillaux and Leujeune 1971, Comings 1978).

Chromosome Classification

Slides were first observed with an optical microscope at objective magnification 10× to estimate the mitotic index (AxioZeiss Scope A1). Slides, showing a higher mitotic index, were analysed and prometaphases and metaphases, showing decondensed and dispersed chromosomes, were photographed (CoolCube1 Metasystems). The first eight macrochromosomes and Z sex chromosomes from Barbary partridge *Alectoris barbara* and Chukar partridge *Alectoris chukar* were classified in G- and R- banding as described in International System of Standardised Avian Karyotypes ISSAK (Ladjali-Mohammedi et al. 1999). Macrochromosomes pairs were classified according to decreasing size and centromere position (Shoffner 1974), whereas microchromosomes were not presented because of their small physical size making very difficult any classification or description. In order to avoid any ambiguity, nomenclature adopted in this article followed the ISSAK (Ladjali-Mohammedi et al. 1999) adapted from ISCN (1978).

Chromosome measurement

Analyses measurements of fifteen first pairs of chromosomes were undertaken using KARYOTYPE 2.0 software (Altinordu et al. 2016). Measured parameters were: Long (q) and short (p) arms, total chromosome length (p+q) and arm ratio r: Long/short. In the Results section below, morphometry will be presented of the first eight chromosomes and the Z chromosome, which have been compared to the domestic fowl. Other microchromosomes were physically too small and did not give significant values. Partridge's karyotypes have been established manually, considering that software used in the present work was not adapted to birds.

Results

Primary fibroblasts cell lines were obtained a few hours after incubation and constituted a good source for obtaining chromosome preparations. The younger the embryos, the more mitotic divisions were obtained. The strict follow up of cell divisions after inhibition removal enabled the estimation of half cycle time to 7–8 hours for Barbary partridge *Alectoris barbara* and 6–7 hours for Chukar partridge *Alectoris chukar*. Important mitotic indices with high resolution chromosomes were obtained with simple synchronisation for *A. barbara* and double synchronisation for *A. chukar* during 18h. Furthermore, observation of cell cultures of both species showed that *A. barbara* cells were much more sensitive than *A. chukar* to the different drugs added during incubation. Trypsinisation and synchronisation steps caused important Barbary partridge cell death compared to Chukar partridge. In fact, we have incubated an average of 3×10^6 cells/ml (Ladjali 1994). After a confluence, we estimate that cells have divided four times (12.10^6 cells/ml). Following the trypsinisation, cells divided twice (24×10^6 cells/ml). A continuous observation of cultures after *in vitro* treatments shows an average decrease of 30% of live cells of Barbary partridge, equivalent to 7.2×10^6 cells/ml for all four embryos. Whereas, no diminution of mitotic power was observed in Chukar partridge regardless of trypsination, addition of BSS^+, BrdU/FdU or colchicine.

Diploid numbers of Barbary partridge *Alectoris barbara* and Chukar partridge *Alectoris chukar* were estimated as 2n=78 from most metaphase plates (Fig. 1). Like most of birds, *A. barbara* and *A. chukar* karyotypes are composed of a few pairs of macrochromosomes and several microchromosomes with small physical size, which are very difficult to distinguish.

The authors proposed *Alectoris barbara* partial karyotype in GTG (Fig. 2a) and RBG banding (Fig. 2b), and *Alectoris chukar* partial karyotype in GTG (Fig. 2c) and RHG banding (Fig. 2d). Most metaphases show male genetic sex ZZ for both partridges, wherefore gonosome W was only described in RBG bands for *A.barbara* and GTG bands for *A.chukar*. The success of simple and double synchronisation resulted in high resolution chromosomes. Measurements show that chromosomes of *A. chukar* were more decondensed than those of *A. barbara* (Table 1). In fact, the size of the first eight macrochromosomes ranges from 14 μm to 3μm in *A. chukar* and from 9 μm to 2 μm for *A. barbara*. This is certainly due to the success of double synchronisation and extreme resistance of *A. chukar* cells to drugs added *in vitro*.

Observation of partridge's spreads shows that in *A.barbara* an average of 45 metaphases /100 displayed break points. These breaks seem to appear in sub-terminal regions of macrochromosomes 1 and 3 (Fig. 3). None of *A.chukar* metaphases have shown this phenomenon. Furthermore, the same typical distribution of partridge's chromosomes was observed. In fact, macrochromosomes are preferentially located towards the mitosis periphery, while microchromosomes are clustered within the mitosis interior (Fig. 3).

Partial ideograms of *A. barbara* and *A. chukar* were proposed on the basis of means of 20 metaphases plates following the International System of Standardised Avian Karyotypes (Ladjali-Mohammedi et al. 1999) (Fig. 4 and Fig. 5, Table 2 and 3).

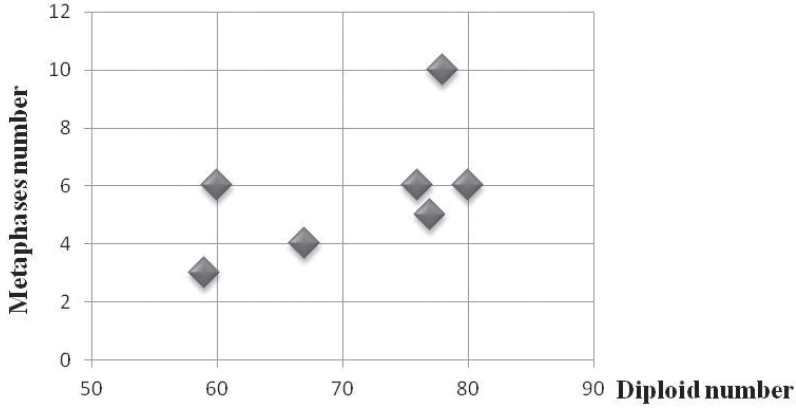

Figure 1. Estimation of diploid number of Barbary and Chukar partridges. Major metaphase plates (10) displayed diploid number 2n= 78 chromosomes.

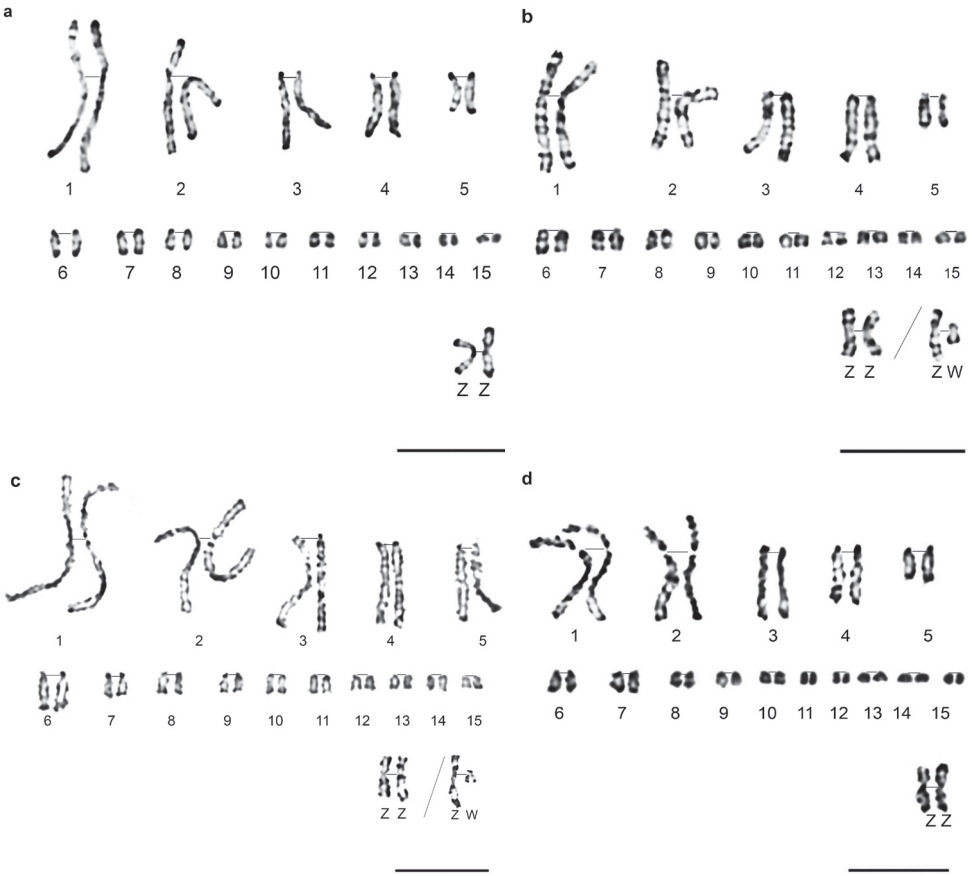

Figure 2. Partial karyotypes of *A. barbara* in GTG bands (**a**), *A. barbara* in RBG bands (**b**), *A. chukar* in GTG bands (**c**), and *A. chukar* in RHG bands (**d**). Gonosomes Z W are classified apart. Scale bars: 5 μm.

Table 1. *A. barbara* and *A. chukar* morphometry of the first eight macrochromosomes and gonosomes. Means are obtained at least from 10 prometaphases/metaphases (from 10 to 20). Chr: chromosome, q: long arm, p: short arm, t: total (p+q), r: ratio (q/p), lengths are given in micrometer (μm).

	A. barbara				*A. chukar*			
Chr	*p*	*q*	*t*	*r*	*p*	*q*	*t*	*r*
1	3.78	5.98	**9.76**	1.58	5.63	8.82	**14.45**	1.56
2	2.89	4.71	**7.6**	1.62	3.83	6.76	**10.59**	1.76
3	1.03	5.57	**6.6**	5.4	1.2	7.5	**8.7**	6.25
4	1.02	4.33	**5.35**	4.24	1.15	6.19	**7.34**	5.38
5	0.75	2.85	**3.6**	3.8	0.78	4.9	**5.68**	6.28
6	0.68	2.32	**3**	3.41	0.75	3.35	**4.1**	4.46
7	0.7	1.7	**2.4**	2.42	0.7	3	**3.7**	4.28
8	0.53	1.57	**2.1**	2.96	0.63	2.37	**3**	3.76
Z	2.5	3.1	**5.6**	1.24	3.1	3.5	**6.6**	1.12
W	0.75	1.03	**1.78**	1.37	0.93	1.37	**2.3**	1.47

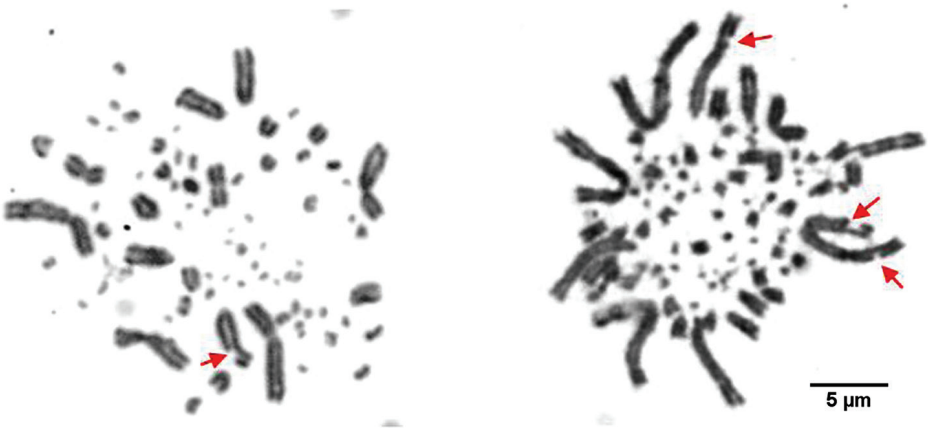

Figure 3. Partridges' metaphases showing spatial distribution of chromosomes (*A. barbara* on the left and *A. chukar* on the right). Macrochromosomes are located towards metaphases periphery, microchromosomes are confined to the central area. *Arrows* indicates break points in chromosomes. Bar = 5 μm.

Partial ideograms of *Alectoris barbara* and *Alectoris chukar* described in GTG bands (Fig. 4, Table 2)

Chromosome 1

P arm

Barbary partridge: two regions. 11 G bands with a visible negative band (21) which divides the p arm into two regions. A large terminal positive band is also visible (26).

Chukar partridge: three regions. 17 G bands with a predominant terminal negative band (33).

Q arm

Barbary partridge: Five regions. 21 bands, four negative bands divide the q arm into four regions with one predominant negative band (41). The centromeric region is positively banded.

Chukar partridge: Five regions. 23 G bands, with a wide terminal negative band (51).

Chromosome 2

P arm

Barbary partridge: three regions. 11 G bands with a large negative proximal band (21).

Chukar partridge: three regions. 13 G bands with large negative terminal band (31).

Q arm

Barbary partridge: three regions. 19 G bands with two wide negative bands (21 and 31).

Chukar partridge: three regions. 21 G bands with a large negative subtelomeric band (31).

Chromosome 3

P arm

Barbary partridge: one region with 3 G bands.

Chukar partridge: one region with 2 G bands.

Q arm

Barbary partridge: four regions. 23 G bands with two wide proximal negative bands (13 and 21).

Chukar partridge: four regions. 23 G bands with two large negative bands (31 and 41).

Chromosome 4

P arm

Barbary partridge: one region.

Chukar partridge: one region with 2 G bands.

Q arm

Barbary partridge: four regions. 19 G bands with a wide proximal negative band (21).

Chukar partridge: four regions. 25 G bands with a visible central positive band (26).

Chromosome 5

Q arm

Barbary partridge: three regions. 12 G bands with a wide central negative band (21).
Chukar partridge: three regions. 19 G bands with a visible central positive band (22).

Chromosome 6

Q arm

Barbary partridge: two regions. 8 G bands with a wide central negative band (21).
Chukar partridge: two regions. 9 G bands with two central positive bands (22 and 24).

Chromosome 7

Q arm

Barbary partridge: two regions. 6 G bands.
Chukar partridge: two regions. 6 G bands with a visible central negative band (21).

Chromosome 8

Q arm

Barbary partridge: two regions. 7 G bands with a wide central negative band (21).
Chukar partridge: two regions. 7 G bands with a large terminal negative band (21).

Chromosome Z

P arm

Barbary partridge: two regions. 7 G bands showing a large negative band (21).
Chukar partridge: two regions. 9 G bands with a visible negative band (21).

Q arm

Barbary partridge: two regions. 11 G bands with a large negative band (21) and a positive land mark (22).
Chukar partridge: two regions. 11 G bands with two large negative bands (15 and 21).

Chromosome W

P arm

Chukar partridge: one region. 2 G bands with terminal positive band.

Q arm

Chukar partridge: two regions. 5 G bands with one positive subcentromeric band (11) and a telomeric positive band (22)

Table 2. Values summarized from partial ideograms of *A. barbara* and *A. chukar* described in GTG bands. Chr: chromosome, p: short arm, q: long arm, R: region, B: bands, LM: Landmark (all positions show negative landmarks except when (+) is added), empty boxes indicate that there is no particular landmark.

	Alectoris barbara						***Alectoris chukar***					
Chr	***P arm***			***Q arm***			***P arm***			***Q arm***		
	R	**B**	**LM**	**R**	**B**	**LM**	**R**	**B**	**LM**	**R**	**B**	**LM**
1	2	11	(21), (26)	5	21	(41)	3	17	(33)	5	23	(51)
2	3	11	(21)	3	19	(21), (31)	3	13	(31)	3	21	(31)
3	1	3	-	4	23	(13) (21)	1	2	-	4	23	(31), (41)
4	1	1	-	4	19	(21)	1	2	-	4	25	(26) +
5	1	1	-	3	12	(21)	1	2	-	3	19	(22) +
6	1	1	-	2	8	(21)	1	2	-	3	9	(22), (24)
7	1	1	-	2	6	-	1	1	-	2	6	(21)
8	1	1	-	2	7	-	1	1	-	2	7	-
Z	2	7	(21)	2	11	(21), (22)+	2	9	-	2	11	(15), (21)
W	-	-	-	-	-	-	1	2	-	2	5	(11)+(22)+

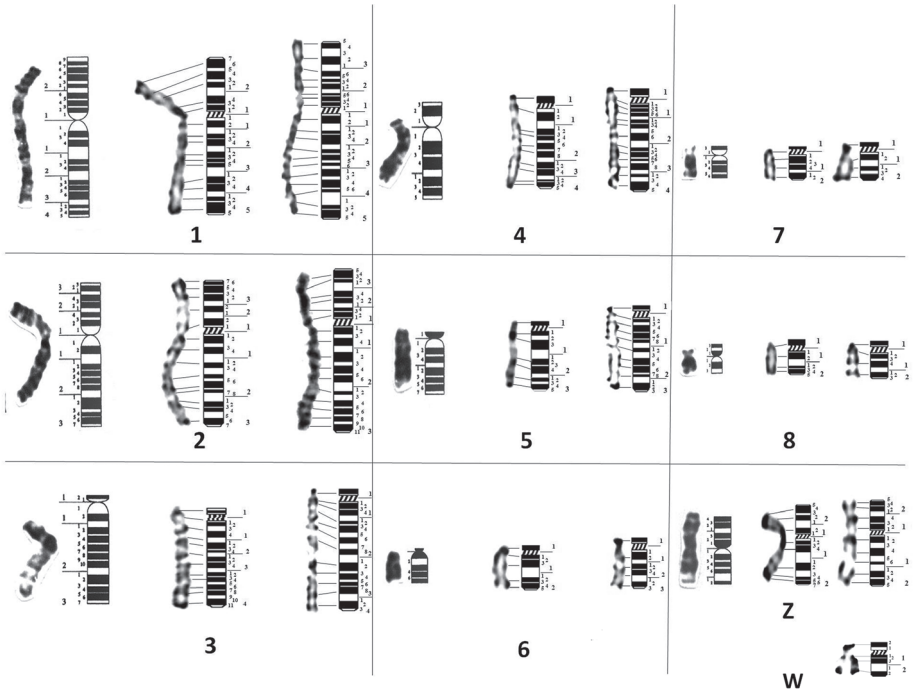

Figure 4. GTG partial ideograms of (from left to right) *G. domesticus* (Ladjali-Mohammedi et al. 1999), *A. barbara* and *A. chukar*. W chromosome is represented only in *A.chukar*. Horizontal traits indicate correspondence of positive bands between chromosomes and ideograms. Along ideograms: Large numbers indicate regions, smallest numbers indicate positive and negative bands.

Partial ideograms of *Alectoris barbara* and *Alectoris chukar* described in RBG / RHG bands (Fig. 5, Table 3)

Chromosome 1

P arm

Barbary partridge: three regions. 13 RBG bands with a large terminal negative band (31).

Chukar partridge: Three regions. 18 RHG bands with two principal negative bands (21 and 31).

Q arm

Barbary partridge: Four regions. 20 bands with two wide terminal respectively negative and positive bands (41 and 42). The centromeric region is positively banded.

Chukar partridge: Four regions. 25 bands with three large negative bands which divided the q arm (13, 31 and 45).

Chromosome 2

P arm

Barbary partridge: two regions. 10 bands with a large negative telomeric band (25).

Chukar partridge: three regions. 17 bands with large negative proximal band (21).

Q arm

Barbary partridge: three regions. 15 bands with two wide negative bands (21 and 31).

Chukar partridge: four regions. 25 bands with a large negative telomeric band (31).

Chromosome 3

P arm

Barbary partridge: one region with 2 bands.

Chukar partridge: one region with 2 bands.

Q arm

Barbary partridge: three regions. 16 bands with a central positive band (22) and a telomeric negative band (31).

Chukar partridge: four regions. 27 bands with a large submedian negative band (31).

Chromosome 4

P arm

Barbary partridge: one region and 2 bands.

Chukar partridge: one region with 3 bands.

Q arm

Barbary partridge: three regions. 14 bands with two visible negative bands (21 and 31).
Chukar partridge: four regions. 21 bands with two proximal positive bands (14 and 16) and two central positive bands (22 and 24).

Chromosome 5

P arm

Barbary partridge: one region. 2 RBG bands.
Chukar partridge: one region. 3 RHG bands.

Q arm

Barbary partridge: three regions. 8 bands with two wide negative bands (21 and 31).
Chukar partridge: three regions. 15 bands with two large proximal positive bands (12 and 14).

Chromosome 6

P arm

Barbary partridge: one region showing 2 RBG bands.
Chukar partridge: one region presenting 3 RHG bands.

Q arm

Barbary partridge: two regions. 7 bands with a wide central negative band (21).
Chukar partridge: two regions. 8 bands and a large negative band (21).

Chromosome 7

P arm

Barbary partridge: one region.
Chukar partridge: one region with 3 RHG bands.

Q arm

Barbary partridge: two regions. 5 bands showing a large distal negative band (21).
Chukar partridge: two regions. 7 bands with a central positive band (14).

Chromosome 8

P arm

Barbary partridge: one region with one band.
Chukar partridge: one region with 3 RHG bands.

Q arm

Barbary partridge: two regions. 5 bands with a central positive band (13).
Chukar partridge: two regions. 7 bands and a central negative band (21).

Chromosome Z

P arm

Barbary partridge: two regions. 7 R bands and a wide terminal negative band (21).
Chukar partridge: two regions. 10 R bands with a large negative band (21).

Q arm

Barbary partridge: three regions. 9 R bands with a positive terminal land mark (24) and a large negative band (31).
Chukar partridge: two regions. 13 R bands with a visible terminal negative band (21).

W chromosome

P arm

Barbary partridge: one region. 2 RBG bands with terminal positive band.

Q arm

Barbary partridge: one region. 2 RBG bands with a large positive telomeric band. Centromeric region is negatively stained.

Table 3. Values summarized from partial ideograms of *A. barbara* and *A. chukar* described in RBG/RHG bands. Chr: chromosome, p: short arm, q: long arm, R: region, B: bands, LM: Landmark (all positions show negative landmarks except when (+) is added), empty boxes indicate that there is no particular landmark.

	Alectoris barbara						***Alectoris chukar***					
Chr	***P arm***			***Q arm***			***P arm***			***Q arm***		
	R	**B**	**LM**	**R**	**B**	**LM**	**R**	**B**	**LM**	**R**	**B**	**LM**
1	3	13	(31)	4	20	(41) (42)	3	18	(21) (31)	4	25	(13), (31), (45)
2	2	10	(25)	3	15	(21)	3	17	(21) (31)	4	25	(31)
3	1	2	-	3	16	(22)+(31)	1	2	-	4	27	(31)
4	1	2	-	3	14	(21) (31)	1	3	-	4	21	(14)+, (16)+ (22)+ , (24)+
5	1	2	-	3	8	(21) (31)	1	3	-	3	15	(12)+, (14)+
6	1	2	-	2	7	(21)	1	3	-	2	8	(21)
7	1	1	-	2	5	(21)	1	3	-	2	7	(14)+
8	1	1	-	2	5	(13)+	1	3	-	2	7	(21)
Z	2	7	(21)	3	9	(24)+, (31)	2	10	(21)	2	13	(21)
W	1	2	(12)+	1	2	(12)+	-	-	-	-	-	-

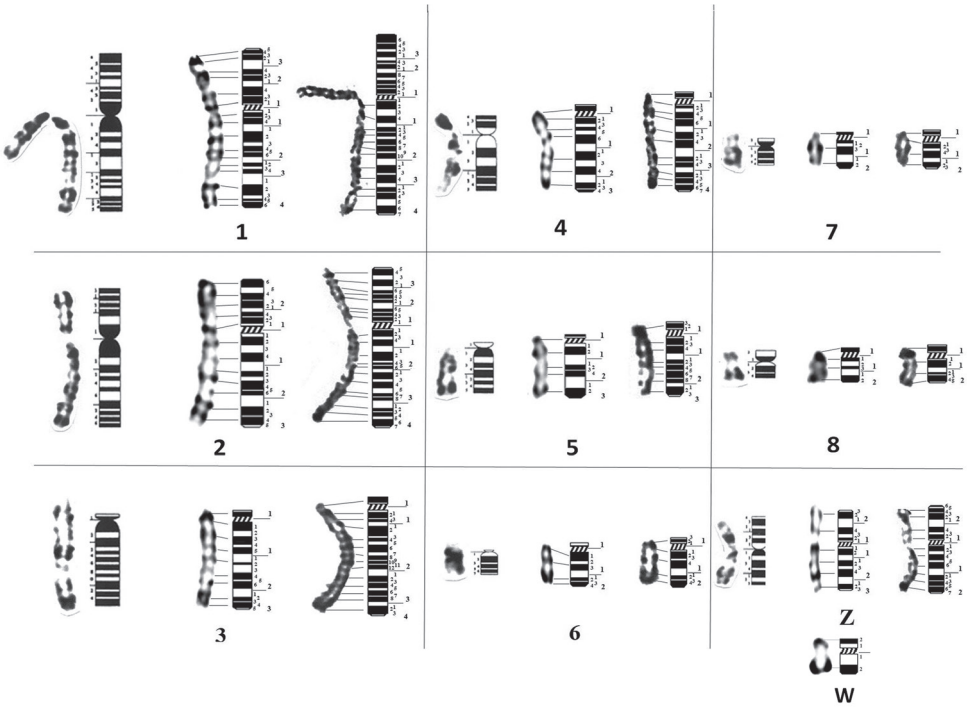

Figure 5. RBG and RHG partial ideograms of (from left to right) *G. domesticus* (Ladjali-Mohammedi et al. 1999), *A. barbara* and *A. chukar.* W chromosome is represented only in *A.barbara.* Horizontal traits indicate correspondence of positive bands between chromosomes and ideograms. Along ideograms: Large numbers indicate regions, smallest numbers indicate positive and negative bands.

