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Banding cytogenetics of the vulnerable species Houbara bustard (Otidiformes) and comparative analysis with the Domestic fowl

Leila Mahiddine-Aoudjit[1,2], Ouahida Boucekkine[3], Kafia Ladjali-Mohammedi[1]

1 *University of Sciences and Technology Houari Boumediene (USTHB), Faculty of Biological Sciences, Laboratory of Cellular and Molecular Biology, Team of Developmental Genetics, PO box 32 El-Alia, Bab-Ezzouar, 16110 Algiers, Algeria* **2** *University of M'hamed Bougara of Boumerdes, Faculty of Sciences, Department of Biology, Avenue de l'Indépendance, 35 000 Boumerdès, Algeria* **3** *The General Direction of Forests, Ben Aknoun, Algiers, Algeria*

Corresponding author: *Leila Mahiddine-Aoudjit* (mahiddinel1@gmail.com)

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Abstract

The Houbara bustard *Chlamydotis undulata* (Jacquin, 1784) is an emblematic and endangered bird of steppes and desert spaces of North Africa. This species belonging to Otidiformes is recognized as vulnerable by the International Union for Nature Conservation.

The critical situation of this species and the revision of its classification on the tree of birds encouraged the authors to start accumulating chromosome data. For that, we propose the GTG- and RBG-banded karyotypes of the Houbara bustard prepared from primary fibroblast cell cultures. The first eight autosomal pairs and sex chromosomes have been described and compared to those of the domestic fowl *Gallus domesticus* (Linnaeus, 1758). The diploid number has been estimated as 78 chromosomes with 8 macrochromosomes pairs and 30 microchromosomes pairs, attesting of the stability of chromosome number in avian karyotypes.

The description of the karyotype of the Houbara is of crucial importance for the management of the reproduction of this species in captivity. It can be used as a reference in the detection of chromosomal abnormalities, which would be responsible of the early embryonic mortalities.

Keywords

Chlamydotis undulata undulata, endangered species, GTG- and RBG- banded karyotypes, interspecific comparison

Introduction

With approximately 10,699 species, birds represent the class of Tetrapoda with the highest number of species (http://www.worldbirdnames.org). This class presents a certain number of particularities such as the presence of feathers, flight and a small genome (about 1.45 pg that represents 1/3 of the mammalian genome). As well, the avian karyotypes are very particular, with a very consistent diploid number. The range of variation is very wide, between 40 and 138 chromosomes, the average being from 76 to 82 for most species (Takagi and Sasaki 1974, De Boer 1976, De Boer and Sinoo 1984, Christidis 1990). About 18–23% of the avian genome is represented by microchromosomes (Smith and Burt 1998). However, they contain more than 50% of the genes (Smith et al. 2000), they are GC-rich (Auer et al. 1987) and enriched for CpG islands (McQueen et al. 1996). Moreover, the female represents the heterogametic sex named ZW and the male the homogametic sex ZZ (Christidis 1990).

Despite the diploid number that seems to be stable in birds, the avian genome has undergone multiple evolutionary events. Chromosome fission has previously been reported as being a factor of evolutionary change (Takagi and Sasaki 1974, Tegelström et al. 1983, Perry et al. 2004). Nevertheless, other regions of the genome can be subject to frequent breakage (Skinner and Griffin 2012). The analysis of macrochromosomes of chicken, turkey and zebra finch has provided evidence that the presence of hotspots facilitates chromosomal rearrangements (Kretschmer et al. 2018a).

Besides, phylogenetic analysis of 48 bird species representing all Neoaves orders was conducted and the analysis identified a first divergence of the Neoaves into two independent lines named Passerea and Columbea, without forgetting the emergence of the new order Otidiformes to which the endangered species Houbara bustard is now affiliated (Jarvis et al. 2014). In fact, before this study, and for a long time, the Houbara bustard was affiliated to the order of Gruiformes whose classification has been revised (Wetmore 1960, Roselaar 1980, Cracraft 1981, Olson 1985, Sibley and Ahlquist 1990, Sibley et al. 1993, Houde et al. 1997, Livezey 1998, Fain and Houde 2004).