Alectoris barbara, Alectoris chukar and *Gallus domesticus* chromosome comparison

Comparison of morphological and dynamic G and R banding of *A. barbara* and *A. chukar* with domestic fowl (Ladjali-Mohammedi et al. 1999) shows a wide conservation of patterns in macrochromosomes. However, some rearrangements in partridges chromosomes 4 and Z were observed (Fig. 4 and Fig. 5). All centromeric regions of partridge chromosomes were positively stained in G and R banding. Chromosomes 1 and 2 are submetacentric in both Barbary and Chukar partridges, like in the domestic fowl. Despite the difference in chromosome 1 length, the arm ratio is quite similar (r = 1.58 / 1.56) (Table 1). On the other hand, the *A. chukar* long arm of chromosome 2 is longer than for *A. barbara* (r = 1.76 / 1.62). In both partridges, the centromere position is more submedian in chromosome 2 compared to chromosome 1. Chromosome 3 is acrocentric in partridges and domestic fowl. The banding pattern of the first three chromosomes is apparently widely conserved in all three species. Chromosome 4 is acrocentric in partridges and telocentric in chicken. The banding pattern is, however, conserved in *A. barbara* and *G. domesticus*, while in *A. chukar*, the subcentromeric region presents a different profile (Fig. 6). Chromosomes 5 and 6 are acrocentric in each

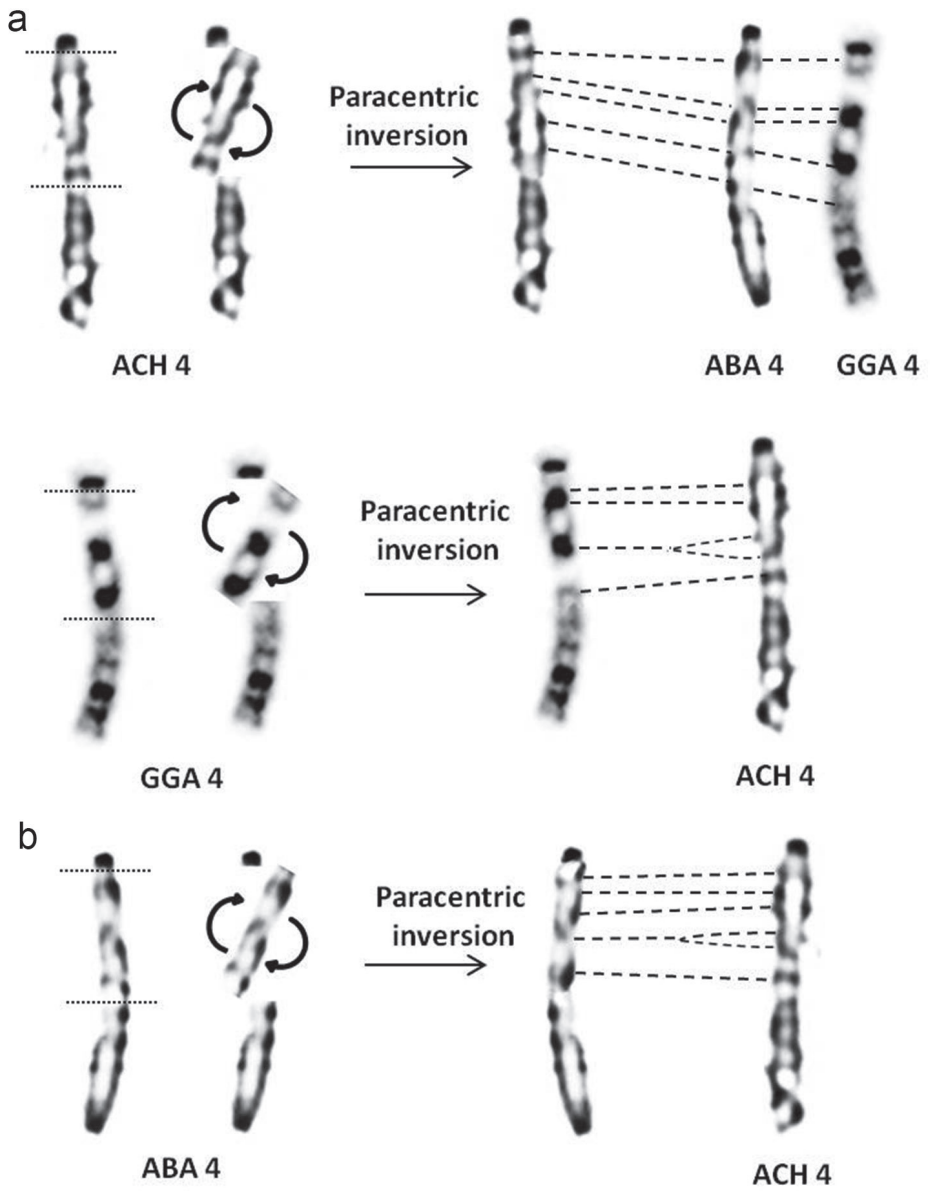

Figure 6. Schematic representation of a paracentric inversion in *A. chukar* (**a**), *G.domesticus* and *A. barbara* (**b**) chromosome 4. Corresponding bands are indicated by dashes. (ACH: *A. chukar*, GGA: *G.domesticus* and ABA: *A. barbara*).

species and present a similar pattern distribution, although, *A. chukar* presents higher number of bands due to decondensation. Chromosome 7 and 8 are acrocentric in both partridges and respectively, telocentric and submetacentric in the domestic fowl (Ladjali-Mohammedi et al. 1999). Surprisingly, the distribution of bands is conserved

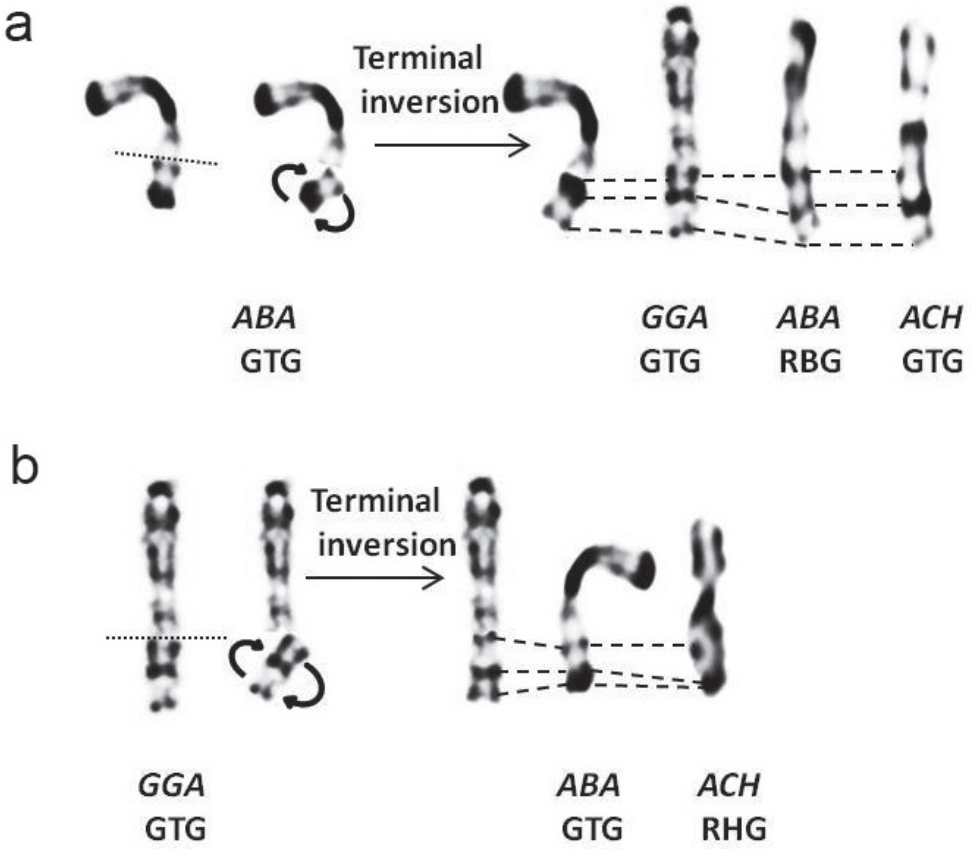

Figure 7. Schematic representation of a terminal paracentric inversion in chromosome Z of *A. barbara* in GTG (**a**) and chromosome Z of *G. domesticus* in GTG banding (**b**). Corresponding bands are indicated by dashes. (ACH: *A. chukar*, GGA: *G.domesticus* and ABA: *A. barbara*). Rearranged ABA Z in GTG corresponds to GGA Z and ACH Z in GTG. Rearranged GGA Z in GTG corresponds to ABA in GTG and ACH in RHG.

through these three Galliformes (comparison of chromosomes at the same decondensation stage). Sex chromosomes Z and W are submetacentric and morphologically conserved in all three species. However, the terminal region of the Z chromosome long arm presents a different pattern in *A. barbara* compared to that of *A.chukar* and the domestic fowl (Fig 7). In the present work, we found in *A. barbara* a total of 145 G/123 R-bands and in *A. chukar* 173 G/187 R-bands only for the first eight chromosomes (Table 2 and 3).

Discussion

Implementation of fibroblasts was observed in all cultures and confluence was quickly reached in all eight embryos, mainly in the youngest ones (6 days). This is due to the

important mitotic power of cells at early embryonic stages (Ladjali et al. 1995). The high mortality in cell cultures of *A.barbara* (for the four embryos) is interestingly reflected in breeders' observations regarding the Barbary partridge's high vulnerability in breeding areas, unlike the Chukar partridge (personal communication of the Centre Cynégétique de Zéralda). Indeed, the Barbary partridge is a vulnerable endemic species, whereas Chukar partridge is usually introduced to reinforce the low local densities populations because of its easy practical prolificacy in captivity compared to other partridges (Rojas et al. 2011).

Distribution of partridges' macrochromosomes and microchromosomes in metaphases is similar to that reported in several studies on chicken fibroblasts and neurons nucleis (Habermann et al. 2001, Federico et al. 2005) and mammalian fibroblasts nuclei (Cremer et al. 2000) (Fig. 3). In fact, it was reported that gene dense and early replicating chromatin, represented by microchromosomes (McQueen et al. 1996, Schmid et al. 1989, Ponce de Leon et al. 1992, Burt 2002, Skinner et al. 2009) were located in the nuclei central area, surrounded by gene-poor and later replicating chromatin (macrochromosomes) (Cremer et al. 2000). These results indicate that the radial position of chromosome territories is correlated with their size, their gene-density and replication timing (Habermann et al. 2001, Federico et al. 2005). Further, this specific distribution was assumed to be evolutionarily conserved in Galliformes (Maslova and Krasikova 2011) and also between mammals and birds despite their highly divergent karyotypes (Tanabe et al. 2002). The typical distribution of macro-and microchromosomes in metaphases could explain the particularly low rate of interchromosomal rearrangements in Galliformes (Shibusawa et al. 2002).

Fortuitously, 45% of *A. barbara* metaphase plates show breaks on some macrochromosomes which could be identified as fragile sites (Fig. 3). In birds, breakpoint regions of fragile sites are frequently associated with chromosomal rearrangements (Zlotina et al. 2010, Itoh et al. 2011, Skinner and Griffin 2012). Chromosomal fragile sites are loci prone to breakages within metaphase chromosomes (Fungtammasan et al. 2012). In mammals and birds, these breaks are assumed to occur in repetitive DNA clusters (Zlotina et al. 2010). Nevertheless, recent works in humans show that chromosomal rearrangements could appear in early replicating and actively transcribed gene clusters (Mortusewicz et al. 2013). It can be assumed that Barbary partridge chromosomes are particularly vulnerable to breakages, which could be favorable to intrachromosomal reshuffling. It would be very interesting to explore such genomic regions by molecular tools.

The diploid number of *Alectoris barbara* and *Alectoris chukar* was estimated as 2n = 78. This result is concordant with the exceptional stability of avian karyotype, i.e. about 65% of karyotyped birds displayed 76 to 82 chromosomes, including 7 to 8 pairs of macrochromosomes (Christidis 1990, Rodionov 1997). The diploid number of partridges emphasizes the conservation of karyotypes in the order of Galliformes (Stock and Bunch 1982, Shibusawa et al. 2002, Shibusawa et al. 2004). This is the case for the Chukar partridge described by Ishishita et al. (2014), as well as domestic fowl *Gallus domesticus* (Pollock and Fechheimer 1976, Ladjali-Mohammedi et al. 1999); Red-legged partridge *Alectoris rufa* (Arruga et al. 1996) and Japanese quail

Coturnix japonica Temminck & Schlegel, 1849 (Stock and Bunch 1982). Interchromosomal conservation of partridges karyotype was also shown in previous studies. In fact, cross species painting using chicken macrochromosomes DNA probes (Zoo-FISH) has shown a perfect homology with, respectively, *A.chukar* and *Alectoris rufa* macrochromosomes (Kasai et al. 2003, Ishishita et al. 2014). Karyotypes of *A. barbara* and *A. chukar* show 8–10 pairs of macrochromosomes that have been measured and 30–28 pairs of microchromosomes whose morphology was difficult to determine despite obtaining high uncondensed chromosomes. Number of microchromosomes of partridges is quite similar to that of Galliformes (Stock and Bunch 1982, Shibusawa et al. 2002, Shibusawa et al. 2004). Microchromosomes were classified arbitrarily by decreasing size, their identification will be possible only by molecular cytogenetics (Zoo-FISH) using chicken microchromosomes specific markers (Fillon et al. 1998, Romanov et al. 2005).

Structural and dynamic R-bands obtained in the present work show similarities in pattern. However, dynamic RBG bands seem well delimited than morphological R-bands even if these latter present a higher number (Fig. 5). Pioneer studies have reported that RHG and RBG-bands are 75 to 85% congruent, and GTG and RHG-bands are 90% complementary, meaning that morphological G and R bands have a reverse pattern (Drouin et al. 1991). Dynamic and morphological R-bands are not totally stackable but correspond quite well and can be compared (Lemieux et al. 1990, Drouin et al. 1991).

Simple and double synchronisation of partridge cell cultures have offered the possibility to obtain important rate of prometaphasic chromosomes presenting high number of bands (Table 1 and 2). Comparatively, size of chicken macrochromosomes was ranged from 7 to 3 μm (Hammar 1966) and the first ten macrochromosomes of chicken haploid karyotype presented 209 G-bands and 182 R-bands (Ladjali et al. 1995). High resolution chromosomes allow detection of intrachromosomal changes that are not always visible at the metaphasic stage (Pollock and Fechheimer 1976, Ladjali et al. 1995, Ladjali-Mohammedi et al. 1999).

A. barbara and *A. chukar* chromosome 4 is acrocentric, while in *G. domesticus* it is telocentric. Furthermore, comparison of bands showed conservation of patterns in *A. barbara* and *G. domesticus* but not in *A. chukar*. This morphological difference could suggest repositioning of the centromere during the speciation event of partridges 6 million years ago (Randi et al. 1992). The difference in banding pattern in *A. chukar* could be explained by a paracentric inversion occurrence (4q11-4q31 in GTG) (Fig. 6). This result is supported by a previous study performed on red-legged partridge *A. rufa* chromosome 4, which is acrocentric (Arruga et al. 1996) and suggested that the morphological difference between *A. rufa* and *G. domesticus* was due to an inversion occurrence (Ramos et al. 1999). Later, Kasai et al. (2003) showed a perfect conservation of chicken BAC clones order on *A. rufa* chromosome 4 and introduced, for the first time in bird class, the term neocentromere (Kasai et al. 2003). Repositioning of the centromere or evolutionary new centromeres (ENC) is the movement of a centromere along the chromosome with the inactivation of the old one but without marker order

alteration during evolution (Rocchi et al. 2012). Interestingly, this phenomenon is not so scarce and has been well described. In fact, several cases of *de novo* centromere formation have been reported in Japanese quail *Coturnix japonica* and Peking duck *Cairina moschata* Linnaeus, 1758 (Galkina et al. 2006, Skinner et al. 2009, Zlotina et al. 2012). Nevertheless, the hypothesis of double inversion occurrence should not be excluded as it was reported in the Japanese quail (Zlotina et al. 2012). High conservation of chromosome 4 in chicken and human over 300 million years has so far been reported (Chowdhary and Raudsepp 2000, Groenen et al. 2000). Conversely, the most common fusion reported in birds is between ancestral chromosome 4 and an ancestral microchromosome (Schmid et al. 2000, Shibusawa et al. 2002; 2004). In fact, in the chicken, whose karyotype is considered as the most similar to the ancestral bird karyotype, chromosome 4 is suggested to have arisen from a fusion of ancestral acrocentric chromosome 4 and ancestral microchromosome 10 (Belterman and De Boer 1984, Schmid et al. 2000, Griffin et al. 2007).

The morphological difference of chromosome 7 and 8 between partridges and the chicken, despite conservation of banding range, could be explained by repositioning of the centromere. However, double pericentric inversion cannot be excluded and only molecular investigations could elucidate such evolutionary events. Several studies show that chromosomes 7 and 8 are quite conserved in Galliformes and hybridize respectively to their homologous when using chicken chromosomal painting (Kasai et al. 2003). Exceptionally, in Guinea fowl *Numida meleagris* Linnaeus, 1758 belonging to Galliformes, Zoo-FISH with chicken DNA specific probes reveals a pericentric inversion in chromosome 7 which corresponds to chicken chromosome 8 (Shibusawa et al. 2002).

The Z chromosome in partridges shows a different terminal region. In fact, *A. barbara* Z gonosome presents an inversion of banding pattern in the terminus of long arm q compared to that of *A.chukar* and *G. domesticus*. Z gonosome of *A.barbara* in RBG corresponds to *G.domesticus* and *A.chukar* Z gonosome in GTG bands (Fig. 7). This result suggests occurrence of Z chromosome terminal inversion in the common ancestor of *A. barbara*, *G. domesticus* and *A.chukar* (Zq21 in GTG) (Fig. 7). The terminal region of Z chromosome in chicken is a characteristic heterochromatic band negatively stained in GTG (Ladjali-Mohammedi et al. 1999). Also, avian Z gonosome is particularly subjected to intrachromosomal rearrangements despite conservation of synteny in most species (Griffin et al. 2007, Nanda et al. 2008). In addition, total sequencing and assembly of chicken Z chromosome has confirmed low gene density (compared to autosomes) associated with high interspersed repeat content (Bellott et al. 2010), which is favorable to rearrangements (Völker et al. 2010).

In both partridges and chicken, the W chromosome is submetacentric and highly heterochromatic as reported in other studies on partridges (Ishishita et al. 2014, Arruga et al. 1996) (Fig. 2b, c). The W chromosome is ranked at the ninth position in *A. barbara* and *A. chukar* karyotypes. In different lineages of Neoaves, the W chromosome is supposed to have arisen by the accumulation of repetitive sequences and their conservation during evolution (Graves 2014, Schartl et al. 2016). A recent sequencing

of chicken W chromosome has shown preservation of ancestral genes enriched for expressed dosage-sensitive regulators (Bellott et al. 2017). Therewith, it is well established that repetitive DNA polymorphism plays an important role in recombination, chromosomal instability and avian sex chromosome differentiation (Völker et al. 2010).

Conclusion

Banding cytogenetics performed on high resolution chromosomes allowed the precise description of *Alectoris barbara* Bonnaterre, 1790 and *Alectoris chukar* karyotypes. Comparative chromosomal mapping highlighted a large conservation with domestic fowl *Gallus domesticus* Linnaeus, 1758. However, rearrangements in acrocentric macrochromosomes 4, 7 and 8 were observed. Except for the Z chromosome, the partridge chromosomes share more similarities with the putative Galliform ancestral karyotype (Belterman and De Boer, 1984) than with chicken. Such cytogenetic studies could be of an important contribution to detect eventual chromosomal rearrangements in hybrids, given that *A. barbara* and *A. chukar* share an overlapping area. Obviously, more detailed molecular cytogenetic studies are necessary to refine the results of the present work. Indeed, we have selected clones from Wageningen chicken BAC (Bacterial Artificial Chromosomes) library (Zoorob et al. 1996, Fillon et al. 1998, Crooijmans et al. 2000) and hybridized them on Barbary partridge and Chukar partridge metaphases. The aim of this fluorescence *In Situ* Hybridization (FISH) is to confirm rearrangement events and individually identify each pair of microchromosomes (work in progress). This study shows that, despite the importance of molecular investigation, banding cytogenetics is still an important step that provides basic knowledge on evolution of avian karyotypes.

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The revision of chromosome III (EF) mapping in *Chironomus plumosus* (Linnaeus, 1758) group (Diptera, Chironomidae)

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Abstract

A revision of mapping of main and alternative banding sequences in chromosome III (EF) has been made for 14 species of the *Chironomus plumosus* group. In total, new versions of mapping are presented for 18 banding sequences of arm E and 18 banding sequences of arm F. A new way of tracing the origins of banding sequences in chromosome III of the *Ch. plumosus* group in comparison with basic banding sequences of the genus *Chironomus* is suggested. The presented data indicate that h'pluE2 in arm E and p'borF2 in arm F are the closest to banding sequences of *Ch. piger* Strenzke, 1959 and thus should be considered the most ancient among banding sequences of chromosome III in the *Ch. plumosus* group. Phylogenetic relationships of banding sequences of chromosome III are discussed.

Keywords

Chironomus plumosus, *Chironomus*, Chironomidae, karyotype, polytene chromosome, banding sequence, chromosome III (EF), chromosome mapping, phylogeny, phylogenetic relationship, karyological analysis

Introduction

The *Chironomus plumosus* group of sibling species presents a great opportunity for the study of the genomic reorganization at the chromosome level during speciation as most of the sibling species have wide geographic ranges with high levels of chromosomal

polymorphism in natural populations (Kiknadze 1987, Kiknadze et al. 1987, 2000, Shobanov 1994b, Michailova and Petrova 1991, Petrova et al. 1996, Golygina et al. 1996, Butler et al. 1999, Golygina 1999, Gunderina et al. 1999, Golygina and Kiknadze 2001, Golygina et al. 2007). The possibility of mapping all the karyotypes in the genus *Chironomus* Meigen 1803 against one standard species allows us to detect all chromosomal rearrangements that distinguish different species and reconstruct their phylogenetic relationship on the basis of karyological analysis (Keyl 1962, Wülker et al. 1989, Shobanov and Zotov 2001, Kiknadze et al. 2004b, 2008, 2016, Gunderina et al. 2005a). However, for conducting such studies it is very important to have high-resolution photographic maps of karyotypes and a unified mapping system of polytene chromosomes. In our earlier works (Golygina and Kiknadze 2008, 2012) we extensively discussed the general difficulties facing a researcher who works with the *Ch. plumosus* group and presented a revision of mapping for chromosome I (AB) and II (CD). However, the situation with mapping of banding sequences in chromosome III (EF) has an additional problem.

Arm E is the most conservative arm in karyotypes of *Ch. plumosus* sibling species, as well as in the genus *Chironomus* (Keyl 1962, Wülker 1989, Kiknadze et al. 2004a, Gunderina et al. 2005b, Golygina et al. 2007). Despite the fact that the established relationships between banding sequences of *Ch. plumosus* sibling species in this arm are quite simple, the situation with mapping is rather complicated due to the presence of two versions of mapping of banding sequence h'pluE1 in comparison with h'pigE1 – the standard banding sequence of *Ch. piger* Strenzke, 1959. The first version of h'pluE1 mapping was presented by Keyl (1962). This version of h'pluE1 mapping was used until 1999 by all authors who worked with the Keyl mapping system and h'pluE1 was considered the closest to h'pigE1 among banding sequences of arm E in *Ch. plumosus* group. At the same time in most works on inversion polymorphism in populations of species from *Ch. plumosus* group the Maximova mapping system (Maximova 1976, Shobanov 1994a), designed for mapping of chromosomes only in this group, was used. Because of this no other banding sequences found in *Ch. plumosus* sibling species were actually directly compared to h'pigE1 but rather mapped using h'pluE1 as a reference. In 1999 we performed an extensive analysis of banding sequences h'pluE1 and h'pluE2 and suggested that the true relationships between h'pigE1, h'pluE1 and h'pluE2 are different from those assumed previously (Golygina 1999, Butler et al. 1999, Golygina and Kiknadze 2001). For example, the comparison of h'pluE2 to h'pigE1 and banding sequences in arm E of other *Chironomus* species indicated that h'pluE2 is actually identical to the banding sequence considered basic for the genus, which is present in karyotypes of several species, such as *Ch. acidophilus* Keyl, 1960, *Ch. luridus* Strenzke, 1959, *Ch. yoshimatsui* Martin & Sublette, 1972 etc. (Wülker 1980). This banding sequence differs from h'pigE1 by single inversion and thus h'pluE2 is closer to h'pigE1 than h'pluE1. Moreover, a revision of h'pluE1 breakpoints was suggested (Golygina 1999, Butler et al. 1999), which means that virtually all banding sequences in arm E of other species from *Ch. plumosus* group required a revision as they are either identical to or originating from the h'pluE1.

However, h'pluE1 is also considered to be identical to banding sequences in arm E of many species from the genus *Chironomus* Meigen outside the *Ch. plumosus* group, such as *Ch. aberratus* Keyl, 1961, *Ch. anthracinus* Zetterstedt, 1860, *Ch. cucini* Webb, 1969, *Ch. jonmartini* Lindeberg, 1979 and several others (Keyl 1962, Wülker 1980, Kiknadze et al. 2004a, 2016). Thus, in papers discussing evolution of banding sequences in the genus *Chironomus*, including other *Ch. plumosus* sibling species, Keyl's original version of the mapping of h'pluE1 has been used (Kiknadze et al. 2004a). This situation makes comparison of data from different papers increasingly difficult so complete revision of banding sequences from the arm E in *Ch. plumosus* group was required.

Banding sequences of arm F also show a high level of conservatism among *Chironomus* species, although it is not as high as in arm E (Keyl 1962, Wülker 1980, Kiknadze et al. 2004a, 2016). The first mapping of arm F of *Ch. plumosus* was published by Keyl (1962) for banding sequence h'pluF1 and this version was used by all authors until now. Yet our analysis indicated that changes to this mapping should be made and thus, as for the arm E, banding sequences of several species in the group required a revision.

In this paper we present the results of revision of mapping for main (present in homozygotes in most populations with high frequencies) and alternative (present in homozygotes in some populations with high frequencies and in heterozygotes in most populations) banding sequences in chromosome III (EF) of 14 sibling species belonging to *Ch. plumosus* group.

Material and methods

Revision of chromosome III (EF) mapping was conducted for 14 *Ch. plumosus* sibling species: *Chironomus agilis* Shobanov & Djomin, 1988, *Ch.* sp. prope *agilis* (working name "*Ch. agilis* 2") (Kiknadze et al. 1991a), *Ch. balatonicus* Devai, Wülker & Scholl, 1983, *Ch. bonus* Shilova & Dzhvarsheishvili, 1974, *Ch. borokensis* Kerkis, Filippova, Shobanov, Gunderina & Kiknadze, 1988, *Ch. entis* Shobanov, 1989, *Ch. muratensis* Ryser, Scholl & Wülker, 1983, *Ch. nudiventris* Ryser, Scholl & Wülker, 1983, *Ch. plumosus* (Linnaeus, 1758), *Ch. sinicus* Kiknadze, Wang, Istomina & Gunderina, 2005, *Chironomus* sp. J (Kiknadze et al. 1991b), *Chironomus* sp. K (Golygina and Ueno 2005), *Ch. suwai* Golygina & Martin, 2003, *Ch. usenicus* Loginova & Belyanina, 1994. High-resolution photomaps of all banding sequences created from chromosome slides prepared from the salivary glands of 4th instar larvae by standard aceto-orcein method (Kiknadze et al. 1991b).

Mapping of arms E and F was done according to Keyl-Devai mapping system (Keyl 1962, Devai et al. 1989) with *Ch. piger* chromosomes as the standard.

Each banding sequence in each chromosomal arm is given a short designation as follows: three-letter abbreviation of the species name (for example, agi – for *Ch. agilis*, bal – for *Ch. balatonicus* etc.) is followed by the name of the arm and the serial number of banding sequence in this arm (according to the order of its discovery), and prefixed by a letter that indicates its geographical distribution – p' for

Palearctic sequences, n' for Nearctic sequences, or h' for Holarctic sequences (e.g. p'balE1, h'pluE2, n'entF4 etc.).

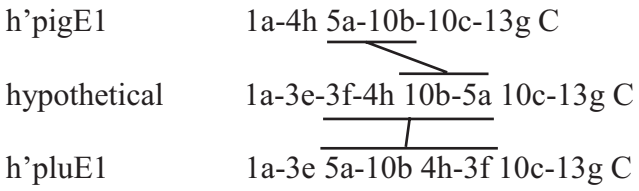
Equipment of the Centre of Microscopical analysis of biological objects SB RAS in the Institute of Cytology and Genetics (Novosibirsk) was used for this work: microscope "Axioskop" 2 Plus, CCD-camera AxioCam HRc, software package AxioVision 4 (Zeiss, Germany).

Results

Arm E

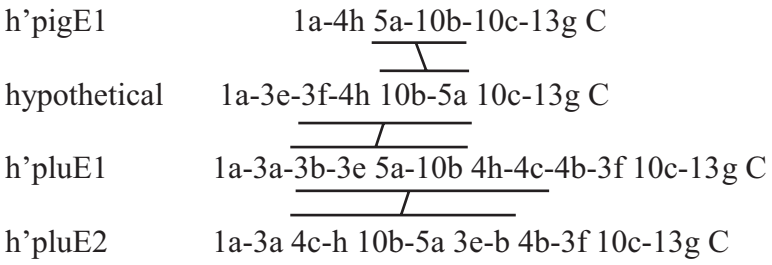
As was mentioned above, two versions of mapping of banding sequence h'pluE1 in comparison with standard banding sequence h'pigE1 are used in different publications.

The Keyl version suggests two inversion steps between h'pluE1 and h'pigE1 as follows (Keyl 1962):

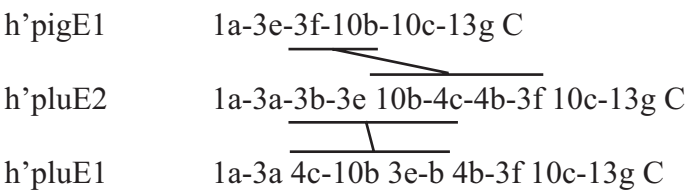


This hypothetical banding sequence has never been found in any studied karyotypes of *Chironomus* species.

As h'pluE2 differs from h'pluE1 by simple inversion but initially was not directly compared to h'pigE1, its previous mapping was a derivative from h'pluE1 mapping (Table 1) and placed it within 3 inversion steps from h'pigE1:



However, our study of these three banding sequences leads us to believe that h'pluE2 is in fact closer to h'pigE1 and h'pluE1 originated from it, which required a slightly different position of inversion breakpoints:



Reasons for the suggested change in mapping of h'pluE1 and h'pluE2 are shown on Figure 1a, b where comparison of regions 3 and 4 of arm E of *Ch. plumosus*, *Ch. agilis* and *Ch. piger* are presented.

As all banding sequences in arm E of other species from *Ch. plumosus* group are either identical to or originated from the h'pluE1 it was required to make a revision of all of them.

Mapping of banding sequences according to the Keyl-Devai system for *Ch. plumosus* sibling species published up to now, is shown for both versions in Table 1. In total 18 banding sequences (14 main and 4 alternative) are considered in this study. A dendrogram of banding sequences constructed on the basis of published mapping using Keyl's version of h'pluE1 is shown in Fig. 2a, where main banding sequences are written in bold and alternative banding sequences in italics. As can be seen, 12 banding sequences were considered to be identical to h'pluE1 with five other banding sequences originating from h'pluE1 by one simple inversion (three of which – h'entE1, h'murE1 and h'nudE2 – were considered identical to each other).

Table 1. Mapping of arm E main and alternative banding sequences in *Ch. plumosus* group before the revision.

Designation of banding sequence	Mapping of banding sequence
h'agiE1*†	=h'pluE1 KV‡: (Shobanov and Djomin 1988, Kerkis et al. 1989a, Kiknadze et al. 1991b, 1996b, 2004a, Shobanov and Zotov 2001, Michailova et al. 2002) §
h'agi2E1*	=h'pluE1 KV: (Kiknadze et al. 1991a, 2004a)
p'balE1*	=h'pluE1 KV: (Devai et al. 1983, Kiknadze and Kerkis 1986, Kiknadze 1987, Kiknadze et al. 1991b)
h'bonE1*	=h'pluE1 KV: (Kerkis et al. 1989, Kiknadze et al. 1991b, 2004a, Shobanov and Zotov 2001)
h'borE1*	=h'pluE1 KV: (Kerkis et al. 1988, 1989a, Kiknadze et al. 1991b, 1996b, 2004a, Shobanov and Zotov 2001)
h'entE1*	KV: 1a-2e 10g-10c 3f-4h 10b-5a 3e-a 11a-13g C (Golygina 1999, Kiknadze et al. 2000, 2004a, Proviz and Bazova 2013) ǀ GV: 1a-2e 10g-10c 3f-4b 3b-e 10b-4c 3a 11a-13g C (Golygina 1999, Kiknadze et al. 2000)
h'entE2	=h'pluE1 KV: (Dyomin and Shobanov 1990, Golygina 1999, Kiknadze et al. 2000, Proviz and Bazova 2013) GV: (Golygina 1999, Kiknadze et al. 2000)
h'murE1*	=h'entE1¶ KV version 1: 1a-3e 4a-h 10b-5a 11d-10c 3f 12a-13g C (Ryser et al. 1983, Kiknadze and Kerkis 1986, Kiknadze 1987, Wülker et al. 1989) KV version 2: 1a-2e 10g-10c 3f-4h 10b-5a 3e-a 11a-13g C (Kiknadze et al. 2004a)

Designation of banding sequence	Mapping of banding sequence
h'nudE1*	=h'pluE1 KV: (Ryser et al. 1983, Kiknadze et al. 1987, 1991b, 2004a)
h'nudE2	=h'murE1[#] KV: 1a-3e 4h-a 10b-5a 11d-10c 3f 12a-13g C (Kiknadze et al. 1987)
h'pluE1*	KV: 1a-3e 5a-10b 4h-3f 10c-13g C (Keyl 1962, Kiknadze 1987, Wülker et al. 1989, Kiknadze et al. 1991b, 1996b, 2004, Butler et al. 1999, Golygina 1999, Golygina and Kiknadze 2001, Michailova et al. 2002) GV: 1a-3a 4c-10b 3e-b 4b-3f 10c-13g C (Butler et al. 1999, Golygina 1999, Golygina and Kiknadze 2001)
h'pluE2	KV: 1a-3a 4d-h 10b-3b 4c-3f 10c-13g C (Butler et al. 1999, Golygina 1999, Golygina and Kiknadze 2001) GV: 1a-3e 10b-3f 10c-13g C (Butler et al. 1999, Golygina 1999, Golygina and Kiknadze 2001)
h'sinE1*	=h'pluE1 KV: (Kiknadze et al. 2005) GV: (Kiknadze et al. 2005)
h'spJE1*	=h'pluE1 KV: (Kiknadze et al. 2004a)
h'spKE1*	=h'pluE1 GV: (Golygina and Ueno 2008)
h'suwE1*	=h'pluE1 KV: (Golygina et al. 2003, Kiknadze et al. 2004a) GV: (Golygina et al. 2003)
p'useE1*	KV: 1a-3e 5a 3f-4h 10b-5b 10c-13g C (Loginova and Belyanina 1994)
h'useE3	=h'pluE1 KV: (Loginova and Belyanina 1994)

[†] – main banding sequences are marked by *, [‡] – KV – variant of mapping done according to Keyl's version of mapping of banding sequence h'pluE1 (Keyl 1962), GV – variant of mapping done according to Golygina's version of mapping of banding sequence h'pluE1 (Golygina 1999) [§]– papers with given version of the mapping are shown in parenthesis, [|] – shown only the last version of mapping of this banding sequence as there were several other papers published earlier – Kerkis et al. 1989, Dyomin and Shobanov 1990, Kiknadze et al. 1991c – with different mapping variants, [¶] – the fact that h'murE1 is identical to h'entE1 was not known until 2004 so these banding sequences were mapped separately in earlier works and their mapping differed from one another, [#]– while h'nudE2 is identical to h'murE1 and h'entE1 the mapping of h'nudE2 published by Kiknadze and coauthors in 1987 differs from the mapping published later for h'murE1 (Kiknadze et al. 2004).

According to our analysis, the true phylogenetic relationships are shown on Figure 2b. Eleven banding sequences are indeed identical to h'pluE1 so the changes in their mapping had to be made in accordance with h'pluE1 mapping (Table 2, Fig. 3a). Four banding sequences – h'entE1, h'murE1, h'nudE2 and p'useE1 – required minor corrections of inversion breakpoints, which differentiate them from h'pluE1. One banding sequence – p'balE1 – required a major revision.

The revision of arm E mapping of *Ch. balatonicus*

It was believed previously that the main banding sequence of *Ch. balatonicus* is identical to h'pluE1. However, our analysis had shown that this species differs from all other

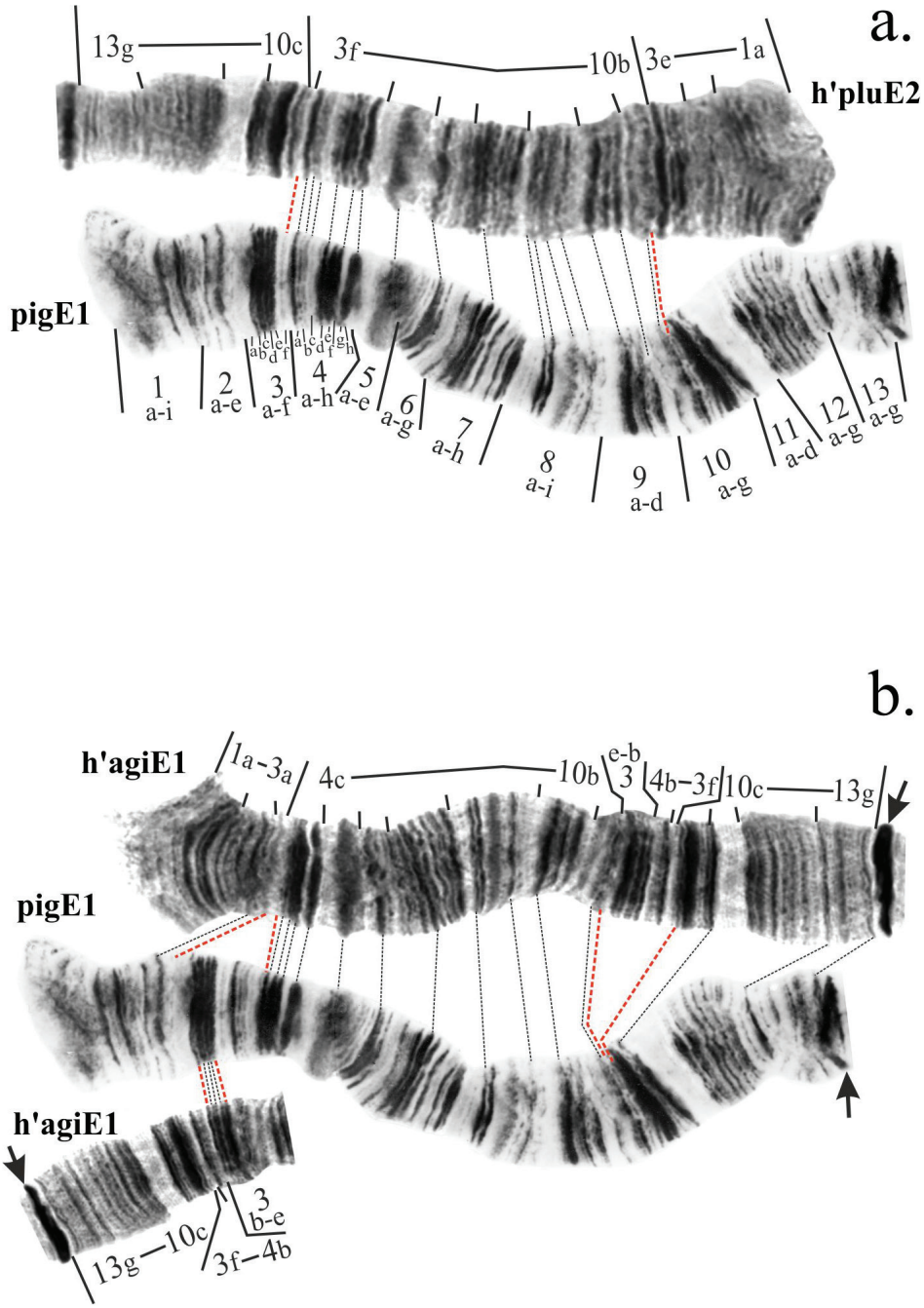


Figure 1. Mapping comparison of banding sequences h'pigE1, h'pluE2 and h'agiA1 (identical to h'pluE1). a – comparison of h'pigE1 and h'pluE2, b – comparison of h'pigE1 and h'agiE1=h'pluE1. Centromeric bands are designated by arrows. Individual band in regions 3 and 4 of h'pigE1 are marked by small letters. Dotted lines connect identical discs in compared banding sequences. Red dotted lines indicate borders of regions, where banding patterns of compared banding sequences are identical.

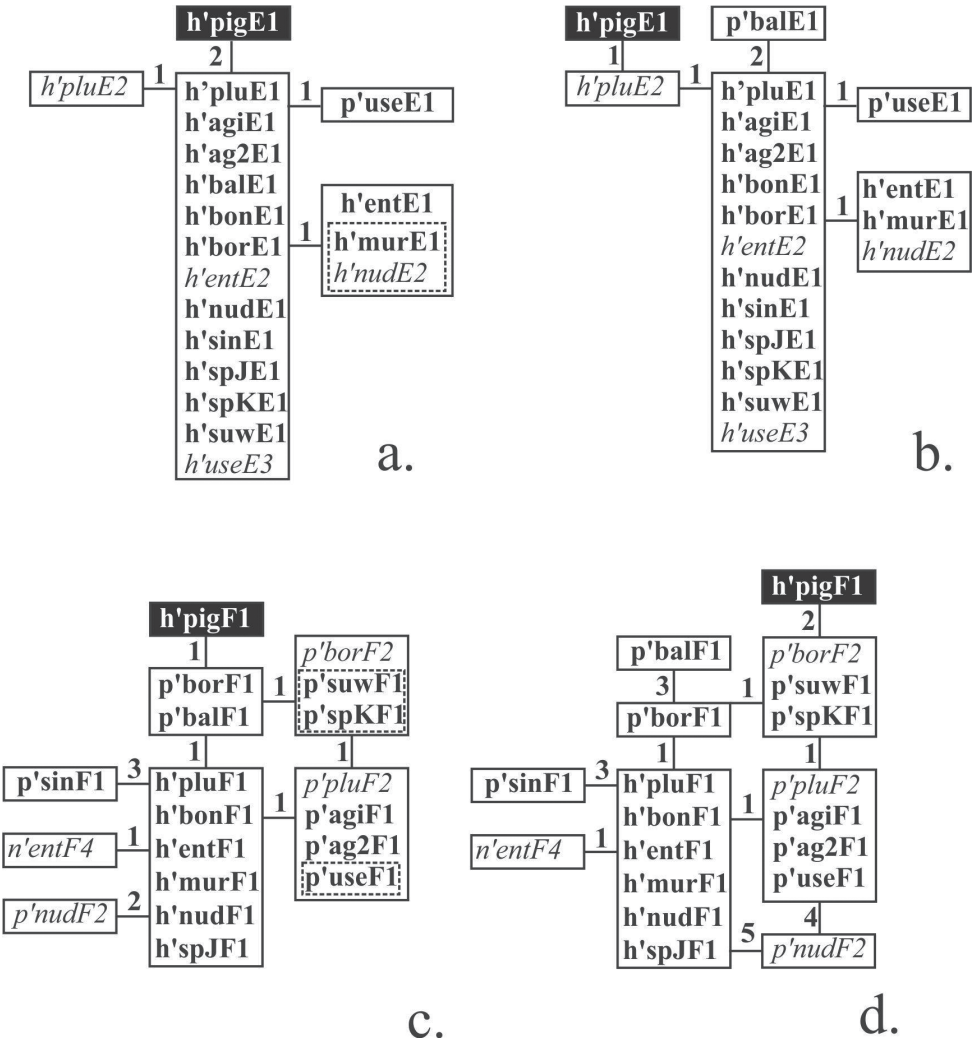


Figure 2. Phylogenetic relationship of main and alternative banding sequences in arms E and F before (**a, c**) and after (**b, d**) the revision. Main banding sequences are written in bold, alternative – in italic. Identical banding sequences enclosed in boxes, figures near the lines that connect banding sequences indicate numbers of inversion steps between them. Dotted lines enclosing some banding sequences inside a block indicate that mapping presented for these banding sequences differ from mappings of other banding sequences in the block, yet all banding sequences in the block were considered identical.

species of *Ch. plumosus* group by the presence of complex pericentric inversion in chromosome EF (Figs 3b, 4). As a result of this inversion bands 13fg transferred from arm E into arm F of *Ch. balatonicus* so while on the most length of the arm banding pattern of p'balE1 is indeed identical with h'pluE1, *Ch. balatonicus* arm E is shorter than arm E of the rest of *Ch. plumosus* group species by 2 bands (Table 2). The more detailed analysis of this inversion is presented in revision of arm F below (Fig. 4).

Table 2. Mapping of arm E main and alternative banding sequences in *Ch. plumosus* group after the revision.

Designation of banding sequence	Mapping of banding sequence
h'agiE1*†	=h'pluE1
h'agi2E1*	=h'pluE1
p'balE1*	KV: 1a-3e 5a-10b 4h-3f 10c-**13e** C ‡ GV: 1a-3a 4c-10b 3e-b 4b-3f 10c-**13e** C§
h'bonE1*	=h'pluE1
h'borE1*	=h'pluE1
h'entE1*	KV: KV: 1a-2e **11a**-10c 3f-4h 10b-5a 3e-a **11b**-13g C GV: 1a-**2e 11a**-10c 3f-4b 3b-e 10b-4c **3a 11b**-13g C
h'entE2	=h'pluE1
h'murE1*	=h'entE1
h'nudE1*	=h'pluE1
h'nudE2	=h'entE1
h'pluE1*	KV: 1a-3e 5a-10b 4h-3f 10c-13g C GV: 1a-3a 4c-10b 3e-b 4b-3f 10c-13g C
h'pluE2	KV: 1a-3a 4d-h 10b-3b 4c-3f 10c-13g C GV: 1a-3e 10b-3f 10c-13g C
h'sinE1*	=h'pluE1
h'spJE1*	=h'pluE1
h'spKE1*	=h'pluE1
h'suwE1*	=h'pluE1
p'useE1*	KV: 1a-3e 5ab 3f-4h 10b-5c 10c-13g C GV: 1a-3a 4c-**5b** 3f-4b 3b-e 10b-**5c** 10c-13g C
h'useE3	=h'pluE1

† – main banding sequences are marked by *, ‡ – KV – variant of mapping done according to Keyl's version of mapping of banding sequence h'pluE1 (Keyl 1962), GV – variant of mapping done according to Golygina's version of mapping of banding sequence h'pluE1 (Golygina 1999), § – parts of the sequences highlighted in bold indicate regions which mapping had been changed as a result of the revision.

The revision of arm E mapping of *Ch. entis*, *Ch. muratensis*, and *Ch. nudiventris*

Banding sequences h'entE1, h'murE1, and h'nudE2 are identical and differ from h'pluE1 by one simple paracentric inversion. We believe that minor revision should be made for its breakpoints (Fig. 3c, Table 2).

The revision of arm E mapping of *Ch. usenicus*

Banding sequence p'useE1 differs from h'pluE1 by simple inversion. Loginova and coauthors (Loginova et al. 1994) placed the left inversion breakpoint between bands 5a and 5b but closer analysis shows that the real breakpoint is situated between bands 5b and 5c as the latter – the wide fuzzy dark band – closely adjoins region 10c-g (Fig. 3e, Table 2).