The Houbara bustard is an emblematic bird of the large steppe areas and desert spaces of North Africa and the Middle East (Heim de Balsac and Mayaud 1962). Two monophyletic sister groups of bustards are considered (Tobias et al. 2010, Del Hoyo et al. 2014). Firstly, the Asian Houbara bustard *Chlamydotis macqueenii* (Gray, 1832) is found in the east of Egypt, from the Arabian Peninsula and Pakistan to Central Asia. On the other hand, the North African Houbara bustard *Chlamydotis undulata* is subdivided into two subspecies: *Chlamydotis undulata undulata* (Jacquin, 1784) extending from Morocco and northern Mauritania through Algeria, to the west of Egypt, and *Chlamydotis undulata fuerteventurae* (Rothschild & Hartert, 1894) which is endemic to the Canary Islands. Over the past 30 years, illegal harvesting of bustards and degradation of their environment has increased throughout its range. This has led to significant population decline in Africa and in Asia (Le Cuziat et al. 2005, Azafzaf et al. 2005). Therefore, the International Union for the Conservation of Nature has listed this species on the Red List as vulnerable (http://www.iucnredlist.org).

No description of the karyotype of the Houbara bustard has been reported to date. The only known cytogenetic data are a metaphase of this species, without a precise description of the chromosomes, which was reported in the study that allowed the development of chromosome paints and BACs for the characterization of inter- and intrachromosomal rearrangements of avian microchromosomes (Lithgow et al. 2014). However, several molecular studies based on the characterisation of microsatellites have been conducted in the Houbara bustard for the genotyping of individuals (Chbel et al. 2002, Pitra et al. 2004, Arif et al. 2012).

Here we describe the macrochromosomes of the Houbara bustard in morphologic GTG bands and dynamic RBG bands. Morphometric measurements were used to facilitate the classification of smaller macrochromosomes. The obtained banding pattern in Houbara bustard chromosomes was compared with that of the chicken chromosomes, in order to determine the presence of chromosomal rearrangements that would have occurred during speciation.

Material and methods

Embryos

Fifteen Houbara bustard embryos aged between 8 and 19 days were collected from Emirati Bird Breeding Centre for Conservation EBBCC (32°55'40.54"N, 0°32'33.71"E) in the region of Abiod Sidi Cheikh (Wilaya d'El-Bayadh, south of Algeria) during the breeding season. The embryos were obtained in accordance with the authorization from the General Direction of Forests of Algeria (N°30BOG/N°80DPFF/DGF-18).

Cell culture and chromosomes preparations

Fibroblasts were isolated from Houbara bustard embryos by trypsinisation (trypsin solution 0.05%, Sigma) and incubated at 41 °C with RPMI 1640 culture medium (20 mM HEPES, Gibco) supplemented with 10% of fetal calf serum (FCS) (Gibco), 1% L-Glutamine (Sigma), 1% penicillin, streptomycin and fungizone (Sigma). Cultures of fibroblasts were synchronised as described by Ladjali et al. (1995), using a double thymidine block during S phase in order to increase the yield of metaphase and early metaphase cells. The 5-bromo-2'-deoxyuridine (BrdU) (final concentration: 10 µg/ml, Sigma) was added to prepare chromosomes to the RBG staining (Zakharov and Egolina 1968, Ladjali et al. 1995).

As a sufficient number of refractive mitotic cells was observed (after 6–8 h), they were treated with colchicine (final concentration: 0.05 µg/ml, Sigma) for 5 min at 37 °C. Cells were harvested by the addition of 0.05% trypsin-EDTA (Gibco). Hypotonic treatment was performed. In fact, cells were suspended for 13 min at 37 °C in hypotonic solution 1:5 (FCS- distilled water). Fixation and spreading were performed using standard methods (Dutrillaux and Couturier 1981, Ladjali et al. 1995).

Staining procedures

The revelation of the structural GTG bands is based on enzymatic digestion with proteolysis (Seabright 1971, Ladjali et al. 1995). Aged (3–10 days) slides were incubated for 14 seconds in a fresh trypsin (Sigma) solution (final concentration: 0.25%) and stained for 10 min with 6% Giemsa (Fluka) solution (Ladjali et al. 1995).

The RBG-FPG staining (R-bands obtained with BrdU by Fluorochrome-photolysis and Giemsa staining) was performed as previously described (Romagnano and Richer 1984, Schmid et al. 1989, Viegas-Péquignot et al. 1989, Ladjali et al. 1995). The slides were incubated in a solution of Hoechst 33258 (final concentration: 0.01 mg/ml) during 20 min, followed by an incubation in fresh 2×SSC solution at a distance of 15 cm from blacklight blue (NARVA, LT18W/073) during 90 min. The slides were immersed in Earle's buffer (pH= 6.5) at 87 °C for 10 minutes and stained with 6% Giemsa (Fluka) for 10 min.

To make a comparison with chromosomes of the chicken, GTG banding was also performed on previously frozen chicken chromosome preparations.

Chromosome classification and measurement

Slides were analysed using Axio Scope A1 (Zeiss) and thirty metaphases with decondensed chromosomes were selected and photographed with CoolCube1 (Metasystems). Houbara bustard chromosomes were classified according to the International System of Standardised Avian Karyotypes (ISSAK) (Ladjali-Mohammedi et al. 1999).

The first eight pairs of chromosomes of the Houbara bustard and the domestic fowl and their sex chromosomes were measured using KARYOTYPE 2.0 software (Altinordu et al. 2016). The rest of the chromosomes were not measured because of their very small size. Different parameters of morphometry are presented: length of the long (*q*) and the short (*p*) arms, total length (p+q), arm ratio (r= q/p) and the centromeric index (CI% = p/p+q × 100).

Results

The diploid number of the Houbara bustard has been estimated as 78 chromosomes by examination of full metaphases (Fig. 1). Although the use of double synchronisation can produce decondensed chromosomes, classic cytogenetic techniques alone do not accurately count and describe microchromosomes. Often, they are dispersed outside the metaphases during spreading or hidden by other chromosomes (Tegelström and Ryttman 1981).

In this study, we propose the karyotype of the Houbara bustard with morphological GTG-banded chromosomes (Fig. 2A) and dynamic RBG-banded chromosomes (Fig. 2B). Partial ideograms of the Houbara bustard have been established (Fig. 3A, B) to describe precisely the chromosomes (Table 1).