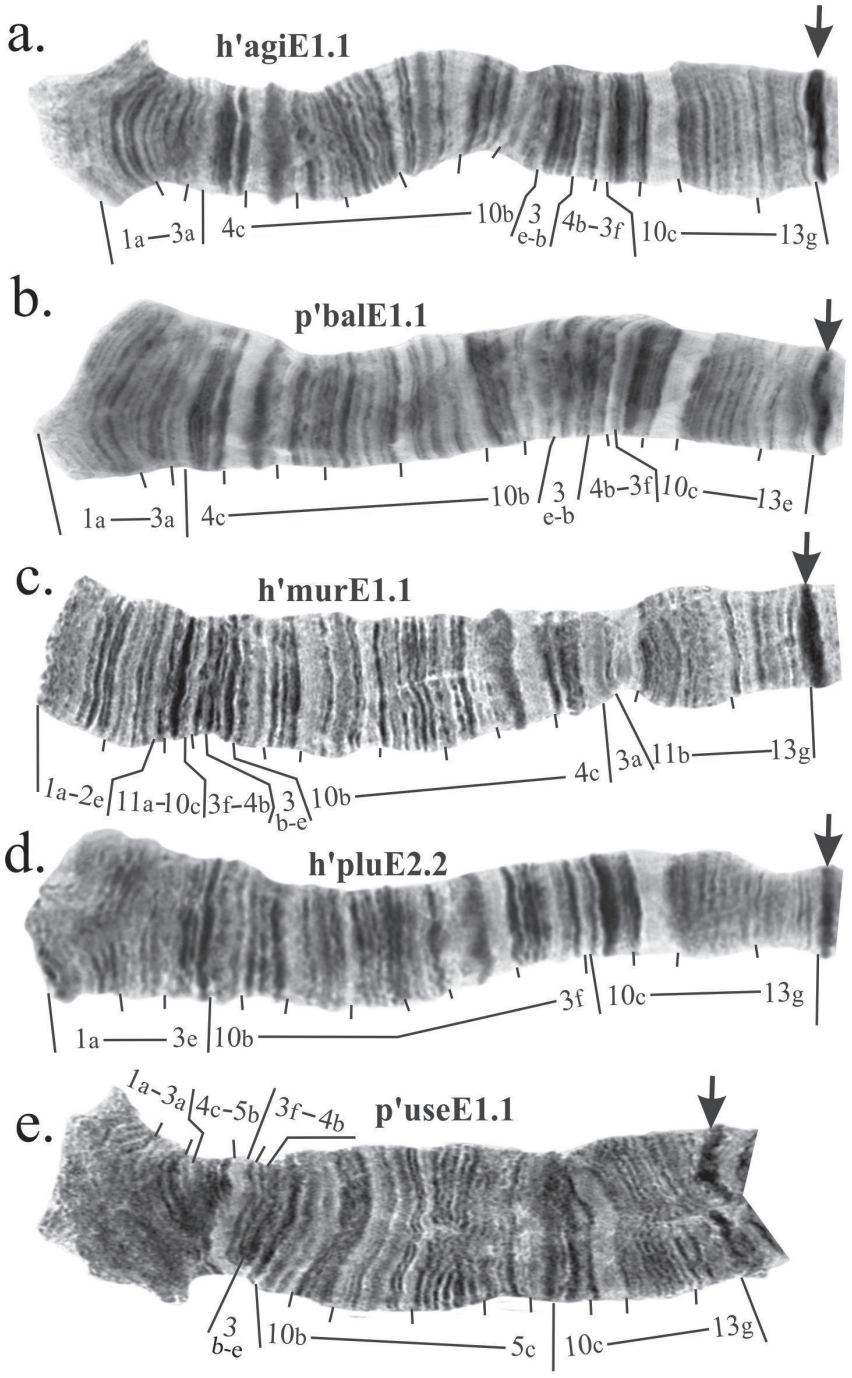


Figure 3. Mapping of banding sequences of *Ch. plumosus* sibling species in arm E after the revision. **a** h'agiE1.1 (identical to h'pluE1, h'ag2E1, h'bonE1, h'borE1, h'entE2, h'nudE1, h'sinE1, h'spJE1, h'spKE1, h'suwE1, h'useE3) **b** p'balE1.1 **c** h'murE1.1 (identical to h'entE1, h'nudE2) **d** h'pluE2.2 **e** p'useE1.1. Centromeric bands are designated by arrows.

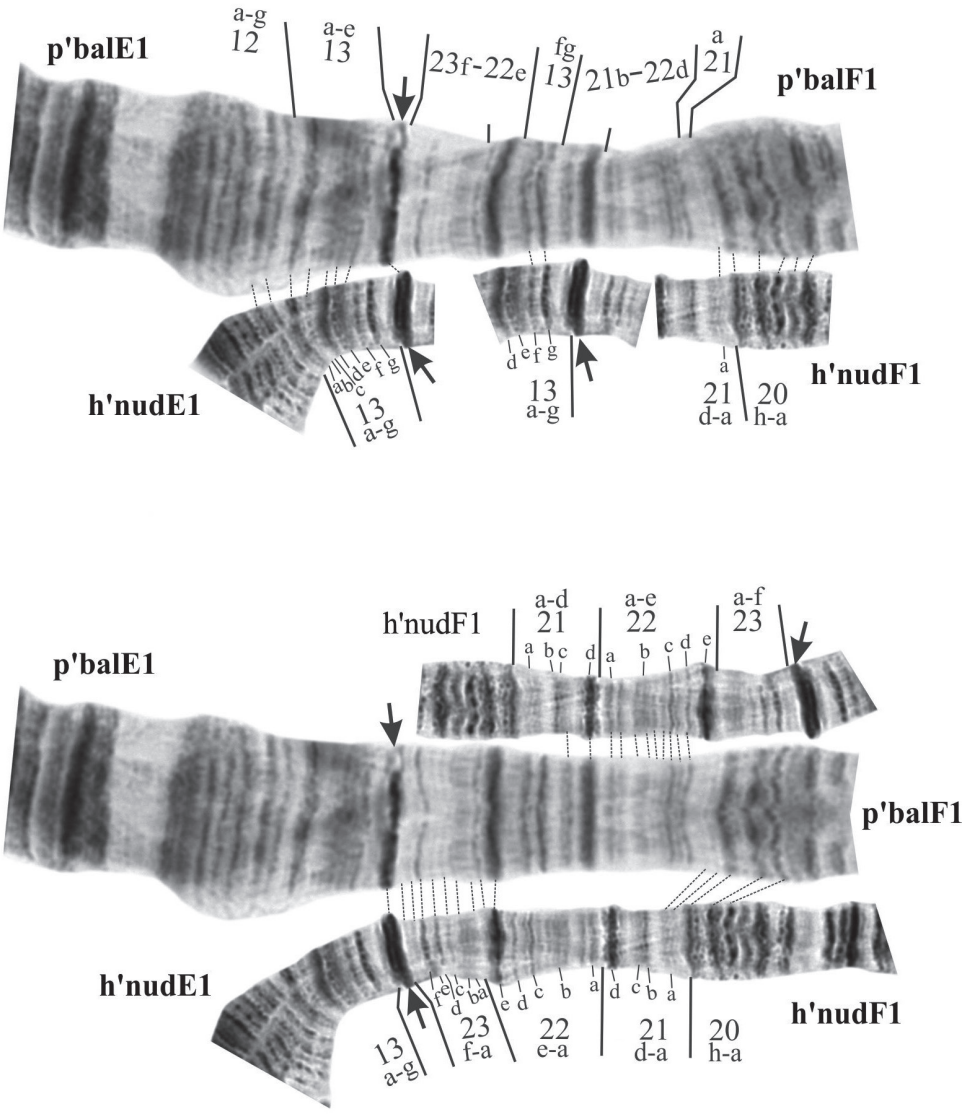


Figure 4. Mapping of pericentric inversion on chromosome EF of *Ch. balatonicus*. Individual bands in regions are marked by small letters.

Arm F

Banding patterns in arm F of *Chironomus* species are not as conservative as in arm E, but the arm is still considered to have a low level of polymorphism with many species sharing the same banding sequences and a lot of species that differ from each other by single inversion steps (Keyl 1962, Wülker 1989, Kiknadze et al. 2004a, Gunderina et al. 2005b, Golygina et al. 2007). *Ch. plumosus* was the first species in the *Ch. plumosus* group which arm F's banding sequence was mapped so it became the template to map

all other species in the group. Mapping of h'pluF1 was first presented by Keyl (1962) and according to it h'pluF1 differs from h'pigF1 by two non-overlapping paracentric inversions – one in the region 1e-6e and the other in the region 11a-17d. According to this version of mapping region 10 remain unbroken. When karyotype of *Ch. borokensis* was described (Kerkis at al. 1988) the mapping of p'borF1 presented in the paper placed it as an intermediate banding sequence between h'pigF1 and h'pluF1, which differs from h'pigF1 by single inversion in the region 11a-17d (Table 3). Later when banding sequences of other species in the group were described, their mapping was based on these two assumptions so in all of them region 10 was mapped as whole. Thus basically all main banding sequences of *Ch. plumosus* group species differ from each other by combination of presence or absence of inversions in regions 1e-6e and 11a-17d (Table 3). The relationships of banding sequences in arm F as they were presumed to be up until now are shown on Figure 2c.

However, our analysis had clearly shown that the inversion that was previously defined as 11a-17d actually has different breakpoints, which in turn required re-evaluating relationships both between banding sequences inside the *Ch. plumosus* group and with the standard h'pigF1.

Photos in Figure 5 show the comparison of regions where breakpoints of this inversion occur between banding sequences p'agiF1 and p'borF1 as they had the best banding structure and their mapping could resolve mapping for the rest of banding sequences in the arm. As can be clearly seen, on the left the real inversion breakpoint occurs after band 10b, and on the right – before band 19a. Now it was necessary to determine in which banding sequences region 10 remains whole and in which it breaks and what happens with region 18. To answer these questions it was necessary to compare these banding sequences to h'pigF1. The comparison of p'agiF1 and h'pigF1 is shown on Figure 6. After analysis of arm F from many preparations of *Ch. piger* we concluded that in *Ch. piger* groups of bands 10ab and 10cd have about the same intensity, they are both rather dark and group 10cd is slightly wider than group 10ab. At the same time group 18ed is less dark and defined and often these two bands are so close that it is hard to distinguish them. As can be seen of Figure 6 it is viable to conclude that region 10 stays whole in banding sequence p'agiF1 while region 18 breaks so that bands 18de stay in place before region 19 while 18a-c falls inside the inversion that differentiates it from h'pigF1 and so are transferred to the distal part of the arm near region 10. At the same time region 11a-17d is affected by second inversion in p'agiF1 which results in final banding sequence 1a-d 6e-1e 7a-10d 18c-a 11a-17d 18d-23f (Figs 6, 7a, Table 4). This means that in p'borF1 and, thus, in h'pluF1 and all its homologous and derivatives banding sequences region 10 breaks (Figs 5, 7b-e, g, h, Table 4). It also means that the banding sequence that is closest to h'pigF1 is p'borF2 (and its homologous banding sequences) as it is identical to p'agiF1 in the proximal part of the arm but also have intact banding pattern in region 1a-10d (Fig. 7f, Table 4).

Aside from the general revision that affects all banding sequences in arm F of all species in the group, we have found that arm F of *Ch. balatonicus* required a major revision due to the presence of the pericentric inversion and several banding sequences of different species were in need of breakpoint correction.

Table 3. Mapping of arm F main and alternative banding sequences in *Ch. plumosus* group before the revision.

Designation of banding sequence	Mapping of banding sequence
p'agiF1*†	=p'pluF2 While all authors considered it to be identical to p'pluF2, the presented mapping of the banding sequence was different in different papers: 1a-d 6e-1e 7a-10d 18c-a 11a-17d 18d-23f C (Shobanov and Djomin 1988, Shobanov and Zotov 2001, Kiknadze et al. 2004a) ‡ 1a-d 6e-1e 7a-10d 18e-a 11a-17d 19a-23f C (Kerkis et al. 1989a, b, Kiknadze et al. 1991b?, 1996b, Michailova et al. 2002)
p'agi2F1	=p'agiF1 While authors stated that it is identical to p'agiF1, the presented mapping of the banding sequence was different in different papers: 1a-d 6e-1e 7a-10d 17d-a 11a-16g 18a-23f C (Kiknadze et al. 1991a) 1a-d 6e-1e 7a-10d 18c-a 11a-17d 18d-23f C (Kiknadze et al. 2004a)
p'balF1	=h'borF1 (Devai et al. 1983, Kiknadze and Kerkis 1986, Kiknadze 1987, Kiknadze et al. 1991b, Michailova and Krastanov 2000, Michailova et al. 2002)
p'bonF1	=h'pluF1 (Kerkis et al. 1989b, Kiknadze et al. 1991b, 2004, Shobanov and Zotov 2001)
p'borF1	1a-10d 17d-11a 18a-23f C (Kerkis et al. 1988, 1989a, Kiknadze et al. 1991b?, 1996a, 2004a)
p'borF2	no published mapping according to Keyl-Devai system
h'entF1	=h'pluF1 (Kerkis et al. 1989a, b, Dyomin and Shobanov 1990, Golygina 1999, Kiknadze et al. 2000, 2004a, Proviz and Bazova 2013, Shobanov and Zotov 2001)
n'entF4	1a-d 6e-1e 19d-18a 11a-17d 10d-7a 20a-23f C (Kiknadze et al. 2000)
h'murF1	=h'pluF1 (Ryser et al. 1983, Kiknadze and Kerkis 1986, Kiknadze 1987, Kiknadze et al. 2004a)
h'nudF1	=h'pluF1 (Ryser et al. 1983, Kiknadze et al. 1987, 1991b, 2004a)
p'nudF2	1a-d 14a-15i 19d-18a 11a-13d 6e-1e 7a-10d 17d-16a 20a-23f C (Kiknadze et al. 1987)
h'pluF1	1a-d 6e-1e 7a-10d 17d-11a 18a-23f C (Keyl 1962, Kiknadze 1987, Wülker et al. 1989, Kiknadze et al. 1991b, 1996b, Butler et al. 1999, Golygina 1999, Michailova and Krastanov 2000, Golygina and Kiknadze 2001, Michailova et al. 2002, Kiknadze et al. 2004a, Proviz and Bazova 2013)
p'pluF2	1a-d 6e-1e 7a-10d 18c-a 11a-17d 18d-23f C (Butler et al. 1999) 1a-d 6e-1e 7a-10b 18e-a 11a-17d 10dc 19a-23f C (Golygina 1999, Golygina and Kiknadze 2001)
p'sinF1	1a-d 6e-5d 10d-7a 5c-1e 14f-17d 14e-11a 18a-23f C (Kiknadze et al. 2005)
h'spJF1	=h'pluF1 (Kiknadze et al. 1991b)
p'spKF1	=p'suwF1 (Golygina and Ueno 2008)
p'suwF1	=p'borF2 1a-10b 18e-a 11a-17d 10dc 19a-23f C (Golygina et al. 2003, Kiknadze et al. 2004a)
p'useF1	1a-d 6e-1e 7a-10d 18e-a 11a-17d 19a-23f C (Loginova and Beljanina 1994)

† – main banding sequences are marked by *, ‡ – papers with given version of the mapping are shown in parenthesis.

The revision of arm F mapping of *Ch. balatonicus*

As was mentioned above, thorough analysis of the centromeric region of chromosome EF of *Ch. balatonicus* had shown that this arm had undergone complex pericentric inversion that differentiates it from the rest of *Ch. plumosus* group and thus p'balF1 is

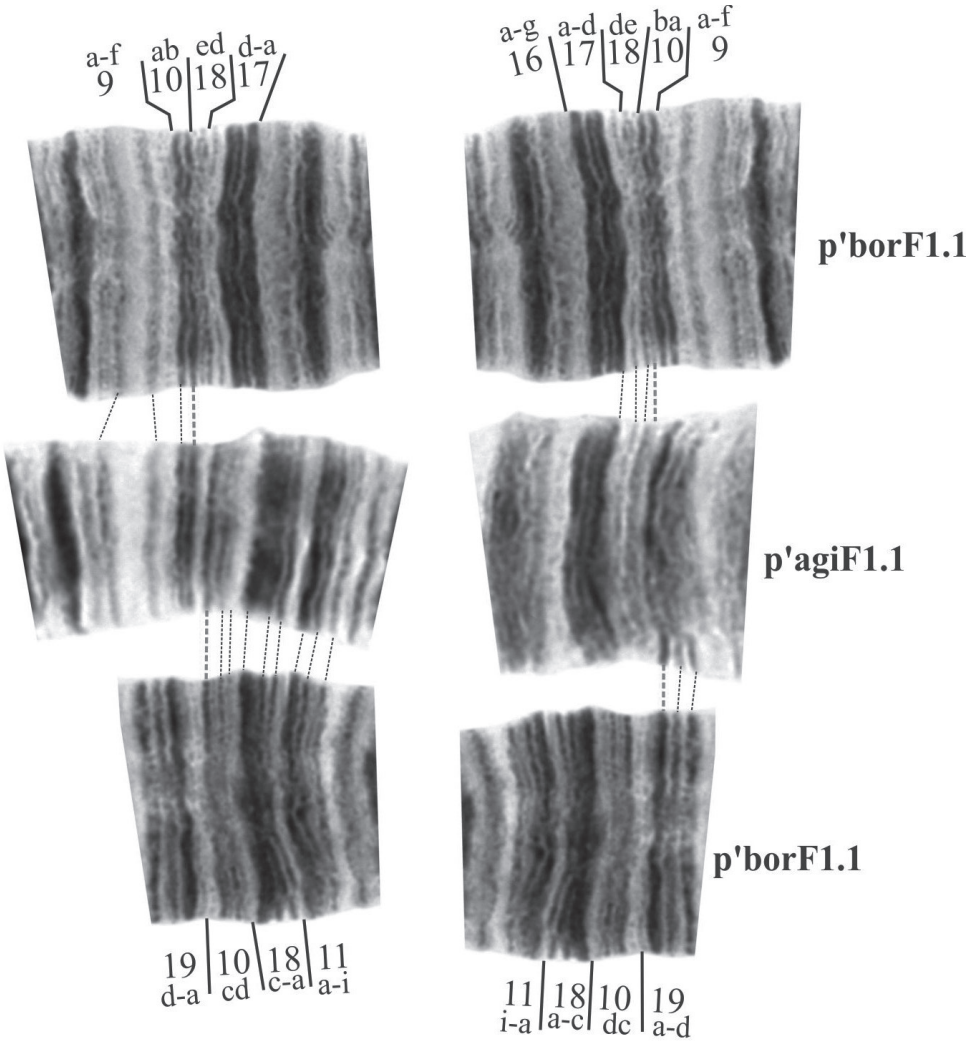


Figure 5. Comparison of regions of inversion breakpoint between banding sequences p'borF1 and p'agiF1.

not identical to p'borF1 as was supposed previously. Mapping of this inversion proved to be very difficult due to the complexity of the rearrangement along with the fact that bands in the pericentromeric region are often weak, not well defined and can be very similar in appearance. The comparison of inversion region between p'balE1, p'balF1, h'nudE1 and h'nudF1 is shown on Figure 4 (photos of h'nudE1 and h'nudF1 were used instead of h'borE1 and p'borF1 as *Ch. nudiventris* has much better structure in the pericentromeric region where the inversion of interest is located). We believe that p'balF1 is a result of three consecutive inversions from p'borF1 so that arm F of *Ch. balatonicus* became longer by addition of bands 13gf from arm E and regions 21 and 22 had rearranged in a complex pattern (Figs 4, 7b, Table 4).

p'borF1 (GV) 1a-10b 18ed 17d-11a 18a-c 10dc 19a-21a-21b-23f C [13gf-13e-a]

hypothetical 1 1a-10b 18ed 17d-11a 18a-c 10dc 19a-21a [13fg] C 23f-21b [13e-a]

hypothetical 2 1a-10b 18ed 17d-11a 18a-c 10dc 19a-21a [13fg] 21b-22d-22e-23f C [13e-a]

p'balF1 (GV) 1a-10b 18ed 17d-11a 18a-c 10dc 19a-21a 22d-21b [13gf] 22e-23f C [13e-a]

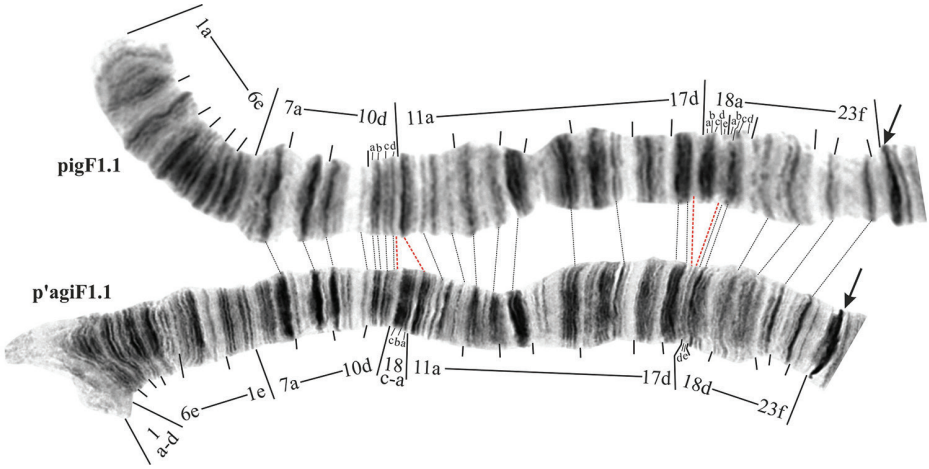


Figure 6. Mapping comparison of banding sequences h'pigF1 and p'agiF1. Centromeric bands are designated by arrows. Individual band in the regions 10, 18 and 19 of h'pigF1 are marked by small letters. Dotted lines connect identical discs in compared banding sequences. Red dotted lines indicate borders of regions, where banding patterns of compared banding sequences are identical.

The revision of arm F mapping of *Ch. entis* and *Ch. nudiventris*

Main banding sequences h'entF1 and h'nudF1 are identical to h'pluF1 (Fig. 2d) so their mapping was changed in accordance with new mapping of h'pluF1. Banding sequence n'entF4 differs from h'entF1 by one simple inversion and we believe that correction should be made for the inversion breakpoints as the inversion divides region 7 (Figs 7d, Table 4). Banding sequence p'nudF2 was previously mapped only in the Maximova system with two inversion steps suggested between it and h'nudF1. However, our analysis has shown that it is much more complex and could be derived from h'nudF1 only by five inversion steps (Figs 2d, 7g, Table 4). What we find very unusual is that so far we found no intermediary banding sequences in the banding sequence pool of *Ch. nudiventris* yet this inversion differs by only four inversion steps from p'pluF2, i.e. it is closer to banding sequence of a sibling species than to main banding sequence of its own species. It is possible that banding sequences identical to p'pluF2 either exists in *Ch. nudiventris* banding sequence pool but was not found yet due to insufficient number of populations studied or existed previously but was lost during speciation. It may also be an evidence that this banding sequence is very old and originated before species speciation of *Ch. nudiventris*.

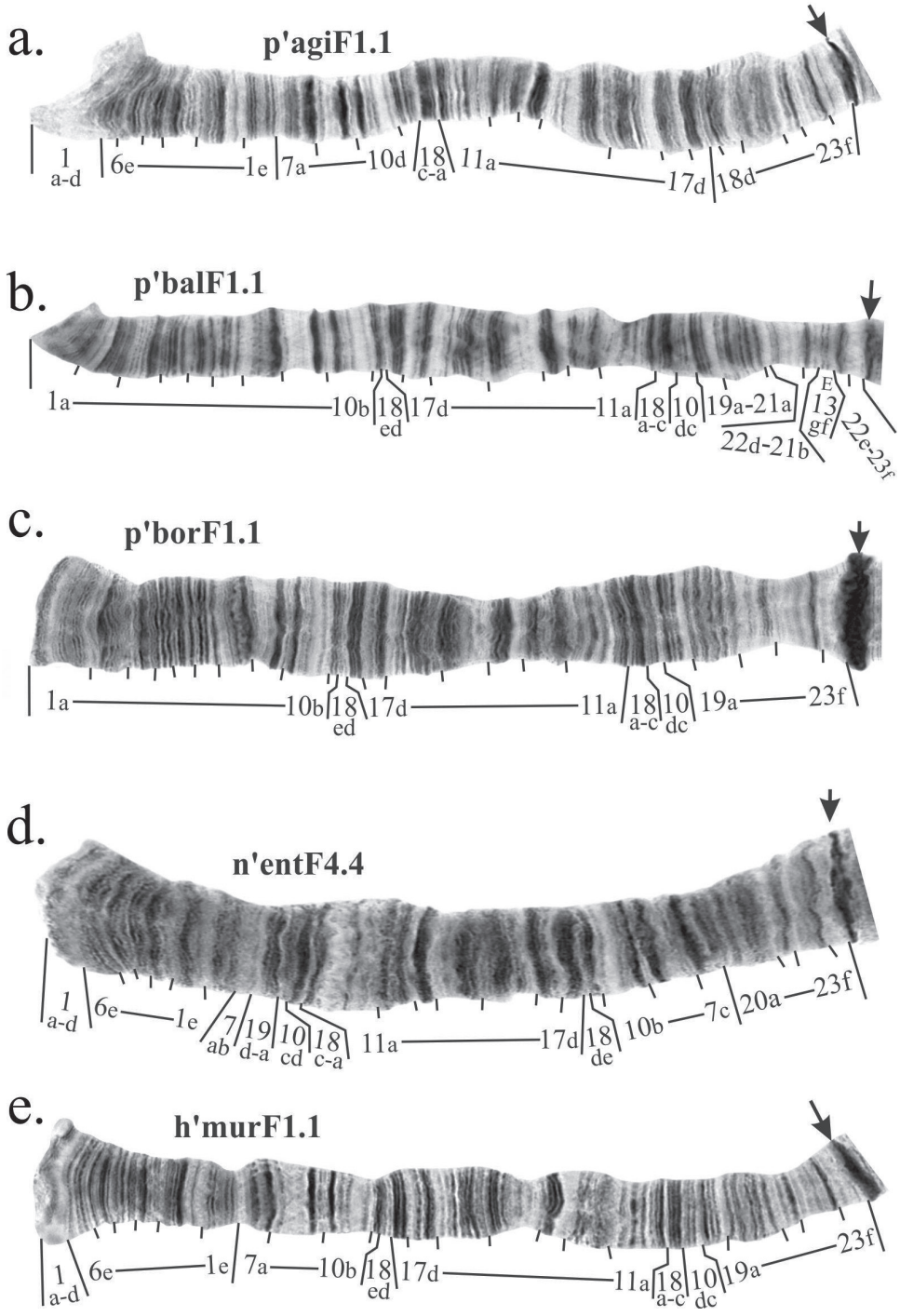


Figure 7. Mapping of banding sequences of *Ch. plumosus* sibling species in arm F after the revision. **a** p'agiF1.1 (identical to p'pluF2, p'ag2F1, p'useF1) **b** p'balF1.1 **c** p'borF1.1 **d** n'entF4.4 **e** h'murF1.1 (identical to h'pluF1, h'bonF1, h'entF1, h'nudF1, h'spJF1).