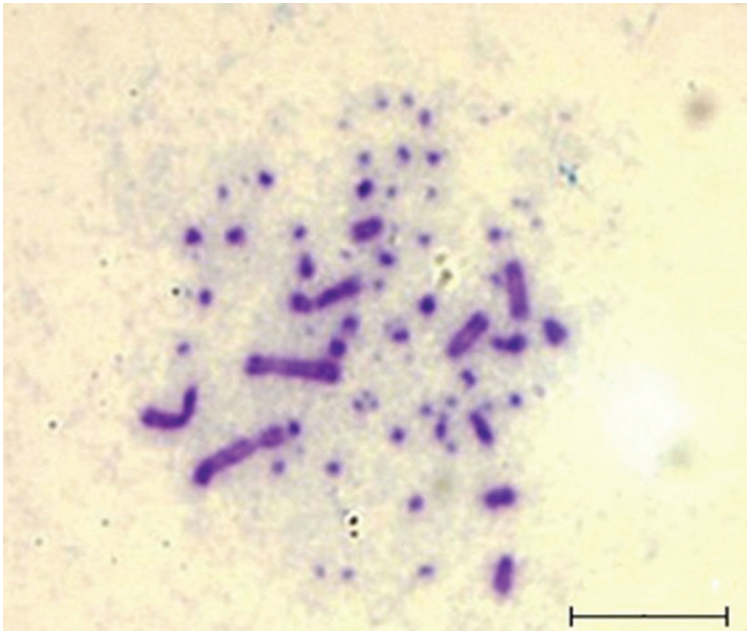


Figure 1. Metaphase of the Houbara bustard showing macrochromosomes and microchromosomes with Giemsa staining. Scale bar: 5 μm.

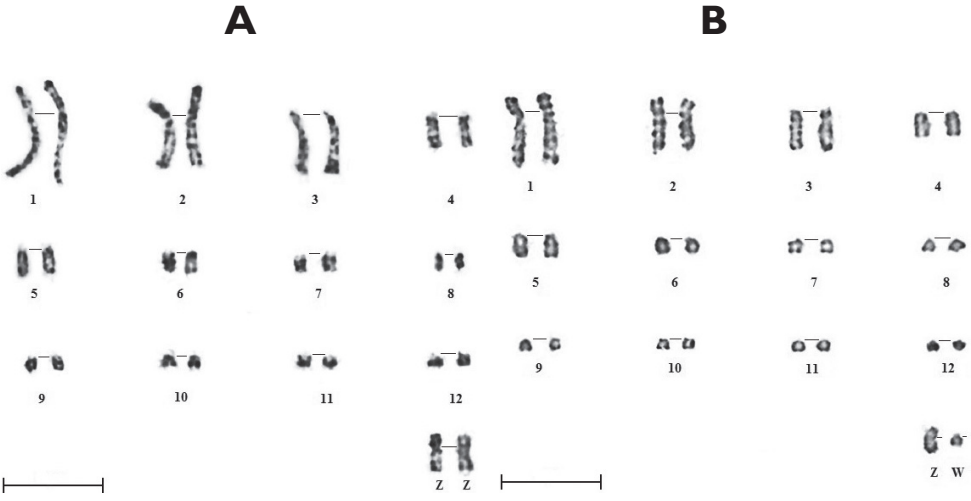


Figure 2. GTG (**A**) and RBG (**B**) karyotypes of the first 12 and sex chromosomes of Houbara bustard *Chlamydotis undulata undulata*. Scale bar: 5 μm.