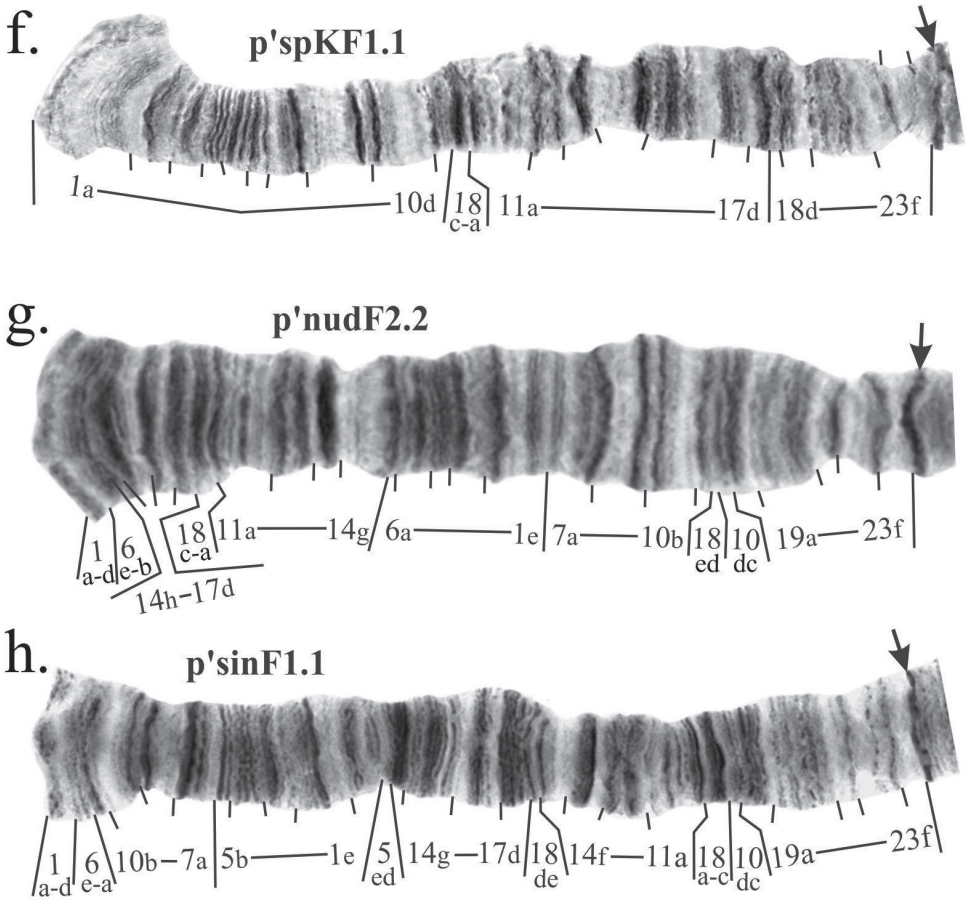


Figure 7. Continued: **f** p'spKF1.1 (identical to p'borF2, p'suwF1) **g** p'nudF2.2 **h** p'sinF1.1. Centromeric bands are designated by arrows.

The revision of arm F mapping of *Ch. sinicus*

Main banding sequence p'sinF1 differ from h'pluF1 by 3 inversion steps and aside from corrections that follow from changes made to h'pluF1 require a minor revision of inversion breakpoints (Figs 2d, 7h, Table 4).

Discussion

As was observed in many previous studies, arm E remains the least polymorphic arm of the karyotype in the group. Out of 14 species 10 have identical main banding sequences with another 2 having the same banding sequence as alternative. Only one species – *Ch. balatonicus* – doesn't share any banding sequences with other species due

Table 4. Mapping of arm F main and alternative banding sequences in *Ch. plumosus* group after the revision.

Designation of banding sequence	Mapping of banding sequence
p'agiF1	=p'pluF2
p'agi2F1	=p'pluF2
p'balF1	1a-10b 18ed 17d-11a 18a-c 10dc 19a-**21a 22d-21b [13gf] 22e-23f** C
p'bonF1	=h'pluF1
p'borF1	1a-**10b 18ed** 17d-11a **18a-c 10dc 19a**-23f C
p'borF2	**1a-10d 18c-a 11a-17d 18d-23f C**
h'entF1	=h'pluF1
n'entF4	1a-d 6e-1e **7ab 19d-a 10cd 18c-a** 11a-17d **18de 10b-7c** 20a-23f C
h'murF1	=h'pluF1
h'nudF1	=h'pluF1
p'nudF2	1a-d 6e-b 14h-17d 18c-a 11a-14g 6a-1e 7a-10b 18ed 10dc 19a-23f C
h'pluF1	1a-d 6e-1e 7a-**10b 18ed** 17d-11a **18a-c 10dc 19a**-23f C
p'pluF2	1a-d 6e-1e 7a-**10d 18c-a** 11a-**17d 18d**-23f C
p'sinF1	1a-d **6e-a 10b**-7a **5b**-1e **5cd 14g**-17d **18de 14f**-11a **18a-c 10dc 19a**-23f C
h'spJF1	=h'entD1
p'spKF1	=p'borF2
p'suwF1	=p'borF2
p'useF1	=p'pluF2

† – parts of the sequences highlighted in bold indicate regions which mapping had been changed as a result of the revision.

to the presence of pericentric inversion in the chromosome EF. This species is also the only one that differs from others by more than one inversion step in arm E.

The revision in arm F has also mostly provided minor changes in the mapping of inversion breakpoints without affecting phylogenetic relationship of banding sequences inside the group. Aside of the placement of h'balF1 due to the presence of pericentric inversion the only significant change has come from the correction of inversion breakpoint of p'nudF2 which made it related to both h'nudF1 and p'pluF2. In general banding sequences in arm F show a moderate level of divergence comparable with what we observe in arm A, with three species that have species specific main banding sequences and only two species – *Ch. balatonicus* and *Ch. sinicus* – that don't share any banding sequence with other species.

Considering the level of banding sequences divergence in both arms it can be stated that chromosome EF is the least divergent among the three big chromosomes of *Chironomus*.

At the same time the revision has shown the phylogenetic relationship of banding sequences of *Ch. plumosus* group sibling species and the rest of the genus are different from what was assumed previously as the closest to h'pigE1 and h'pigF1 are h'pluE2 and p'borF2. As both arm E and F are low polymorphic in the genus, many species share the same banding sequences. As was mentioned previously, h'pluE1 and p'borF1 were believed to be identical to banding sequences of many other species as their banding pat-

terns are considered basic for the genus *Chironomus* (Keyl 1962, Wülker 1980, Kiknadze et al. 2004a, 2016). Because of the revision presented for these banding sequences in this paper we are now facing two possibilities. First, it is possible that they are indeed identical to banding sequences of other species in the genus, in which case a revision of banding sequences in arms E and F required for the most species of the genus. Second, there is a possibility that these banding sequences are actually different from the basic banding sequences, in which case only mapping of banding sequences in the *Ch. plumosus* group is affected. To answer the question of which assumption is correct it is necessary to compare these banding sequences to basic banding sequences found in other species of the genus *Chironomus*.

Acknowledgements

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RESEARCH ARTICLE

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Comparative cytogenetics and derived phylogenic relationship among *Sitophilus* grain weevils (Coleoptera, Curculionidae, Dryophthorinae)

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Abstract

Cytogenetic characteristics and genome size are powerful tools for species characterization and identification of cryptic species, providing critical insights into phylogenetic and evolutionary relationships. *Sitophilus* Linnaeus, 1758 grain weevils can benefit from such tools as key pest species of stored products and also as sources of archeological information on human history and past urban environments. Moreover, the phylogenetic relationship among these weevil species remains controversial and is largely based on single DNA fragment analyses. Therefore, cytogenetic analyses and genome size determinations were performed for four *Sitophilus* grain weevil species, namely the granary weevil *Sitophilus granarius* (Linnaeus, 1758), the tamarind weevil *S. linearis* (Herbst, 1797), the rice weevil *S. oryzae* (Linnaeus, 1763), and the maize weevil *S. zeamais* Motschulsky, 1855. Both maize and rice weevils exhibited the same chromosome number (2n=22; 10 A + Xyp). In contrast, the granary and tamarind weevils exhibited higher chromosome number (2n=24; 11 A + Xyp and 11 A + neo-XY, respectively). The nuclear DNA content of these species was not proportionally related to either chromosome number or heterochromatin amount. Maize and rice weevils exhibited similar and larger genome sizes (0.730±0.003 pg and 0.786±0.003 pg, respectively),

followed by the granary weevil (0.553±0.003 pg), and the tamarind weevil (0.440±0.001 pg). Parsimony phylogenetic analysis of the insect karyotypes indicate that *S. zeamais* and *S. oryzae* were phylogenetically closer than *S. granarius* and *S. linearis*, which were more closely related and share a more recent ancestral relationship.

Keywords
karyotypes, C-banding, fluorochromes, heterochromatin, stored products, evolutionary history

Introduction

Closely related species usually exhibit similar karyotypes concerning chromosome number and morphology. However, other characteristics such as the amount, size and distribution of heterochromatic blocks and/or nucleolus organizing regions (NORs) can vary considerably, even among cryptic species, which makes cytogenetic analyses powerful tools for species characterization and identification (Holecová et al. 2002, Rozek et al. 2004, Lachowska et al. 2004, 2006, 2008, 2009, Angus et al. 2011). As a consequence, these analyses can lead to important insights into phylogenetic relationships and evolutionary history, contributing to the understanding of species context and relevance. Although seldom used, such knowledge is particularly appealing for economically important insect pest species, and/or species that shed light on human history/past urban environments, and grain trade and trade routes, as exemplified by stored product insect pest species (Levinson and Levinson 1994, Kenway and Carrott 2006, Smith and Kenward 2011, Corrêa et al. 2017).

Interspecific divergence is also associated with chromosome variation (Goodisman et al. 2008), encouraging the use of cytogenetic analysis for inferences about the process of chromosome evolution (Sumner 2003). In this context, base-specific fluorochromes and fluorescent *in situ* hybridization (FISH) with different ribosomal DNA probes allow a more detailed analysis of the molecular structure of chromosomes, and reveal many more differences among closely related species than conventional techniques (Bione et al. 2005, Silva et al. 2009, Cabral-de-Mello et al. 2010, 2011). As an example, the identification of rRNA clusters in different species has been widely used in comparative cytogenetics to understand the patterns of karyotypic evolution in different taxonomic groups (Cuadrado et al. 2008, Cabral-de-Mello et al. 2011, Cioffi et al. 2011, Grozeva et al. 2011, Golub et al. 2015, Palacios-Gimenez and Cabral-de-Mello 2015).

Genome size is another trait useful in comparative studies in a variety of taxonomic levels (Gregory and Shorthouse 2003, Tsutsui et al. 2008, Tavares et al. 2012). Such information is also important to clarify the relationship between variation in genome size and chromosome number (Tsutsui et al. 2008, Cardoso et al. 2012, Jacobson et al. 2012), and direct the selection of species for genome sequencing projects (Hardie et al. 2002, Gregory 2005, Geraci et al. 2007).

Curiously, cytogenetic studies are non-existent for several taxa and species groups that have recognized importance as pest species, and exhibit archaeological relevance, such as grain weevils of the genus *Sitophilus* Linnaeus, 1758 (Kenway and Carrott

2006, Plarre 2010, Smith and Kenward 2011, Corrêa et al. 2017). A few species of *Sitophilus* weevils were karyotyped to date, mainly in the 1970's and 1980's (Inkmann 1933, cited in Smith and Virkki 1978, Takenouchi 1958, cited in Smith and Virkki 1978, Smith and Brower 1974, Smith and Virkki 1978, Barrion et al. 1988, Zhi-Yua et al. 1989, Moraes et al. 2003, Silva et al. 2015). However, the results of these earlier efforts involving grain weevils were discrepant, emphasizing the need for further and more reliable analysis. Only a single recent karyotype analysis of the maize weevil *Sitophilus zeamais* Motschulsky, 1855 used more refined cytogenetic techniques (Silva et al. 2015). Knowledge of genome size is even scarcer, since no data are currently available in the literature for any species of *Sitophilus*.

The genus *Sitophilus* comprises fourteen species, three of which (the rice weevil *S. oryzae* (Linnaeus, 1763), the maize weevil *S. zeamais* and the granary weevil *S. granarius* (Linnaeus, 1758)), are of greater scientific interest because of their broadly recognized status as primary pest species of stored products throughout the world (Rees 1996, Danho et al. 2002, Ojo and Omoloye 2012). However, a congeneric fourth species, the tamarind weevil *S. linearis* (Herbst 1797), is also of scientific interest due to its devastating seed damage to tamarind crops (*Tamarindus indica* L.) (Adebayo et al. 2011, Ojo and Omoloye 2015).

The phylogenetic relationship among these weevils is controversial (Khan and Musgrave 1968, Plarre 2010). Sequencing-based molecular analyses of individual gene fragments, particularly those encoding cytocrome oxidase I, the elongation factor 1-alpha, and ribosome 28S provided the basis for the initial suggestion that *S. granarius* and *S. zeamais* form a sister taxon to *S. oryzae,* with *S. linearis* more distantly related (O'Meara 2001, Plarre 2010). Alternatively, the granary weevil was reported as a sister species of *S. oryzae*/*S. zeamais* (Lefevre et al. 2004), while in another study, *S. oryzae* and *S. granarius* form the sister group of *S. zeamais* (Conord et al. 2008). *Sitophilus linearis* was also considered a sister group of *S.oryzae*/*S. zeamais,* not *S. granarius*, in a recent study (Devi et al. 2017). Considering these difficulties and the resulting controversy, cytogenetic analyses and genome size determinations are needed to shed light on the phylogenetic relationship among these *Sitophilus* species.

The aims of this study were to: 1) perform a comparative cytogenetic characterization among *S. granarius*, *S. linearis*, *S. oryzae* and *S. zeamais*); 2) quantify the genome size of these four species; and 3) perform a more complete karyotype-based phylogenetic analysis with these species. The data will contribute to the understanding of the genomic organization and the taxonomic status of these species.

Materials and methods

Biological material

Sitophilus granarius were obtained from wheat kernels in Manhattan (Kansas, USA; 39°11'18"N; 96°36'21"W); *S. linearis* was obtained from tamarind seeds in Piraci-

caba (São Paulo, Brazil; 22°43'31"S; 47°38'57"W) and Montes Claros (Minas Gerais, Brazil; 16°44'06"S; 43°51'42"W); and *S. oryzae* was obtained from rice kernels in Cascavel (Paraná, Brazil; 24°57'21"S; 53°27'19"W) and São Borja (Rio Grande do Sul; Brazil; 28°39'38"S; 56°00'16"W). Samples of *S. zeamais* were obtained from maize kernels in Cruzeiro do Sul (Acre, Brazil; 07°37'52"S; 72°40'12"W) and Porto Alegre (Rio Grande do Sul, Brazil; 30°01'59"S; 51°13'48"W).

The last larval instars of each weevil species (i.e., *Sitophilus granarius*, *S. linearis*, *S. oryzae* and *S. zeamais*) were used for karyotyping and adult insects were used for genome size determination. Insects of each species were reared in glass containers (0.5 L) in an environmentally controlled rearing room (18 ± 2 °C, 70 ± 10% relative humidity and a photoperiod of 12:12 h L:D), containing grains of either wheat (*S. granarius*), tamarind fruits (*S. linearis*) or maize grains (*S. oryzae* and *S. zeamais*). The larvae were extracted from their respective hosts after inspection of different substrate grains with a LX-60 specimen radiography system equipped with a 14-bit digital camera (Faxitron X-Ray Corp., Wheeling, IL, USA). The adults were sieved from the grains, snap-frozen in dry ice and maintained under –80 °C until genome size determination.

Cytogenetic analyses

The cerebral ganglia of individuals of the last larval stage were processed according to Imai et al. (1988) after incubation in a hypotonic solution of colchicine (1% sodium citrate plus 0.005% colchicine) for 1 h 45 min. Conventional staining of the slides was performed with 4% Giemsa in Sörensen`s phosphate buffer pH 6.8, for 12 min. Slides were then washed in water and allowed to dry at room temperature. The C-banding technique was performed according to Lachowska et al. (2005), with modifications to the time of the HCl treatment (0.3M, for 4 min) and the $Ba(OH)_2$ incubation (5%, for 3 min). Sequential staining with the fluorochrome DAPI/CMA_3 was performed according to Schweizer (1980), with modifications related to the order of use of fluorochromes and the processing times. DAPI was used first for 30 min, followed by CMA_3 for 1 h. The use of distamycin was omitted.

Mapping of ribosomal DNA was performed with probes for 18S rDNA obtained by PCR amplification using primers F (5' TCATATGCTTGTCTAAAGA-3') and R (3'-TCTAATTTTTTCAAAGTAAACGC-5') designed for *Melipona quinquefasciata* Lepeletier, 1836 (Pereira 2006). During the amplification, the 18S rDNA probes were labeled by the indirect method using digoxigenin-11-dUTP (Roche, Mannheim, Germany). Fluorescent in situ hybridization (FISH) was performed using the method proposed by Pinkel et al. (1986), with modifications concerning the use of pepsin instead of proteinase K, before the dehydration and denaturation steps. The detection of the probe signal was achieved with antidigoxigenin-rhodamine. At the end, the slides were mounted with antifading mounting media containing DAPI (Vectashield).

The sex chromosomes were identified by comparing female and male karyotypes. Ten male karyotypes of each species were mounted in order to establish which chro-

mosomes do not form an exact pair. These chromosomes were considered the sex ones and, by comparison, it was possible to establish the chromosomes corresponding to the sex pair, in females. The sex determination system of the four species, in turn, was recognized by analysing meiotic figures from the testes following Dias et al. (2012). Males were identified by the rostrum morphology, which is smaller, thicker and more punctured than the female rostrum (Khan and Musgrave 1968).

An average of 20 metaphases per slide were evaluated with an Olympus BX60 microscope coupled to an image capturing system (Image-Pro Plus Version 6.3, Media Cybernetics 2009). The slides stained with fluorochromes (CMA_3/DAPI) were analyzed with an epifluorescence light microscope using excitation filters WB (λ = 330–385 nm) and WU (λ = 450–480 nm) under oil immersion at 100× magnification. The chromosomes were classified according to Levan et al. (1964), and the karyotypes were mounted by pairing chromosomes in decreasing order of size.

Flow cytometry analysis

Genome size was estimated by flow cytometry as described in Hare and Johnston (2011), except that the mean fluorescence of the sample and standard were determined using a Beckman Coulter Cytoflex cytometer and the concentration of propidium iodide was 25μg/ml, rather than 50μg/ml. In brief, a single frozen weevil head plus a single frozen head of a *Drosophila virilis* Sturtevant, 1916 standard (1C = 328 Mbp) were placed into 1ml of Galbraith buffer in a 2 ml Kontes tissue grinder and ground with 15 strokes of the "A" pestle at a rate of 3 strokes per 2 seconds. The nuclei released by grinding were filtered through a 40μ nylon filter and stained with 25 ug/ml of propidium iodide for at least 30 minutes in the cold and dark. The relative fluorescence of the 2C nuclei from each of the four *Sitophilus* species and the standard were determined using the flow cytometer indicated above. The 1C amount of DNA was calculated as the ratio of the mean fluorescence of the diploid nuclei of the sample and standard times 328 Mbp.

Phylogenetic analysis

The relationship among the four species of *Sitophilus* grain weevils was determined using a matrix with a total of 20 karyotype characters, where five characters were parsimony informative (exhibiting at least two characters distinct among operation taxonomic units [OTUs]; i.e., the weevil species studied) (Table 2). A maximum parsimony (MP) was consequently built using the heuristic search option in the TNT software (Goloboff et al. 2008). Node support was estimated by 100,000 bootstrap replicates using absolute frequency and search tree with implicit enumeration. The vine weevil *Otiorhynchus bisulcatus* (Fabricius, 1781) (Coleoptera: Curculionidae) was the outgroup (Holecová et al. 2013). The maximum parsimony tree shows only nodes

with bootstrap support > 50. For the phylogenetic analysis of the chromosomal data each structural rearrangement identified was considered a character and scored for variation among four species and the respective outgroup.

Results

Cytogenetics

Sitophilus granarius:
The karyotype of *S. granarius* showed 2n=24 chromosomes, including 11 pairs of autosomes and a pair of sex chromosomes. Most autosomal pairs, except pairs 1, 4 and 5, exhibited a metacentric morphology. The first autosomal pair was longer than the remaining and the other pairs gradually decrease in size. The submetacentric X chromosome was similar in size to the 11th chromosome pair, while the metacentric Y chromosome was the smallest element in the set (Figures 1a). The heterochromatin, based on the C-banding staining, was restricted to the centromeric region of the 6th autosomal pair (Fig. 1a), to the short arm of the X chromosome and to one of the Y arms.

Sequential staining with fluorochromes, in turn, allowed the identification of CMA_3^+ regions only in the centromere of the sixth autosomal pair and in one of the Y arms, whereas DAPI stained the short arm of the X chromosome and the complementary arm of the Y chromosome (Fig. 2a, b). The FISH technique using an 18S rDNA sequence probe showed a positive hybridization signal in the centromeric region of the sixth autosomal pair, both in males and females (Fig. 2c, d).

The analysis of male meiotic cells revealed a sex chromosome system of the Xyp type (Fig. 3a), and the meioformulae n=11 + XX and n=11 + Xyp, observed in females and males respectively.

Sitophilus linearis:
The karyotype of this species also exhibited 2n=24 chromosomes, which gradually decrease in size. Most autosomal chromosomes were metacentric, except pairs 1, 2, 10 and 11, which were submetacentric. The submetacentric X chromosome was the longest element in the karyotype, while the Y showed a subtelocentric morphology equal in size to one of the medium-sized chromosomes (Fig. 1b). The C-banding technique showed small heterochromatic blocks in the centromeric region of all chromosomal pairs (Fig. 1b), including the sexual ones, similar to DAPI staining (Fig. 2f). The chromosomal staining with CMA_3 revealed positive regions located in the telomeric region of pair 10 and in the short arm of the Y chromosome (Fig. 2e).

The chromosomal mapping of major rDNA clusters (18S) confirmed that ribosomal genes were located in the telomeric region of pair 10 and in the short arm of the Y chromosome. So, with both CMA_3 and FISH, females showed two positive signals, while males showed three positive signals (Fig. 2g, h).

Figure 1. Karyotypes of *Sitophilus granarius* (**a**), *S. linearis* (**b**), *S. oryzae* (**c**) and *S. zeamais* (**d**). The first and the second lines for each species represent female karyotypes stained with Giemsa and C-banding, respectively, while the third line represents male karyotypes stained with Giemsa (**a, b, c**) or C-band (**d**). Bar = 5 μm.

The typical parachute association of the sex chromosomes present in *S. granarius* was not observed, despite the analysis of several metaphase I cells. Instead, analysis of these cells showed an XY association in all cells evaluated (Fig. 3b). Therefore, its meioformulae were n=11 + neo-XX and n=11 + neo-XY, for females and males, respectively.