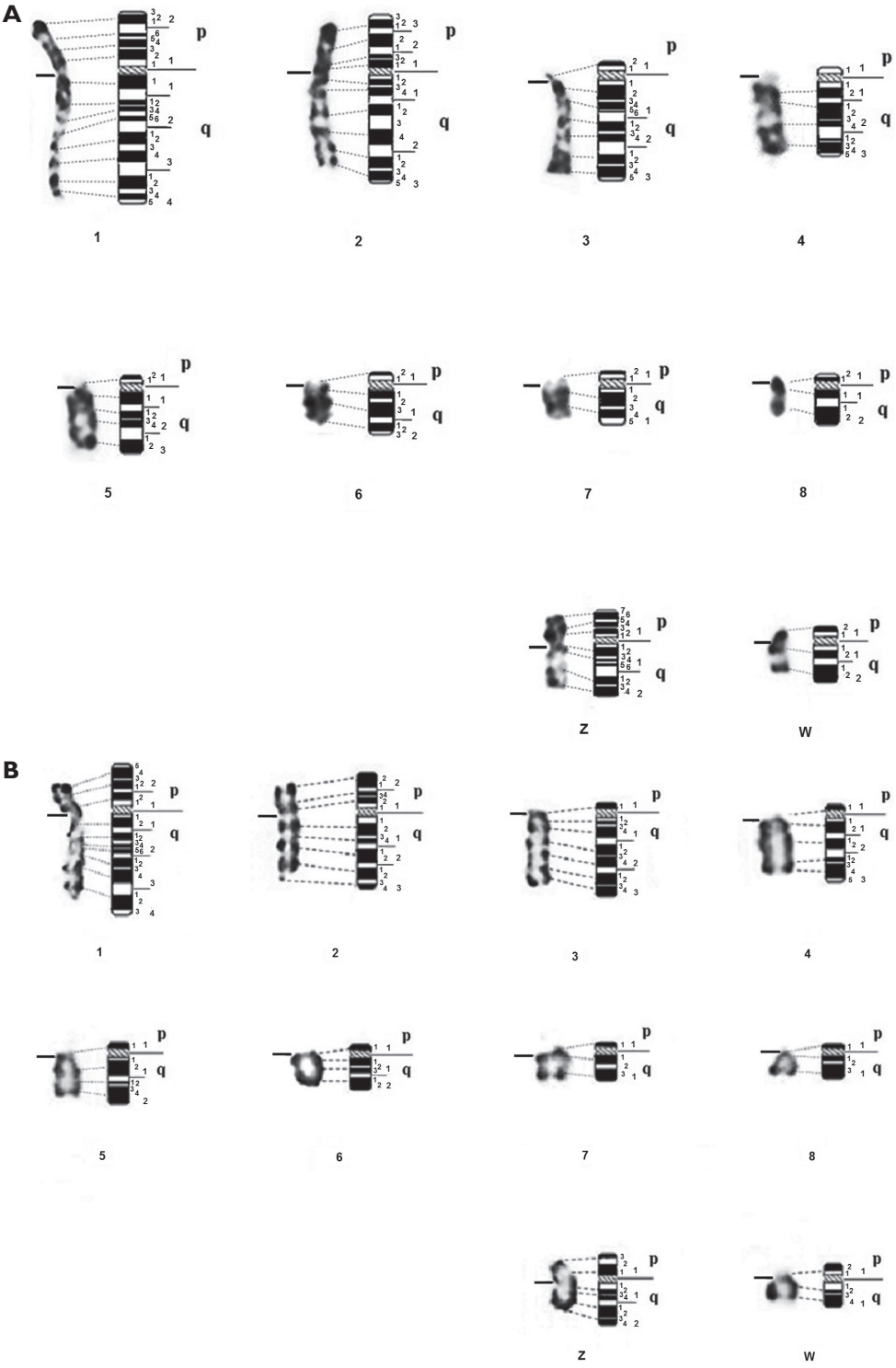
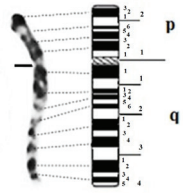
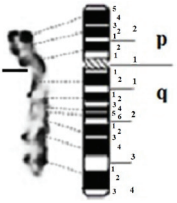
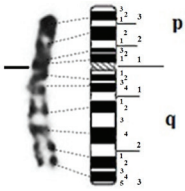
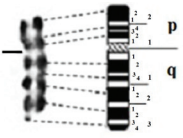
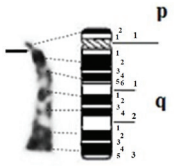
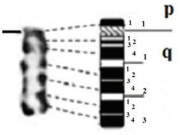
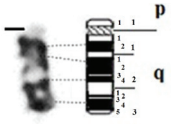
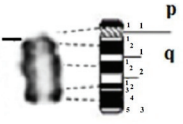
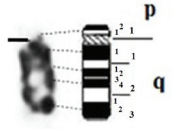
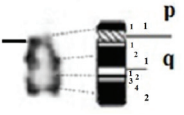
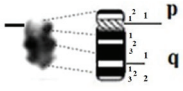
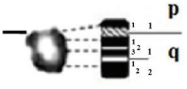
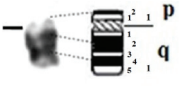
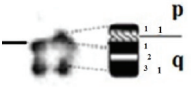
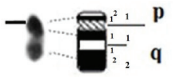
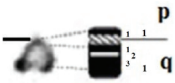
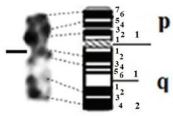
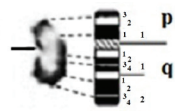
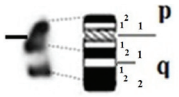


Figure 3. GTG-banded (**A**) and RBG-banded (**B**) macrochromosomes, sex chromosomes ZW and their corresponding ideograms of Houbara bustard *Chlamydotis undulata undulata*.

Table 1. Description of GTG and RBG bands on macrochromosomes and sex chromosomes ZW of the Houbara bustard.

Chromosomes	GTG bands / ideograms	Description	RBG bands / ideograms	Description
1 **submetacentric**		**– Short arm (p)** 2 regions 9 bands **– Long arm (q)** 4 regions 16 bands		**– Short arm (p)** 2 regions 7 bands **– Long arm (q)** 4 regions 15 bands
2 **submetacentric**		**– Short arm (p)** 3 regions 8 bands **– Long arm (q)** 3 regions 13 bands		**– Short arm (p)** 2 regions 6 bands **– Long arm (q)** 3 regions 10 bands
3 **acrocentric**		**– Short arm (p)** 1 region 2 bands **– Long arm (q)** 3 regions 14 bands		**– Short arm (p)** 1 region 1 band **– Long arm (q)** 3 regions 12 bands
4 **acrocentric**		**– Short arm (p)** 1 region 1 band **– Long arm (q)** 3 regions 11 bands		**– Short arm (p)** 1 region 1 band **– Long arm (q)** 3 regions 9 bands
5 **acrocentric**		**– Short arm (p)** 1 regions 2 bands **– Long arm (q)** 3 regions 7 bands		**– Short arm (p)** 1 regions 1 band **– Long arm (q)** 2 regions 6 bands
6 **acrocentric**		**– Short arm (p)** 1 region 2 bands **– Long arm (q)** 2 regions 6 bands		**– Short arm (p)** 1 region 1 band **– Long arm (q)** 2 regions 5 bands
7 **acrocentric**		**– Short arm (p)** 1 region 2 bands **– Long arm (q)** 1 region 5 bands		**– Short arm (p)** 1 region 1 band **– Long arm (q)** 1 region 3 bands

Chromosomes	GTG bands / ideograms	Description	RBG bands / ideograms	Description
8 **acrocentric**		**– Short arm (p)** 1 region 2 bands **– Long arm (q)** 2 regions 3 bands		**– Short arm (p)** 1 region 1 band **– Long arm (q)** 1 region 3 bands
Z **submetacentric**		**– Short arm (p)** 1 region 7 bands **– Long arm (q)** 2 regions 10 bands		**– Short arm (p)** 1 region 3 bands **– Long arm (q)** 2 regions 8 bands
W **submetacentric**		**- Short arm (p)** 1 regions 2 bands **- Long arm (q)** 2 regions 4 bands		**- Short arm (p)** 1 regions 2 bands **- Long arm (q)** 2 regions 4 bands

The haploid karyotype of the first 10 autosomes and the sex chromosomes Z and W of the Houbara bustard corresponds to 130 GTG bands and 104 RBG bands. The number of bands obtained for this species is lower than that reported in the chicken for the same chromosome number (209 bands G and 182 bands R) (Ladjali et al. 1995).

In order to compare the chromosomes of the Houbara bustard and those of the domestic fowl, the first eight macrochromosomes and the sex chromosomes ZW of these two species have been measured (Table 2).