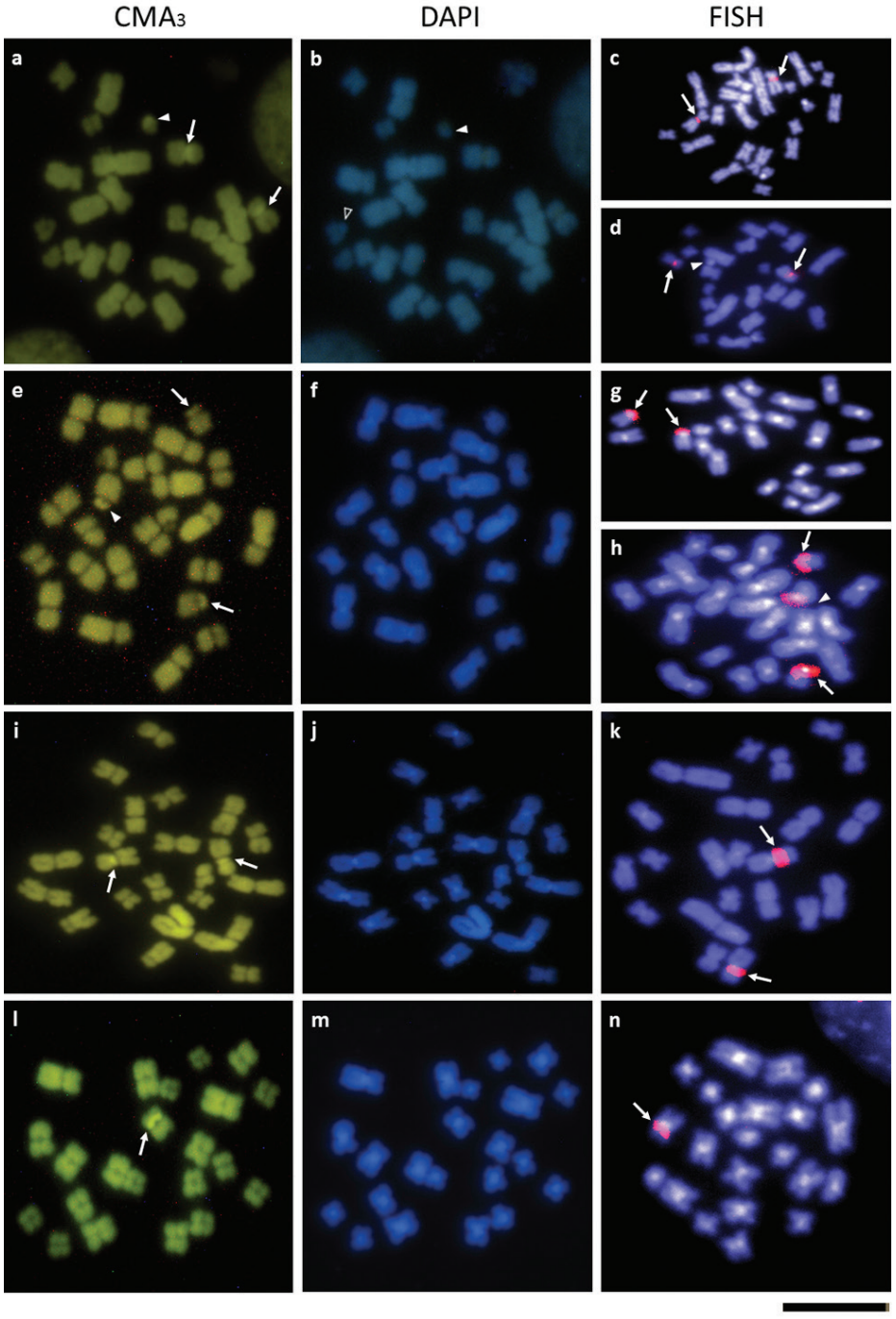

Figure 2. Metaphases of *Sitophilus granarius* (**a–d**), *S. linearis* (**e–h**), *S. oryzae* (**i–k**) and *S. zeamais* (**l–n**) stained with CMA_3 and DAPI or submitted to rDNA 18S FISH. Pictures **a, b, d, e, f, h** represent male cells, while the remaining ones are from females. The arrows indicate the rDNA location, while blank and solid arrowheads indicate the X and the y chromosomes, respectively. Bar = 5 μm.

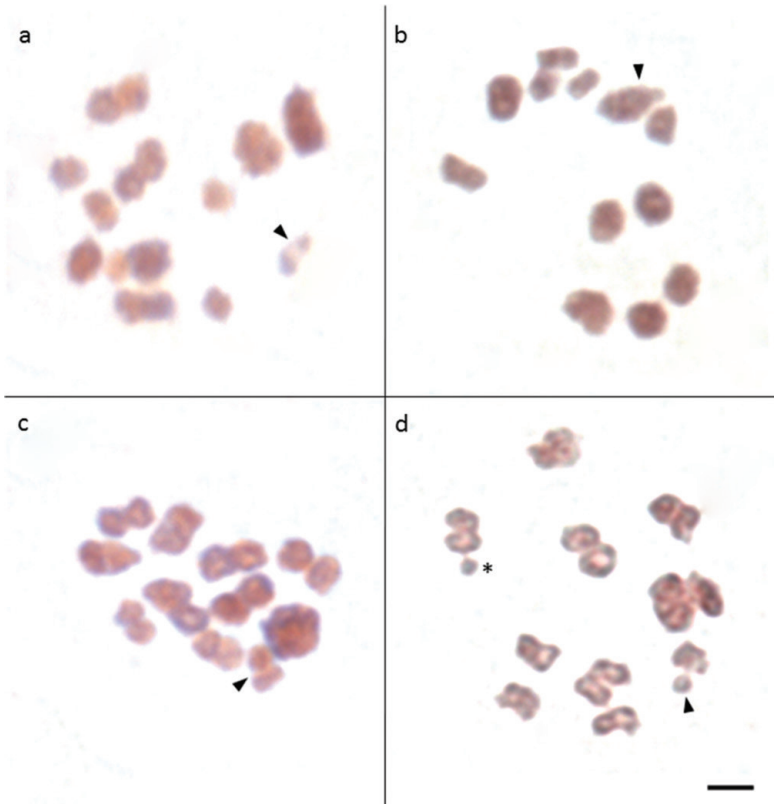

Figure 3. Meiotic male metaphase cells of *Sitophilus granarius* (**a**)*, S. linearis* (**b**), *S. oryzae* (**c**) and *S. zeamais* (**d**), stained with Giemsa, showing the typical parachute association of the sex chromosomes (arrowhead) in all species, except in *S. linearis*. The asterisks indicate a B chromosome. Bar = 5 µm.

Sitophilus oryzae:

This species exhibited a karyotype consisting of 2n=22 chromosomes that gradually decreased in size. Nine autosomal pairs showed a metacentric morphology; only the autosomal pair 6 was submetacentric (Fig. 1c). The X chromosome was metacentric, presenting an intermediate size between the 7th and 8th chromosome pairs. The Y chromosome was also metacentric, but belonged to the group of the small chromosomes (Fig. 1c). All autosomal chromosomes and the sexual pair possessed small heterochromatic blocks, rich in AT bases in the centromeric region, as showed by the C-banding and the DAPI staining (Figures 1c, 2j). The CMA_3 staining and the FISH with 18S rDNA indicated that the ribosomal genes were located in the pericentromeric region of the 5th autosomal pair (Figures 2i, k).

Observation of meiotic cells indicated the sex pair exhibiting a parachute configuration, as in *S. granarius*. Therefore, its meioformulae were n=10 + XX and n=10 + Xyp, for females and males, respectively (Fig. 3c).

Sitophilus zeamais:
As described by Silva et al. (2015), the karyotype of this species had 2n = 22 chromosomes. All autosomal chromosomes of this species exhibited metacentric morphology and a gradual reduction in size. The X chromosome was also metacentric and presented an intermediate size between the first and second pair of autosomes, while the Y chromosome presented a dot-like morphology (Fig. 1d).

Autosomes and the X chromosome exhibited small heterochromatic blocks in the centromeric region after C-banding and DAPI staining, while the Y chromosome was entirely euchromatic (Figures 1d, 2m). Populations of *S. zeamais* from Viçosa (MG), Unai (MG) and Porto Alegre (RS) showed 0-4 B chromosomes that were partially or completely heterochromatic (Fig. 1d). Bright signals were observed in the pericentromeric region of one chromosome of the third autosomal pair after CMA_3 staining and hybridization with 18S rDNA probe (Figures 2l, n).

Analysis of meiotic cells confirmed that the sex pair exhibited the parachute configuration, as in *S. granarius* and *S. oryzae*. Therefore, their meioformulae were n=10 + XX and 10 + Xyp, for females and males respectively (Fig. 3d).

Flow cytometry and Phylogenetic Analysis

The mean genome size (1C) estimates for the four *Sitophilus* species analysed in the present study and their chromosome numbers are in Table 1. Genome size was similar between sexes within each species, except when B chromosomes were present in one of the sexes, as in males of the maize weevil *S. zeamais* (Table 1). In contrast, genome size exhibited marked differences among species, which can be clustered in two distinct groups. The 1st group, encompassing *S. granarius* and *S. linearis*, exhibited smaller genome sizes (0.4395–0.5533 pg), while the 2nd group, encompassing *S. oryzae* and *S. zeamais*, exhibited larger genome sizes (0.7296–0.7865 pg). The technique indicated significant variation in genome size of the maize weevil confirming the presence of variable numbers of B chromosomes among specimens of this species and others not possessing them.

The phylogenetic analysis showed that *S. zeamais* and *S. oryzae* were phylogenetically closer than *S. granarius* and *S. linearis,* supported for the clade with bootstrap = 66 (Table 2, Fig. 4). Furthermore, *S. granarius* and *S. linearis* have common and recent ancestry within the genus *Sitophilus*.

Discussion

Comparative karyotype characterization

The chromosome number of 2n=22, the parachute configuration, and the prevalence of metacentric chromosomes that we found in *S. oryzae* and *S. zeamais* represent cy-

Table 1. Genome size estimates for the grain weevils *Sitophilus granarius*, *S. linearis*, *S. oryzae* and *S. zeamais;* the number of individuals analyzed (N) and chromosome number are indicated.

Species	Haploid genome size pg ± SE (Mbp ± SE) Female (F)	Male (M)	N (F/M)	Chromosome number
Sitophilus granarius	0.5533 ± 0.003 (541.1 ± 2.9)	0.5561 ± 0.003 (543.9 ± 3.0)	5/4	2n=24
Sitophilus linearis	0.4395 ± 0.001 (429.8 ± 0.6)	0.4351 ± 0.001 (425.5 ± 1.4)	2/4	2n=24
Sitophilus oryzae	0.7865 ± 0.002 (769.2 ± 1.9)	0.7852 ± 0.003 (768.0 ± 3.1)	4/6	2n=22
Sitophilus zeamais	0.7296 ± 0.008 (713.5 ± 7.5)	0.7252 ± 0.003 (709.2 ± 2.8) 0.7860 ± 0.006 (768.7 ± 5.7)	5/3 -/2	2n=22 2n=22 + Bs

Table 2. Matrix data of karyotype features of the *Sitophilus* pest species and outgroup *Otiorhynchus bisulcatus* (Coleoptera: Curculionidae).

Karyotype features	Species				
	S. zeamais	***S. oryzae***	***S. granarius***	***S. linearis***	***O. bisulcatus****
Number of chromosomes	0	0	1	1	0
Presence of B chromosomes	1	0	0	0	0
Sex-chromosome system (Xyp)	1	1	1	0	1
22 metacentric chromosomes	1	0	0	0	0
20 metacentric chromosomes	0	1	0	0	0
18 metacentric chromosomes	0	0	0	1	0
16 metacentric chromosomes	0	0	1	0	1
0 submetacentric chromosomes	1	0	0	0	0
2 submetacentric chromosomes	0	1	0	0	0
8 submetacentric chromosomes	0	0	1	1	0
6 submetacentric chromosomes	0	0	0	1	0
4 submetacentric chromosomes	0	0	0	0	1
1 telocentric chromosome	0	0	0	1	0
Number of the sexual pair	0	1	2	3	?
Morphology of the X chromosome	1	1	0	0	1
Morphology of the y chromosome	0	1	1	2	0
Banda C pattern	0	0	1	0	0
DAPI distribution	0	0	1	0	1
CMA_3 distribution**	0	1	2	3	4
NOR localization (FISH)**	0	1	2	3	4

*Outgroup obtained of Holecová et al. (2013); **non-informative characters; ?: missing data; 1, 2, 3 and 4: number of variables in chromosome characters.

togenetic characteristics already described in most species of Curculionidae surveyed so far (Smith and Virkki 1978, Bárcenas-Ortega 1992, Lachowska et al. 1998, 2006, 2008, Holecová et al. 2002, 2013, Rozek et al. 2009). Except for the chromosome number (2n=24), a third species, *S. granarius*, also exhibited karyotypic characteristics likely representing the plesiomorphic (i.e., ancestral) conditions for the Polyphaga suborder of Coleoptera, which are a sex chromosome system of the parachute type (Xyp) and prevalence of metacentric chromosomes (Smith and Virkki 1978, Lachowska et al.

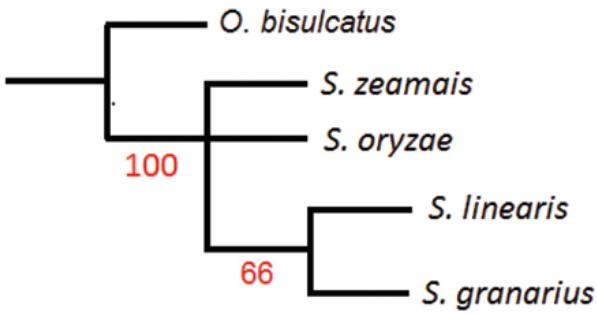

Figure 4. Parsimony tree of *Sitophilus* species with bootstrap values for each node/branch inferred using karyotype traits provided in the Table 2. Node support values below 50% were not recorded in the tree.

1998, 2006, 2008, Holecová et al. 2002, 2013, Rozek et al. 2009). However, the tamarind weevil, *S. linearis,* exhibited a quite different karyotype from the other three species analysed.

First, the higher number of chromosomes observed in *S. linearis* and *S. granarius* (2n =24) suggests that the karyotype of these species may have evolved by centric fission of autosomes. Alternatively, the karyotypes of *S. oryzae* and *S. zeamais,* that have 2n=22 chromosomes, could have originated as a result of pericentric inversions in small pairs followed by fusions between them. The first scenario, however, seems more probable, once 2n=22 is the prevalent and seems to be the ancestral chromosomal number for Curculionidae species (Smith and Virkki 1978, Holecová et al. 1995, Lachowska et al. 1998). Additionally, centric fission has already been described as playing important roles in the karyotype evolution of other Curculionidae species, such as *Peritelus familiaris* (Lachowska et al. 2006), *Cirrorhynchus kelecsenyi* (Lachowska et al. 2008) and for three sibling species of the *Acalles echinatus* group (i.e., *A. echinatus*, *A. fallax* and *A. petryszaki*) (Lachowska et al. 2009).

Secondly, cytogenetic analysis revealed differences among the four species related to the morphology and size of sex chromosomes. For example, in *S. granarius* and *S. linearis*, the X chromosome was submetacentric, but the Y chromosome was metacentric and subtelocentric, respectively. In contrast, *S. oryzae* and *S. zeamais* exhibited metacentric X chromosomes, but whereas the Y chromosome in *S. zeamais* was punctiform, that of *S. oryzae* was metacentric and not so small as in *S. zeamais*. In *S. linearis*, in particular, the X chromosome represents the longest element in the karyotype and the Y is also significantly longer than the four/five small autosomes pairs. They are also much larger than the sexual ones in the other three species analysed. Additionally, B chromosomes were found exclusively in some populations of *S. zeamais*. Together, these characteristics facilitate the identification of this particular species.

Thirdly, as the sex chromosomes of *S. linearis* are large and form a well differentiated figure from the Xyp of the other *Sitophilus* species in first meiosis, we propose that this species has a sex determination system of the neo-XY type. However, translocation(s) between an autosomal pair and the sex chromosomes in an ancestral

species, with increase of the X-Y sizes and reduction in the number of autosomes, does not seem to explain the origin of the neo-XY system in *S. linearis*. Although the(se) translocation(s) were already observe in some insect species (Macaisne et al 2006, Dutrillaux and Dutrillaux 2007, Mamuris and Dutrillaux 2013), *S. linearis* does not exhibit the reduction in the number of autosomes. Thus, the translocation-based explanation of the origin of the neo-XY system in the tamarind weevil seems flawed. In contrast, this species possesses 2n=24 chromosomes, while the chromosome number of 2n=22 represents the plesiomorphic condition for this genus, as already discussed, what allows for an alternative explanation for the neo-XY system.

A more plausible explanation for the neo-XY system in *S. linearis* would be the contributions of more than one autosomal pair to form the large neo-XY chromosomes, with decreases in their sizes, but without reduction in their number, as reported for *Calcosoma atlas* (Dutrillaux and Dutrillaux 2013). In this sense, cytogenetic analysis provided clear evidence of the absence of the first larger autosome pair in the karyotype *S. linearis*, a characteristic easily recognized in the other three *Sitophilus* species and, consequently, its participation in this process. Additionally, considering the actual size of the sex chromosomes of *S. linearis*, the fact that the two/three first pairs of chromosomes of this species are more similar in size than the equivalent chromosomes in the karyotypes of other *Sitophilus* species, and the diminutive size of the sexual chromosomes of its phylogenetically closer species, *S. granarius* (see below), we can suggest that these chromosomes could also be involved in the formation of the neo-XY chromosomes of *S. linearis*, with small reductions in their sizes. The presence of rDNA clusters in the Y chromosomes of *S. linearis*, as discussed above, is another indication of these translocations. However, further studies will be necessary to confirm this mechanism, the autosomal pairs involved in the process and the exact chromosomal rearrangements concerning the evolution of the neo-sex chromosomes of *S. linearis*.

The genus *Sitophilus,* especially *S. granarius*, possesses a small amount of heterochromatin that was located preferentially at the centromeric region, as in most Curculionidae (Holecová et al. 2002, 2013, Rozek et al. 2004, Lachowska et al. 2005, 2008, 2009, Kajtoch and Lachowska-Cierlik 2009). However, as three of the four species analysed exhibited the same heterochromatic distribution pattern, the C-banding patterns obtained did not allow further discrimination. This finding confirms observations by Rozek et al. (2004) that in species with small amounts of heterochromatin, C-banding patterns cannot be used in taxonomic and phylogenetic investigations. Nonetheless, even considering the consistently and uniquely small heterochromatin amount present in the karyotype of *S. granarius*, the heterochromatin distribution pattern obtained for this species clearly allowed its separation from the other *Sitophilus* species.

The coincidence of DAPI staining with the C-banding marks in the chromosomes of *S. granarius*, *S. linearis* and *S. oryzae*, as well as in *S. zeamais* (Silva et al. 2015), demonstrate the occurrence of a higher amount of AT base pairs in the heterochromatic sequences of these species. Positive DAPI signals were present in the majority of weevils previously studied confirming that AT pairs often make up the main part of the heterochromatin in

these species (Lachowska 2008, Lachowska et al. 2008, Holecová et al. 2013). Up to now, *Otiorhynchus* s. str. *bisulcatus* is the only Curculionidade species in which the heterochromatin is rich in AT and GC base pairs (Holecová et al. 2013), as several positive marks for DAPI and CMA_3 were visualized in the majority of its chromosomes.

The analysis of the localization and distribution of rRNA clusters largely contributed toward the cytogenetic characterization of the four *Sitophilus* species analysed. The findings indicate that ribosomal genes are located in a single autosomal pair in three (*S. granarius*, *S. oryzae* and *S. zeamais*) of the four analysed species (different pairs for each species). This corroborates previous reports suggesting that an autosome pair performs as a nucleolus organizer in Coleoptera (Virkki et al. 1984, Colomba et al. 2000, Moura et al. 2003, Gómez-Zurita et al. 2004, Bione et al. 2005, Cabral-de-Mello et al. 2011). This is also the most common pattern observed in the few species of Curculionidae for which the location of the rDNA clusters has been studied, through CMA_3 staining or silver impregnation (Lachowska 2008, Lachowska et al. 2005, 2006, 2008, 2009, Holecová et al. 2013).

In *S. linearis*, however, positive CMA_3 and FISH stainings were also detected in the Y chromosome. Data obtained, therefore, evidenced that in this species, the Y chromosome also bears rDNA clusters. To our knowledge, this is the first time that rDNA genes is mapped directly (FISH) on the Y chromosome in Curculionidae, while the presence of rDNA genes on the X or on both sex chromosome (besides autosomes ones) have already been documented in some species of Coleoptera, by FISH analysis (Gómez-Zurita et al. 2004, Bione et al. 2005, Cabrero and Camacho 2008, Cabral de Mello et al. 2011). Furthermore, centromeric, pericentromeric and telomeric clusters were observed in *S. granarius*, *S. oryzae*/*S. zeamais* and in *S. linearis,* respectively. Transposition of genes to new locations, inversions, translocations, ectopic recombination, transposable elements and hybridization without a change in chromosome number are all mechanisms that have already been used to explain this variation in the localization of rDNA genes (Cabrero and Camacho 2008, Panzera et al. 2012, Pita et al. 2013, Golub et al. 2015, Vershinina et al. 2015). Thus, results presented here show that rDNA loci may be considered an important cytogenetic marker for this genus and that cytogenetic analysis on different populations and/or other *Sitophilus* species will certainly contribute to a better understanding of mechanisms responsible for their ribosomal loci variation.

Additionally, CMA_3 and FISH results revealed fluorescent labels in only one of the homologous of the pair 3 in *S. zeamais*. Although methodological problems cannot be excluded as a source of this variability, it seems unlikely that both techniques would yield the same results, even because they were efficient for the detection of the localization of rDNA genes in the other three *Sitophilus* species. Thus, we believe that this represents a size polymorphism between these homologous and, consequently, that both of them would contain rDNA genes, but that in one of them, the low copy number of ribosomal cistrons (< 10kb [Yiang and Gill 1994]) could not be detected with the probe used here. This suggestion is supported by the fact that this result was found in both populations analysed (Cruzeiro do Sul and Porto Alegre).

Genome size divergence

The flow cytometry analyses provided a preliminary scenario about the haploid genome size variation among the *Sitophilus* species. The genome size of *S. oryzae* (0.7865 pg) was similar to *S. zeamais* (0.7296 pg), whereas *S. granarius* (0.5533 pg) exhibited a small genome size, and an even smaller was found in *S. linearis* (0.4395 pg). These findings also corroborate the reportedly high intra genus variation in arthropods, as *S. oryzae* has 66% more DNA than *S. linearis*. Although genome size variation is mainly due to variation in the amount of non-coding DNA not necessarily reflecting phylogenetic relationship, this does not seem the case for grain weevils, as we reported here. The variation in DNA content among these four weevil species is consistent and reinforces the phylogenetic relationship among them based on the karyotypes reported here and also on their endosymbionts (Lefevre et al. 2004).

Cytometry data also provided evidence that nuclear DNA content is not proportionally related to either the chromosomal number, or the heterochromatin amount in *Sitophilus* species. In the first case, both smaller genome species (i.e., *S. linearis* and *S. granarius*) exhibit higher chromosome numbers than the species with higher genome sizes (*S. oryzae* and *S. zeamais*). In the second case, *S. linearis* exhibited a similar amount of heterochromatin to both *S. oryzae* and *S. zeamais,* and a larger amount than *S. granarius*, despite the smaller genome size of *S. linearis.* The genome sizes of *Sitophilus* males and females were similar, although three species exhibit the Xyp system, while the tamarind weevil exhibits the neo-XY sex determination system. This findings are suggestive that the genome size variation observed in *Sitophilus* grain weevils may be a result of repetitive DNA sequences (e.g., satellite DNA, transposable elements etc.) accounting for a more complex gene regulation in species with larger genome size, as reported for eukaryotes (Comeron 2006, Biscotti et al. 2015). These larger genome sizes correspond to the more ancestral species, *S. oryzae* and *S. zeamais,* among the grain weevil species. The higher specialization and loss of non-coding DNA may account for the smaller genome size of the more recent grain weevil species, *S. granarius* and *S. linearis.*

The obtained genome size of the *Sitophilus* species were within the previously described range for eight other species of Curculionidae, that include four of the genus *Anthonomus* Germar, 1817 (0.62-0.86 pg – Bárcenas-Ortega 2005, Gregory 2017), one *Dendroctonus* Erichson, 1836 (0.21 pg – Gregory et al. 2013), one *Aramigus* Horn, 1876 (3.32 pg – Normark 1996), one *Lissorhoptrus* LeConte, 1876 (1,00 pg – He et al. 2016) and one *Xyleborus* Eichhoff, 1864 (0.24 pg – Hanrahan and Johnston 2011). The values obtained were also within the constrained value proposed for Gregory (2002) for holometabolous insects (2 pg). However, these values are smaller than that of *Aramigus tessellatus* (Say, 1824) (Normark 1996), a parthenogenetic polyploidy species of Curculionidae with DNA content ranging from 3.32 to 5.02 pg, depending on the analysed lineage (Normark 1996).

Worth noting is also the fact that two genome size estimates were obtained for *S. zeamais* males. Considering that this species may possess from 0-4 B chromosomes,

their presence in some individuals explain the difference observed. However, we were unable to carry out cytogenetic and flow cytometry analyses using the same individuals. Consequently, we could neither establish the number of B chromosomes that different individuals possessed nor the contribution of each B chromosome to the whole genome.

Grain weevil phylogeny

Finally, the parsimony phylogenetic analysis had only mild bootstrap support due to the limited number of informative karyotype characters available, but it does agree with the descriptive analysis of *Sitophilus* karyotype, which provides evidence that *S. zeamais* and *S. oryzae* are phylogenetically closer when compared with *S. granarius* and *S. linearis*. The new finding not previously reported is the higher proximity of *S. granarius* to S. *linearis*, suggesting a common and more recent ancestry for both species. This finding is also consistent with the genome size and the number of chromosomes of these species, the closer association of the granary weevil with stored grains losing its flight ability (Plarre 2010), and with the higher host specialization of the tamarind weevil (Adebayo et al. 2011, Ojo and Omoloye 2015).

The ancient origin (ca. 8.7 million years ago) and closer association between the maize and rice weevils were recently reinforced with comprehensive molecular data (Ojo et al. 2016, Corrêa et al. 2017). This finding is consistent with the ancestral karyotype shared by both species and also resemble that of the granary weevil and their fossil records (Plarre 2010, Corrêa et al. 2017), but is significantly distinct from that of the tamarind weevil. The latter species was recently suggested as clustering with *S. oryzae* and *S. zeamais,* not *S. granarius*, but based only on mtCOI sequence fragment (Devi et al. 2017). Nonetheless, this latter report diverges from the available information on karyotype, genome size, endosymbiont association, and life-history traits of these species (O'Meara 2001, Lefevre et al. 2004, Plarre 2010, and present study). Therefore, the current weight of evidence aided by our findings indicate that the origin of the tamarind weevil is more recent and so is its phylogenetic divergence from the granary weevil and the other stored grain weevils, the maize and rice weevils.

The ancient origin of the grain weevils, likely pre-dating the onset of agriculture in Southeast Asia and the India subcontinent, together with their recent adaptation to stored products, make these earlier invader species useful for tracking grain and trade routes since the Neolithic period between 15,200 and 2,000 BC (Levinson and Levinson 1994, Kenway and Carrott 2006, Smith and Kenward 2011, Panagiotakopulu and Buckland 2017). More abundant fossil information is available for the granary weevil, which is more closely associated with stored commodities due to its inability to fly, but the oldest fossil records are from the maize weevil reinforcing the ancient origin of this species (Levinson and Levinson 1994, Kenway and Carrott 2006, Plarre 2010, Panagiotakopulu and Buckland 2017). Again this is in contrast with the tamarind weevil, whose dispersion is more recent and allegedly associated with the Indian palm (i.e., the tamarind) (Plarre 2010).

Conclusion

In summary, we were able to describe the karyotype of the tamarind weevil and extend the karyotypic analysis of the maize weevil, allowing a comparative cytogenetic characterization of the four *Sitophilus* grain weevils (*S. granarius, S. linearis, S. oryzae,* and *S. zeamais*). A more complete karyotype-based phylogenetic analysis of these four species, aided by the quantification of genome size in each, shed light on the conflicting phylogeny of the grain weevil species. The ancestral and closer phylogenetic association between *S. zeamais* and *S. oryzae* was recognized, as was the more recent cluster encompassing *S. granarius* and *S. linearis* and a shared ancestral relationship.

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RESEARCH ARTICLE

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Mysterious meiotic behavior of autopolyploid and allopolyploid maize

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Abstract

This study was aimed to investigate the stability of chromosomes during meiosis in autopolyploid and allopolyploid maize, as well as to determine an association of chromosomes between maize (*Zea mays* ssp. *mays* Linnaeus, 1753) and *Z. perennis* (Hitchcock, 1922) Reeves & Mangelsdor, 1942, by producing a series of autopolyploid and allopolyploid maize hybrids. The intra-genomic and inter-genomic meiotic pairings in these polyploids were quantified and compared using dual-color genomic in-situ hybridization. The results demonstrated higher level of chromosome stability in allopolyploid maize during meiosis as compared to autopolyploid maize. In addition, the meiotic behavior of *Z. perennis* was relatively more stable as compared to the allopolyploid maize. Moreover, ten chromosomes of "A" subgenome in maize were homologous to twenty chromosomes of *Z. perennis* genome with a higher pairing frequency and little evolutionary differentiation. At the same time, little evolutionary differentiation has been shown by chromosomes of "A" subgenome in maize, while chromosomes of "B" subgenome, had a lower pairing frequency and higher evolutionary differentiation. Furthermore, $5I^{M} + 5II^{PP} + 5III^{MPP}$ and $5II^{MM} + 5II^{PP} + 5IV^{MMPP}$ were observed in allotriploids and allotetraploids respectively, whereas homoeologous chromosomes were found between the "A" and "B" genome of maize and *Z. perennis.*

Keywords

Maize, polyploidy, meiosis, GISH, chromosome stability, genome evolution

* Authors contributed equally.

Introduction

Zea Linnaeus, 1753, belongs to the tribe Maydeae Candolle, 1882, and consists of two sections: section Luxuriante and section Zea (Iltis and Doebley 1980, Wang et al. 2011). The domesticated maize (*Zea mays* ssp. *mays* Linnaeus, 1753, 2n=20, is also called corn, (belongs to genus *Zea* and is classified in section *Zea*) is an important economic crop and polyploid genetic model with many duplicated genes in all of its ten chromosomes (Ahn et al. 1993; Gaut et al. 2000; Wang et al. 2006; Wang et al. 2011). There are three models to explain these duplicated genes, which are multiple independent duplications occurring in one genome, autotetraploidy and allotetraploidy (Gaut and Doebley 1997, Molina et al. 2013). Besides maize, the other species of genus *Zea* are called teosinte, which provide an excellent system to study ecological genomics, population genetics and plant breeding (Hufford et al. 2012). *Z. perennis* (Hitchcock, 1922) Reeves & Mangelsdor, 1942 (section Luxuriantes), 2n=40 is one of the perennial teosintes having an inferred octoploid origin (González et al. 2006, Wang et al. 2011). In a previous study, *Z. perennis* was thought to have originated from a *Z. diploperennis* Iltis, Doebley & Guzman, 1979 - like ancestor (Tiffin and Gaut 2001).

From previous research, it is evident that crosses could be made between maize and *Z. perennis;* as a consequence the genomic relationship was assessed by meiotic pairing analysis of hybrids between both species (Longley 1924, Emerson and Beadle 1930, Mangelsdorf 1939, Shaver 1964, Poggio et al. 1999, Cao et al. 2002, TANG et al. 2004, Gonzalez and Poggio 2011). Different meiotic pairings were reported in their allopolyploid hybrids; the allotriploid, synthesized from a cross between diploid maize and *Z. perennis*, and the allotetraploid was developed by a cross between tetraploid maize and *Z. perennis.* Moreover, less meiotic stability and fertility has been observed in allotriploids as compared to allotetraploids (Tang et al. 2005). The common meiotic configurations of two allopolyploids were 5I+5II+5III in allotriploid hybrids and 10II+5IV in allotetraploid hybrids (Longley 1924; Molina and Garcia 1999; González et al. 2006). Maize and *Z. perennis* have basic chromosome number x = 5, and hypothetical formulas for maize and *Z. perennis* are "$A_mA_mB_mB_m$" and "$A_pA_pA_pA_pB_{p1}B_{p1}B_{p2}B_{p2}$", respectively (Naranjo et al. 1994). Besides, the existence of a controversy about the origin of "A" and "B" subgenomes, there might be two possible mechanisms behind the evolution, one mechanism refers to a duplication event that might have occurred in the "A" genome of a diploid species followed by evolutionary differentiation that converted "A" genome into homoeologous sub genomes "A" and "B"; the other proposes that the genome composition of "AABB" hybrids might be the result of an ancestral cross between two closely related "AA" and "BB" genomes followed by evolutionary fractionation (Molina et al. 2013). Furthermore, previous studies demonstrated that "A" subgenome in maize and *Z. perennis* showed higher homology of chromosomes, as well as, suffered fewer gene losses and higher level of gene expression as compared to the "B" subgenome, while "B" subgenome had a faster differentiation that led to species isolation and eventually resulted in the formation of different species of *Zea* (Freeling and Thomas 2006, Swanson-Wagner et al. 2010,

Schnable et al. 2011). However, both hypotheses could not explain differences within "A" and "B" subgenomes of both maize and *Z. perennis* clearly. In all, there is limited understanding about relationship of chromosomes between maize and *Z. perennis,* therefore chromosome stability of both autopolyploid and allopolyploid maize was investigated in current study with the following objectives: (i) to give systematic understanding of chromosome relationship between maize and *Zea perennis*; (ii) to observe the meiotic chromosome stability in autopolyploid and allopolyploid maize (iii) to reveal the origin and differentiation process of "A" and "B" subgenomes (iv) to validate the chromosome paring pattern by using general cytology and dual-color genomic in situ hybridization (GISH) in a number of autopolyploid and allopolyploid hybrids that were synthesized by the cross of maize and *Zea perennis.*

Material and methods

Abbreviations

GISH Genomic *in situ* Hybridization
RCC Relative chaotic coefficient
PMCs Pollen mother cells

Plant material

Plant materials are shown in Table 6. Maize inbred line wf9 (2n=2x=20) and a tetraploid maize Twf9 (2n=4x=40) (derived from chromosome doubling of wf9) were provided by the United States Department of Agriculture (USDA), *Zea perennis* (2n=4x=40, accession no. 9475) was obtained from International Maize and Wheat Improvement Center (CIMMYT). The plant material was raised at experimental farm of Sichuan Agricultural University, Jinghong, China. Three crosses were made by hand pollination that were (1) between diploid maize inbred wf9 and *Z. perennis,* (2) between tetraploid maize Twf9 and *Z. perennis* and (3) between diploid maize inbred line wf9 and tetraploid maize Twf9. In next year, the pre-germinated hybrid seeds were planted in soil filled plastic pots (12 × 12 cm, inner diameter × height) and placed in experimental station of Sichuan Agricultural University for initial identification. The seedlings at 5-leaf stage were transplanted into larger plastic pots (26.5 × 26.5 cm, inner diameter × height) for further root tips collections.

Chromosome and DNA preparation

The roots collected from parents and interspecific hybrids were immediately fixed in a saturated solution of α-bromonaphthalene for three hours, subsequently, transferred

in Carnoy's solution I (3:1 ethanol: glacial acetic acid, v/v) for 24 hours and, finally submerged in 70% ethanol solution after which these were preserved at 4 °C. Premature anthers of hybrids and parents were collected and treated with Carnoy's solution for a minimum of 12 hours and then preserved in 70% ethanol solution at 4 °C.

The preserved root tips and anthers were cleaned with distilled water to remove the effects of ethanol and then treated with an enzymatic solution comprising 6% cellulase (R-10, Yakult, Japan) and 1% pectinase (Y-23, Yakult, Japan) for 2.5–5.0 hours at 37 °C. Root tips and anthers were again thoroughly cleaned with distilled water in order to wash enzyme solution and finally, squashed onto glass slides in a drop of Carnoy's solution I and dried with ethanol flame. The preparations showing well-spread and clean mitotic and meiotic chromosomes were selected by phase-contrast light microscopy (Olympus BX-41, Japan) and stored at -20 °C for *in situ* hybridization. Total genomic DNA from young leaves of maize and *Z. perennis* was extracted according to modified 2 × CTAB methods (Jie et al. 2015).

Genomic *in situ* hybridization

The genomic DNA of maize and *Z. perennis* were labeled with DIG-Nick Translation and BIOTIN-Nick Translation Mix (Roche, Swiss), respectively according to manufacturer's protocol. The selected slides were preheated in an air blowing oven at 60 °C for one hour and then transferred into 0.1ug/ml RNase (Solarbio, China) in 2 × SSC solutions in a thermostat water bath at 37 °C for one hour. Afterwards, slides were washed twice in 2 × SSC for 5 minutes each at room temperature, followed by chromosome denaturation in 70 percent deionized formamide (FAD) solution at 70 °C for 2.5 minutes, then immediately dehydrated in an ice-cold 70 percent, 95 percent and 100 ethyl alcohol series and finally air dried at room temperature. The hybridization mixture comprised 150 µl 50% FAD, 60 µl 10% dextran sulfate (DS), 30 µl 2 × SSC, 15 µl 0.5% sodium dodecyl sulfate (SDS), 30 µg salmon sperm DNA (SSDNA) and 18 µl labeled probes for six slides. Hybridization mixture was denatured in a thermostat at 85 °C for 10 minutes, followed by quick cooling in ice for 10 minutes. A total 46 µl hybridization mixture was loaded on each slide and hybridization was accomplished in an incubator at 37 °C for 20–24 hours. After hybridization slides were immersed in 20% FAD, 2 × SSC, 0.1 × SSC, respectively for 15 minutes each, at 42 °C. After that, the slides were washed in 0.1% Triton X-100 once and in 1 × PBS thrice for 5 minutes each and then air dried, at room temperature. All further steps were performed in dark, 50 µl antibody diluent, which contained anti-digoxigenin-fluorescein (0.6 µg/µl in 1 × PBS, Roche) and streptavidin-Cy-3 fluorescein (0.6% in 1 × PBS, Sigma) was applied onto air dried slides and immunodetection was done at 37 °C for one hour in an incubator. Consequently, slides were washed in 1 x PBS thrice for 5 minutes each at room temperature and air dried finally. The chromosome counterstaining was performed by 4, 6-diamidino-2-phenylindole (DAPI) solution containing

86% 1 × PBS and 14% DAPI 10ug/ml (Solarbio), and slides were observed with fluorescence microscope (Olympus BX-61, Japan) coupled with pre-fixed filter sets named as U-MNAU2 (excitation 360–370nm; emission 420–460nm and dichroic 400nm), MWIBA3 (excitation 460–495nm; emission 510–550nm and dichroic 505nm) and U-MWIG3 (excitation 530–550nm; emission 575nm IF and dichroic 570nm). The images were captured with Media Cybernetics CCD 700 (Charge Coupled Device) and Image Pro Plus 6.0 (Media Cybernetics, Inc.). Captured images were processed by Adobe Photoshop 5.1.

Results

Material synthesis and chromosome identification

Three crosses were made between diploid maize, tetraploid maize and *Z. perennis* (9475) to produce polyploid hybrids, and those synthetics are shown in Fig. 1. MP30 was an allotriploid hybrid, produced by crossing wf9 with 9475. MP40 was an allotetraploid hybrid, derived from a cross between Twf9 and 9475. MM30 was an autotriploid hybrid, produced through crossing between wf9 and Twf9. Carbol fuchsin staining was used to confirm that polyploids had been created with whole set of parental chromosomes accurately, and dual-color genomic in situ hybridization *(GISH)* was followed to authenticate the chromosome complements and composition in hybrids. The results confirmed that autotriploid maize MM30 possessed thirty maize chromosomes with 2n=3x=30, allotriploid maize MP30 (2n=3x=10) had 10 maize and 20 *Z. perennis* chromosomes, while allotetraploid maize MP40 (2n=4x=20) was consisted of 20 maize and 20 *Z. perennis* chromosomes (Fig. 2). Furthermore, we did not observe any chromosomal recombination in F_1 hybrids. The hybrids were subjected to detailed meiotic analysis after confirming their genomic constitutions.

Chromosome pairing in diploid maize and *Z. perennis*

Diploid maize genome exhibited regular meiosis and the most frequently observed meiotic configuration was 10II (Fig. 3a_1; Table 4), but quadrivalents were also seen in a few PMCs, which suggested that a limited homology existed between "A" and "B" sub-genomes. The most prevalent meiotic configuration of *Z. perennis* was 10II+5IV (34.83%), and an average pairing configuration was of 0.18I+10.46II+0.13III+4.62IV (Table 4). Univalents and trivalents were rarely seen in the *Z. perennis* genome, and the prevalent numbers of bivalents and quadrivalents were ten (37.31%) and five (40.30%) with the range of 3–18 and 1–8, respectively (Fig. 3b, b_1; Table 4). The RCC of *Z. perennis* genome was 1.13, and significantly higher than that of the maize genome (0.48), as shown in Table 1.

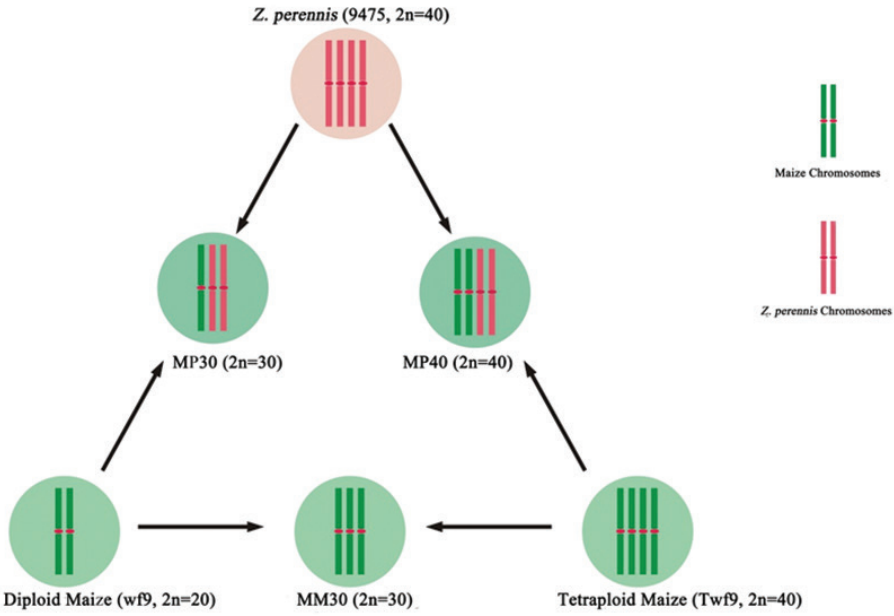

Figure 1. The schematic sketch of "U" triangle presents the production strategy of polyploid hybrids from one-way crosses of diploid and tetraploid parent (wf9, Twf9 and 9475) . The maize and *Z. perennis* cytoplasm are represented by light green and light pink circles, respectively. The dense green and dense pink strips represent maize and *Z. perennis* chromosomes, respectively and central red marks represents centromere of both types of chromosome

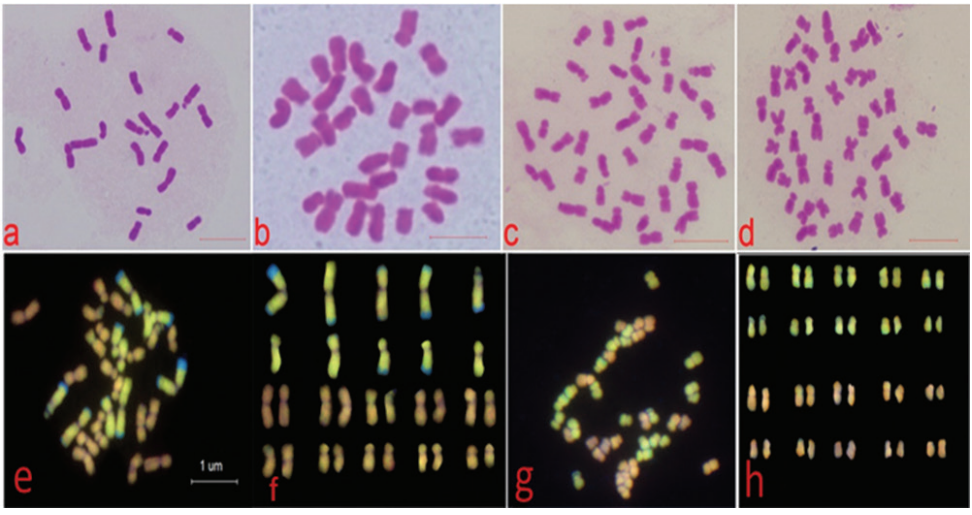

Figure 2. Composition of chromosomes in hybrids revealed by carbol fuchsin staining and GISH. **a, b, c, d** represents chromosome counts of wf9, MM30, Twf9, and *Z. perennis*. **e + f** and **g + h** represent chromosomal composition of MP30 and MP40, respectively. Yellow and pink signals represent maize and *Z. perennis* genome, respectively. All bars = 10 μm. The blue terminal ends of maize chromosomes represent maize knobs (intensely stained with DAPI).

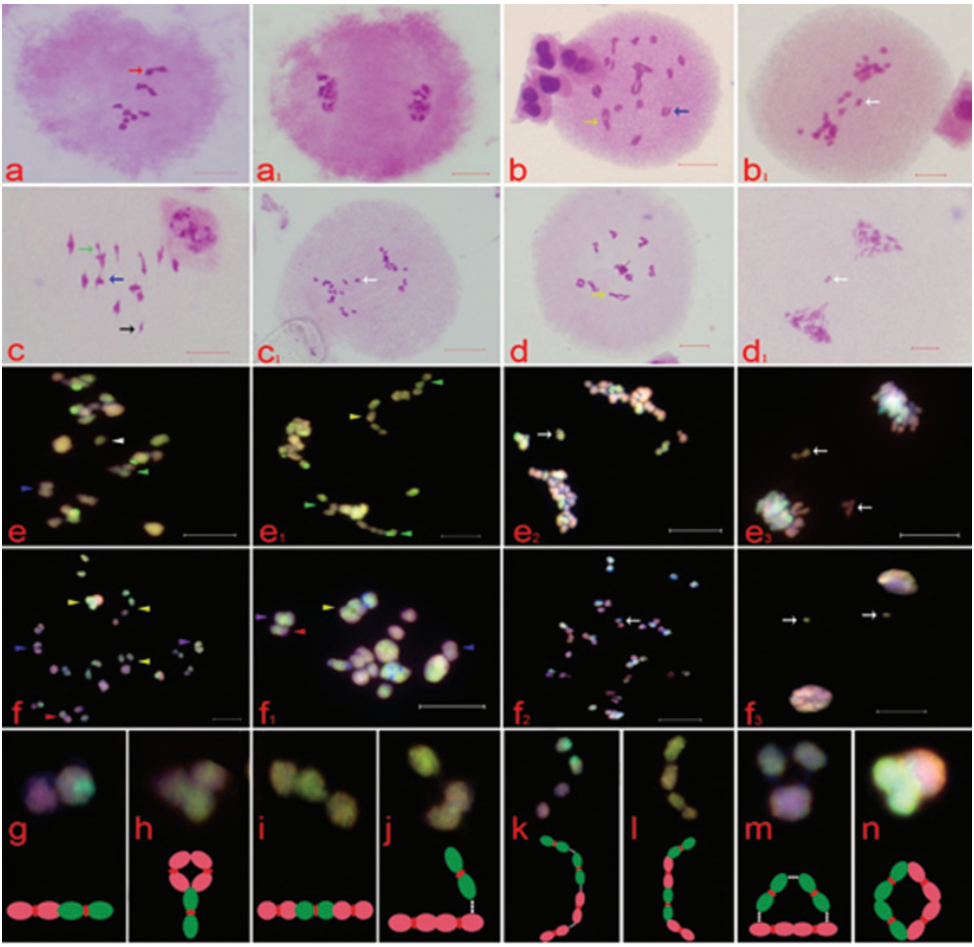

Figure 3. Chromosome pairing analysis of parents and hybrids. **a, b, c, d, e (e_1), f (f_1)** represent diakinesis of wf9, 9475, MM30, Twf9, MP30 and MP40, respectively. **a_1, b_1, c_1, d_1, e_2, f_2** represent meiotic anaphaseIand **e3, f3** represent meiotic telophaseI. Black arrow represents univalent, blue arrow represents bivalent, green arrow represents trivalent, yellow arrow represents quadrivalent, red arrow represents quadrivalent in diploid maize. White triangle represents univalent of maize genome. *Z. perennis* and maize autosyndetic bivalents are shown by blue and purple triangles, respectively. The allosyndetic bivalents, which were composed of one maize and one *Z. perennis* chromosome are represented by red triangles. The allosyndetic trivalents consisting of one maize and two *Z. perennis* chromosomes are represented by green triangles. Allosyndetic quadrivalents composed of two maize and two *Z.perennis* chromosomes are indicated by yellow triangle, while white arrow indicates lagging chromosomes **g, h, i, j, k, l, m, n** show different pairing types with the models below. Yellow and pink signals represent maize and *Z. perennis* genomes, respectively. All Bars = 10 μm.

Chromosome pairing in synthetic triploids

The most prevalent meiotic configuration of MM30 was 1I+4II+7III (29.67%) with the average of 0.71I+3.31II+7.19III+0.28IV, while 10III (11.72%) was also found in some PMCs (Table 4), and the lagging chromosomes were frequently observed at first anaphase stage (Fig. $3c_1$). Nearly half of analyzed PMCs did not show univalents and the average (range) number was 0.71 (0–3). The most frequent number of bivalents was four (24.00%) with an average (range) number of 3.31 (0–9) that suggests some of the paired chromosomes in maize genome didn't share complete homology. MM30 had abundant trivalent, the most repeated number was eight (24.00%) and an average (range) number was 7.17 (2–10). On the contrary with wf9, most of PMCs in MM30 did not contain quadrivalents and an average (range) number was 0.28 (0–3), as shown in Tables 2, 4. The MM30 showed irregular meiotic behavior and its RCC was 4.87, higher than that of wf9 (Table 2).

The PMCs of MP30 frequently showed lagging chromosomes at meiotic anaphase I (Fig. $3e_2$, e_3). Dual-color genomic in situ hybridization was carried out to study cryptic chromosome pairing in synthesized allopolyploid (Fig. 3). An average pairing configuration was 4.56I+5.44II+4.73III+0.07IV (Table 4), and the most common meiotic pairing configuration was 5III+5II+5I (16.9%).Based on the result of GISH, most univalents in MP30 were from maize (I^M), and most common number was five (18.30%) with an average number (range) of 3.79 (0–8). There were also some univalents from *Z. perennis* (I^P) with most repeated number of zero (57.75%) and an average number (range) was 0.77 (0–7). Most autosyndetic bivalents in MP30 were from *Z. perennis* (II^{PP}) with the most common number of five (26.76%) and an average number (range) of 4.34 (1–7). The other autosyndetic bivalents were from maize genome (II^{MM}) with an average number of 0.25 (0–1), whereas allosyndetic bivalents (II^{MP}) were rarely seen in MP30, as their average numbers (range) was 0.85 (0–4). Most of the trivalents were allosyndetic trivalents (III^{MPP}), which were composed of one chromosome from maize and two chromosomes from *Z. perennis*, and most the frequent number was five (36.62%) with an average number of 4.55 (0–7) as shown in Table 2. These results suggest that five chromosomes from maize genome in MP30 were homologous to ten chromosomes of *Z. perennis*. Moreover, quadrivalents and autosyndetic trivalents were rarely seen in MP30 (Table 4).

For more detailed analysis of chromosomes in MP30, the configuration of III^{MPP} was examined. The configuration of III^{MPP} was not similar in all cases (Fig. 3h, i, j and Table 5). The most frequent configuration of III^{MPP} was "frying pan type" with an average number (range) of 3.23 (0–6), Fig. 3h. However, there was also another configuration that was "rod type" with an average number (range) of 1.18 (0–5), suggesting that some paired chromosomes in "A" subgenome of *Z. perennis* were discrepant homologous (Fig. 3i, j and Table 5). Additionally, there were also some allosyndetic trivalents in which maize chromosomes were associated with *Z. perennis* chromosome loosely, which suggested that evolutionary differentiation had occurred in the "A" subgenome

Table 1. Meiotic chromosome pairing in pollen mother cells (PMCs) of parents.

Parents	2n	I	II	III	IV	RCC	PMCs
wf9	20	0.01	7.31	0.01	1.33	0.48^{b}	81
9475	40	0.18	10.46	0.13	4.62	1.13^{a}	201

Note: I, II, III and IV represent univalent, bivalent, trivalent and quadrivalent, respectively. Relative chaotic coefficient (RCC) = (chromosomes number of bivalents) / (total chromosomes number - chromosomes number of bivalents).

[a, b] Groups differed significantly by x^2-test, $p<0.05$.

Table 2. Average number of meiotic chromosomes associations in PMCs of triploid hybrids verified by GISH.

Hybrids	2n	I			II				III			IV	RCC			PMCs
		Total	I^M	I^P	Total	II^{MM}	II^{PP}	II^{MP}	Total	III^{MPP}	Others	Total	Total	wf9	9475	
MM30	30	0.71^{b}	0.71^{b}	–	3.31^{b}	3.31^{a}	–	–	7.19^{a}	–		0.28^{a}	4.78^{a}	4.78^{b}	–	129
MP30	30	4.56^{a}	3.79^{a}	0.77	5.44^{a}	0.25^{b}	4.34	0.85	4.76^{b}	4.55	0.21	0.07^{b}	2.59^{b}	9.00^{a}	1.56	71

Note: I, II, III and IV represent univalent, bivalents, trivalents and quadrivalents. I^M and I^P represent maize and *Z. perennis* univalent, respectively. II^{MM} and II^{PP} represent autosyndetic bivalents of maize and *Z. perennis*, respectively. II^{MP} represents allosyndetic bivalents having one chromosome from maize genome and one chromosome from *Z. perennis*. III^{MPP} represents allosyndetic trivalents, which were composed of one chromosome from maize genome and two chromosomes from *Z. perennis* genome.

[a, b] Groups differed significantly by x^2-test, $p<0.05$.

Table 3. Average number of chromosomes associations in PMCs of tetraploid hybrids revealed by GISH.

Hybrids	2n	I			II				III	IV			RCC			PMCs
		Total	I^M	I^P	Total	II^{MM}	II^{PP}	II^{MP}	Total	Total	IV^{MMPP}	Others	Total	wf9	9475	
MM40	40	0.26^{b}	0.26^{b}	–	3.61^{b}	3.61^{b}	–	–	0.14^{a}	8.03^{a}	–	–	4.83^{a}	4.83^{a}	–	121
MP40	40	1.17^{a}	0.81^{a}	0.36	9.97^{a}	4.30^{a}	4.71	0.96	0.13^{a}	4.62^{b}	4.29	0.33	1.46^{b}	1.60^{b}	1.47	69

Note: The I, II, III and IV symbolize univalent, bivalent, trivalent and quadrivalent, and I^M and I^P represent maize and *Z. perennis* univalents, respectively. II^{MM} and II^{PP} represent bivalent composed of two chromosomes of maize and two chromosomes of *Z. perennis*, respectively. II^{MP} represents allosyndetic bivalent consists of one chromosome from maize and one chromosome from *Z. perennis*. IV^{MMPP} represents allosyndetic quadrivalents composed of two chromosomes from maize and two chromosomes from *Z. perennis*.

[a, b] groups differed significantly by x^2-test, $p<0.05$.

of maize and *Z. perennis* (Fig. 3j). Comparative analysis of MM30 and MP30 showed that the RCC of maize genome in MM30 (4.78) was lower than in MP30 (9.00) and autosyndetic bivalents of maize genome in MP30 were much lower than MM30. The *Z. perennis* chromosomes in MP30 had lower RCC than that of maize in MM30 and MP30. Thus, overall RCC of MP30 was lower than that of MM30.

Chromosome pairing in synthetic tetraploids

The most frequent meiotic configuration of MM40 was 10IV (21.67%) with the average of 0.26I+3.61II+0.14III+8.03IV (Table 4), and the lagging chromosomes in PMCs were found commonly at meiotic anaphase I. More than half of PMCs did not show univalents and trivalents with average number (ranges) for univalents and trivalents being 0.26 (0–2) and 0.14 (0–2), respectively. Moreover, commonest number of bivalents was zero (25.00%) with an average number (range) of 3.61 (0–10). MM40 had abundant quadrivalents and the most frequent number was nine (24.17%) with an average (range) number of 8.03 (4–10), as shown in Tables 3, 4. These results also suggest that some paired chromosomes in maize didn't share complete homology. The MM40 showed anomalous meiosis and its RCC was 4.83, higher than wf9 (Table 3).

The most common meiotic configurations of allotetraploid maize (MP40) were 8II+6IV (15.94%) and 12II+4IV (15.94%), and the lagging chromosomes found at meiotic anaphase I (Fig. 3f2, f3). However, a rare meiotic configuration 10II+5IV (13.04%) was also observed, with an average number of 1.17I+9.97II+0.13III+4.62IV. More than half of PMCs didn't show univalents, and average (range) number of maize and *Z. perennis* genome's univalents were 0.81 (0–8) and 0.36 (0–7), respectively. The autosyndetic bivalents from maize and *Z. perennis* were frequently appeared and the most prevalent number was five (24.64%; 27.54%) for both, while average number (ranges) for maize and *Z. perennis* were 4.30 (2–7) and 4.71 (1–9), respectively. Most PMCs did not possess allosyndetic bivalents and the average number (range) was 0.96 (0–4). The trivalents existed in several PMCs; in addition, maize autosyndetic trivalents were not found. Most PMCs did not contain autosyndetic quadrivalents and most allosyndetic quadrivalents ($IV^{MMPP)}$ consisted of two chromosomes from *Z. perennis* and two chromosome from maize, with most prevalent number of five (24.64%) and an average number was 4.29 (1–7) as shown in Tables 3, 4. These results suggest that ten chromosomes from maize genome in allotetraploid were homologous to ten chromosomes of *Z. perennis* genome.

The detailed chromosome observation of MP40 showed that configurations of IV^{MMPP} were not similar. The most frequent configuration of IV^{MMPP} was of "ring type" with an average number (range) of 0.72 (0–6), while another form of "rod type" also found (Fig. 3k, l, m, n and Table 5). In addition, some allosyndetic quadrivalents were also seen in which maize chromosome were weekly associated with *Z. perennis* chromosomes (Fig. 3k, m). The different configurations of IV^{MMPP} suggested that some paired chromosomes of "B" subgenome of maize and *Z. perennis* were discrepantly homologous. Similarly, the "B" subgenome has undergone considerable evolutionary differentiation in the genus *Zea* but the "A" subgenome has undergone only slight differentiation.

The comparative analysis of MM40 and MP40 revealed that the RCC of maize genome in MM40 was higher than MP40, suggested that a limited homology between maize and *Z. perennis* genomes enhance meiotic stability in maize allotetraploid. Comparative analysis between *Z. perennis* and MP40 showed higher number of bivalents and lower RCC in *Z. perennis* than MP40 and Twf9 that might be due to allopolyploid nature of *Z. perennis* (Naranjo et al. 1994).

Table 4. Meiotic chromosome pairings in PMCs of parents and hybrids.

Materials			wf9		9475		MM30		MP30		MM40		MP40		
Average configuration			0.01I+7.31II +0.01III+1.33IV		0.18I+10.46II +0.13III+4.62IV		0.71I +3.31II +7.19III+0.28IV		4.56I+5.44II +4.73III+0.07IV		0.26I+3.61II +0.14III+8.03IV			1.17I+9.97II +0.13III+4.62IV	
Frequent configurations			10II (35.00)		10II+5IV (34.83)		1I+4II+7III (29.67)	10III (11.72)	5I+5II+5III (16.9)		10IV (21.67)		8II+6IV (15.94)	12II+4IV (15.94)	10II+5IV (13.04)
Frequent valents (Range)	I (%)	Total	0 (98.77)		0 (88.56)		0 (51.9) –		5 (25.3)		0 (80.83)		0 (52.1)		
		I^{M}	0 (98.77)	(0–1)			0 (51.9)	(0–3)	5 (18.3)	(0–8)	0 (80.83)	(0–2)	0(55.07)	(0–5)	
		I^{P}	–	–	0 (88.56) –	(0–4)–	–		0(57.75)	(0–7)	–	–	0(71.01)	(0–2)	
	II (%)	Total	8 (33.33)		10 (37.3)		4 (24.0)		5 (29.58)		0 (25.00)		10 (17.3)		
		II^{MM}	8 (33.33)	(0–10)			4 (24.0)	(0–9)	0(74.65)	(0–1)	0 (25.00)	(0–10)	5(24.64)	(2–7)	
		II^{PP}	–	–	10 (37.31) –	15	–		5(26.76)	(1–7)	–	–	5(27.54)	(1–9)	
		II^{MP}	–	–	–	–	–		0(52.11)	(0–4)	–	–	0(59.42)	(0–4)	
	III (%)	Total	0 (98.77)		0 (90.55)		8 (24.0)		5 (33.8)		0 (86.67)		0(88.41)		
		III^{MMM}	0 (98.77)	(0–1)			8 (24.0)	(2–10)	0 (95.2)	(0–1)	0 (86.67)	(0–2)	0 (100)		
		III^{PPP}	–	–	0 (90.55)	(0–3) –	–		0(90.14)	(0–1)	–	–	0(94.20)	(0–1)	
		III^{MMP}	–	–	–	–	–		0(91.55)	(0–3)	–	–	0(97.10)	(0–1)	
		III^{MPP}	–	–	–	–	–		5(36.62)	(0–7)	–	–	0(94.20)	(0–1)	
	IV (%)	Total	1 (34.57)		5 (40.30)		0 (77.5)		0 (92.96)		9 (24.17)		5 (26.09)		
		IV^{MMMM}	1 (34.57)	(0–5)			0 (77.5)	(0–3)	0 (100)		9 (24.17)	(4–10)	0(81.16)	(0–3)	
		IV^{PPPP}	–	–	5 (40.30) –	(1–8)	–		0(97.18)	(0–1)	–	–	0(89.86)	(0–1)	
		IV^{MMPP}	–	–	–	–	–		0(95.77)	(0–1)	–	–	5(24.64)	(1–7)	

Table 5. Types of allosyndetic trivalents and quadrivalents.

Valente types		MP30	MP40
IIIfry-pan type	Mean (Range)	3.23 (0–6)	–
	Frequency (%)	3 (32.39)	–
		4 (18.31)	–
		5 (22.54)	–
IIIrod type	Mean (Range)	1.18 (0–5)	–
	Frequency (%)	0 (33.80)	–
		1 (30.99)	–
		2 (23.94)	–
IVring type	Mean (Range)	–	2.78 (0–6)
	Frequency (%)	–	2 (26.76)
		–	3 (26.76)
		–	1,4,5 (14.08)
IVrod type	Mean (Range)	–	0.72 (0–6)
	Frequency (%)	–	0 (45.07)
		–	1 (38.03)
		–	2 (11.27)

Table 6. Plant material used in the study.

Scientific name	Source	Accession	Chromosome number
Zea perennis	CIMMYT	9475	2n = 40
Zea mays ssp. *mays*	USDA	wf9	2n = 20
Zea mays ssp. *mays*	USDA	Twf9	2n = 40

Discussion

Dissimilar meiotic stabilities between maize autopolyploids and allopolyploids

Polyploidy is a state in which more than two sets of chromosomes coexist in one nucleus. It is a widespread phenomenon in plants and is considered to be a major force in plant evolution (Comai 2000, Lavania 2013). The autopolyploids have three or more homologous chromosomes and can form multivalents during meiosis so that meiotic stability is a bottleneck for their sexual reproduction (Soltis and Soltis 2000). In allopolyploids, homologous genome causes autosyndesis, while different genome in one nucleus can hardly induce allosyndesis as well. The diploid paring model is strictly enforced in allopolyploids in which parental genomes have limited affinity (Wu et al. 2001). However, to the best of our knowledge, there are also some allopolyploids that possess homologous or homoeologous chromosomes between parental genomes, thus they do not follow diploid paring model strictly. They form univalents and/or multivalents that cause meiotic confusion and genetic instability (Eckardt 2001). Furthermore, meiosis of autopolyploids is generally less stable than allopolyploids. In our study, we also found the consistent observations with those that have been previously reported. The RCC of MP30 and MP40 was lower than MM30 and MM40, respectively that might be as a

result of discrepant homology that exists between maize and *Z. perennis* chromosomes (Wang et al. 2011). The number of autosyndetic bivalents in allotetraploid maize was higher than autotetraploid maize. On the contrary, the RCC of maize allopolyploids was higher than diploid maize. Perhaps the reason for higher RCC of allopolyploids is occurrence of homoeologous chromosomes between maize and *Z. perennis* genome, so that allosyndesis and multivalency can be expected (González et al. 2006).

Genetic relationship between maize and *Z. perennis*

The maize genome has a large number of duplicated genes according to theory of tetraploid origin (Ahn et al. 1993, Wendel 2000, Gaut et al. 2000, Doerks et al. 2002, Wang et al. 2011) (Doerks et al. 2002; Molina et al. 2013). For diploid maize and diploid teosinte hybrids (2n=20), the two groups of five bivalents were observed at meiosis, which suggested that genome can be divided into "AA" and "BB" sections (Naranjo et al. 1994). Quadrivalents were observed in diploid maize (Tables 1, 4) and the same phenomenon was also reported previously (Molina et al. 2013). These results suggested that "A" and "B" subgenomes are homoeologous in maize. The earliest suggested genomic formula for *Z. mays* ssp. *mays* was A_2A_2 B_2B_2 and for *Z. perennis* is $A_1'A_1'A_1''A_1''$ C_1C_1 C_2C_2. Additionally, homoeologous genomes usually do not pair, maybe due to the presence of *Ph*-like gene (Poggio et al. 1990). Hexavalent were not seen in triploid maize, as well as octavalent were also not observed in tetraploid maize (Table 4), which suggested a limited paring between "A" and "B" subgenomes at higher ploidy levels. In addition, the most frequent number (range) of autosyndetic trivalent in PMCs of MM30 was eight (2–10) and common number (range) of autosyndetic quadrivalent in MM40 was nine (4–10), that indicated some paired chromosomes in "A" subgenome or in "B" subgenome have been differentiated. Furthermore, the *Z. perennis* belongs to another section of genus *Zea* (Iltis and Doebley 1980) and has a hypothetical octoploid origin that was also confirmed by genetic linkage maps (Moore et al. 1995). The maximum number of bivalents and quadrivalents in PMCs were 18 and 8, respectively suggesting that "A" subgenome have been subjected to evolutionary differentiation but homologous relationship still exists in "B" subgenomes. Hexavalent and octavalent were not seen in *Z. perennis*, as well as in colchicine treated doubled diploid maize. However colchicine treatment could initiate paring of "B" subgenome with a maximum number of 10IV. These results revealed that homoeologous relationship exists in "B" subgenome of *Z. perennis* (Molina et al. 2013).

Chromosome pairings between maize and *Z. perennis* was observed in PMCs of two allopolyploids. We found univalents, bivalents and multivalents and allosyndetic valents at different levels during meiosis. The meiotic configuration of MP30 was $5I^{M}+5II^{PP}+5III^{MPP}$, while univalents I^{M}, bivalents II^{PP} and allosyndetic trivalents III^{MPP} were common. The meiotic configuration of MP40 was $5II^{MM}$ $+5II^{PP}$ +5IV, while bivalents II^{MM}, bivalents II^{PP} and allosyndetic quadrivalents IV^{MMPP} were frequently observed (Table 4), which reveals that genetic relationship exists in maize and *Z. perennis*. Additionally, ten chromosomes of "A" subgenome in maize are homologous

with twenty chromosomes of "A" subgenome in *Z. perennis*, on the contrary, "B" subgenome has been highly differentiated (Fukunaga et al. 2005; Swanson-Wagner et al. 2010). Comparatively, the levels and frequency of auto- and allosyndesis for each genome as well as meiotic configuration were not in well agreement with previous findings (Naranjo et al. 1994, Poggio et al. 1999, González et al. 2006, Molina et al. 2013). The possible explanations include: (a) Different maize cultivars were used and genomes of those maize cultivars might be slightly different; (b) Different circumstance and different maize cultivar, as well as different genome composition in polyploids might influence the expression of *Phs1* and *Pam1* genes that play an important role in homologous chromosomes pairing (Golubovskaya et al. 2002, Ronceret et al. 2009, Lukaszewski and Kopecký 2010, Feddermann et al. 2010, Ianiri et al. 2014). In addition, autosyndetic bivalents of the maize genome were rarely seen in MP30 and maximum number of III^{MPP} in MPCs was seven. It suggested that homology of ten maize chromosomes in MP30 was extremely low and homologous relationship exists in "B" subgenome, as well as homoeologous relationship existed in "A" subgenome of maize and *Z. perennis.* The *Z. perennis* chromosomes in MP30 had lower RCC than RCC of maize chromosomes in MM30 and MP30, thus, overall RCC of MP30 was lower than MM30, which suggested the limited homology between maize and *Z. perennis* enhance overall meiotic stability in maize allotriploid. In MP40, the maximum number of autosyndetic bivalents, which belong to maize and *Z. perennis* genome, was seven and nine, respectively; otherwise, the maximum number of IV^{MMPP} was also seven. The minimum number of IV^{MMPP} was one, which suggested that "A" subgenome between maize and *Z. perennis* shared partial homology, and maximum number of IV^{MMPP} seven suggested that limited homologous relationship existed in "B" subgenome of maize and *Z. perennis*.

Detailed examination of allosyndetic trivalents (III^{MPP}) revealed that there were not only "frying pan type", but "rod type" also existed in allotriploid with a maximum number of five (Table 5). These results are consistent to previous study (González et al. 2006). In addition, prevalent configuration of allosyndetic quadrivalents (IV^{MMPP}) was not only the "rod type" but "ring type" was also found (Table 5). In III^{MPP} and IV^{MMPP}, the degree of chromosome pairing was variable e.g. relatively tight chromosome pairing between maize and *Z. perennis*, maize and maize, *Z. perennis* and *Z. perennis* were observed, while the loose chromosome pairing between maize and *Z. perennis*, maize and maize, *Z. perennis* and *Z. perennis* was also seen (Figure 3j, k, m). These results suggested that "A" subgenomes in two parents underwent evolutionary differentiation but at lower degree as compared to "B" subgenomes. The schematic genomic formula representation of maize, *Z. perennis* and their hybrids is built (Fig. 4).

The maize and *Z. perennis* cytoplasm are represented by light green and light pink circles, respectively. The blue and green strips represent maize chromosomes, while pink, orange, brown and dark red strips represent *Z. perennis* chromosomes. The centromeres in middle of all chromosomes are labeled red; moreover, both of them have red centromere in the middle. Black parentheses represent paired homologous chromosomes and red parentheses represent expected chromosomes combinations.

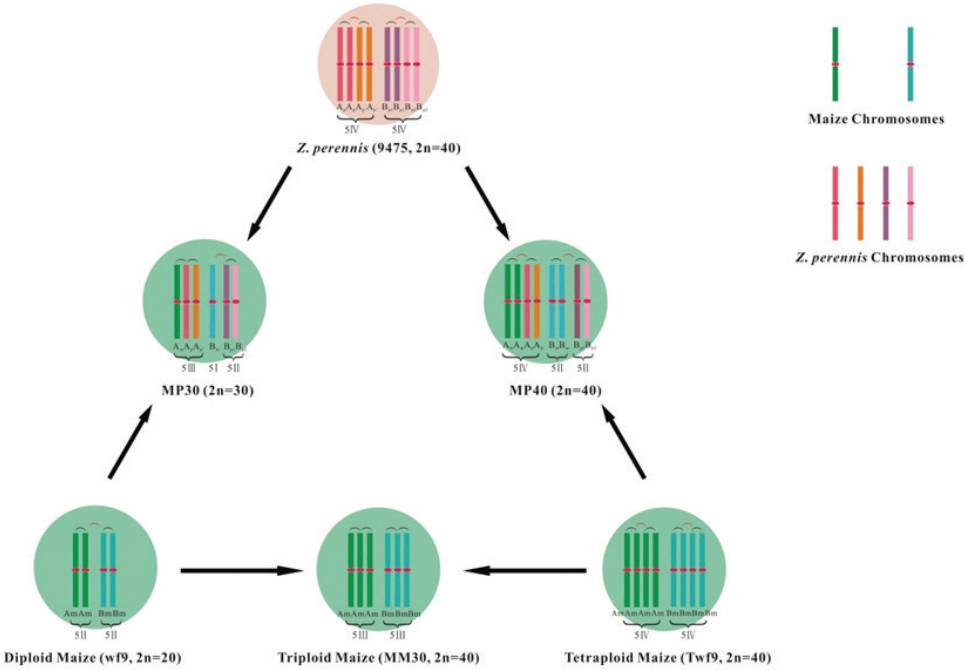

Figure 4. Schematic genomic diagram of maize, *Z. perennis* and hybrids.

Expected evolutionary mechanism of maize and *Z. perennis*

In previous studies, two possible evolutionary mechanisms for maize and *Z. perennis* genome were proposed: First, the genome composition of "AABB" hybrids was an ancestral cross between two closely related "AA" and "BB" genomes that was followed by evolutionary fractionation; Second, *Zea* species were originated through chromosome duplication, followed by homoeologous genomes "A" and "B" differentiation (Molina et al. 2013). However, both hypotheses cannot explain differences within "A" and "B" subgenomes in both maize and *Z. perennis* appropriately. Thus, we put forward a third possible evolutionary mechanism: Firstly, duplication event occurred in two closely related species with "AA" and "BB" genome, as a consequence, autopolyploid of "AAAA" and "BBBB" genome were formed. Secondly, evolutionary fractionation took place in two autopolyploids that turned both genomes into "AAAA" and "BBB$_1$B$_2$". thirdly, crossing between those two autopolyploids followed by probable limited compatible coevolution in "A" and "B" subgenomes led to the formation of "$A_mA_mB_mB_m$" genome with barely deviation of maize intra-subgenomes; Lastly, second duplication event of hybrids "AABB" followed by differential degree of evolutionary fractionation in "A" and "B" subgenomes, led to creation of *Z. perennis* with genome of "$A_pA_pA_pA_pB_{p1}B_{p1}B_{p2}B_{p2}$" (Fig. 5). Moreover, as "A" genome has higher homology between maize and *Z. perennis* than "B" genome and also suffers less genes losses, as well as, has higher expression for genes located in this subgenome, while "B" genome

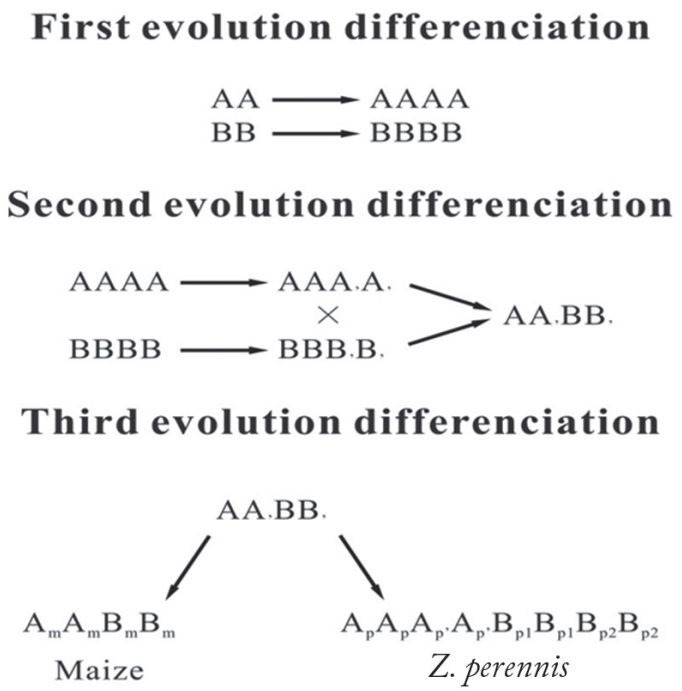

Figure 5. Possible mechanisms of genome differentiation in maize and *Z. perennis.*

has a faster differentiation. So it is concluded that "B" subgenome was responsible for species isolation, domestication, and as well as further speciation in genus *Zea* (Freeling and Thomas 2006, Swanson-Wagner et al. 2010, Schnable et al. 2011).

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Supplementary material 1

Supplementary tables.

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