Discussion

The diploid number has been estimated in the Houbara bustard as 78 chromosomes as in many birds. Indeed, the diploid number is highly conserved with about 63% of birds with a chromosome number that varies between 74 and 86 (Christidis 1990, Rodionov 1997). The relatively unchanged nature of the diploid number amongst the majority of avian species implies that the organisation of bird karyotypes is a highly successful means of genome organisation. The karyotype of the Houbara bustard belonging to the new order of Otidiformes (Del Hoyo et al. 2014) appears very similar to the ancestral karyotype of birds with 8 pairs of macrochromosomes and 30 pairs of microchromosomes.

The size of the first eight pairs of chromosomes of the Houbara bustard varies between 4 µm (chromosome 1) and 0.67 µm (chromosome 8). This average size of Houbara bustard macrochromosomes is lower to the estimated size (3 to 6 µm) for

Table 2. Measurements of eight macrochromosomes and sex chromosomes ZW of Houbara bustard *Chlamydotis undulata undulata* and Domestic fowl *Gallus domesticus*. **Chr**: chromosome, **q**: long arm, **p**: short arm, **p+q**: total length, **r**: arm ratio q/p**, CI %**: centromeric index=$p/p+q \times 100$. Lengths are given in micrometer (μm) ± standard deviation.

Chr	Houbara bustard					Domestic fowl				
	q	p	p+q	r	CI %	q	p	p+q	r	CI %
1	2.93±0.57	1.19±0.29	4.12±0.81	2.46	29 %	6.69±1.26	3.97±0.70	10.66±1.85	1.69	37%
2	2.08±0.41	0.95±0.24	3.02±0.63	2.19	31 %	5.35±0.71	2.76±0.54	8.11±1.24	1.94	34%
3	2.15±0.47	0.12±0.05	2.26±0.49	18.50	5 %	5.42±0.66	0.36±0.05	5.77±0.62	15.18	6%
4	1.33±0.25	0.12±0.05	1.45±0.27	10.98	8 %	3.72±0.59	0.96±0.15	4.69±0.72	3.86	20%
5	1.21±0.20	0.09±0.04	1.29±0.22	13.37	7 %	2.71±0.43	0.29±0.12	3.00±0.51	9.39	9%
6	0.87±0.16	0.06±0.06	0.92±0.18	15.86	6 %	1.53±0.16	0.07±0.10	1.60±0.24	21.83	4%
7	0.79±0.11	0.02±0.03	0.77±0.12	41.89	2 %	1.28±0.23	0.40±0.05	1.68±0.28	3.18	24%
8	0.67±0.13	0.01±0.02	0.67±0.12	92.52	1 %	0.89±0.14	0.61±0.05	1.49±0.15	1.46	41%
Z	0.99±0.20	0.46±0.13	1.44±0.31	2.17	32 %	2.30±0.34	2.04±0.29	4.34±0.63	1.12	47%
W	0.59±0.15	0.20±0.07	0.78±0.17	3.01	25 %	0.95±0.35	0.60±0.28	1.55±0.60	1.59	39%

avian macrochromosomes (Rodionov 1996). A significant decrease in the size of the bustard chromosomes after the third pair has been noted (Table 2).

The comparison of the first eight pairs and sex chromosomes of the Houbara bustard with those of the chicken revealed the presence of similarities as well as differences between these two species. Indeed the karyotype of the chicken conserves the ancestral karyotype of many avian orders (Guttenbach et al. 2003, Shibusawa et al. 2004, Griffin et al. 2007).

The first three chromosomes of the Houbara bustard are morphologically similar to those of the chicken. Chromosome 1 and 2 are submetacentric and chromosome 3 is acrocentric. These results are in agreement with those of Takagi and Sasaki (1974), who showed the conservation of the first three chromosomes in nine different orders of birds and in different species belonging to the family of Gruidae (Order Gruiformes) known to be the family closest to Otididae (Belterman and De Boer 1984).

The comparison of the chromosome 1 of Houbara bustard with that of the chicken revealed a difference in the size of the p- arm of chromosome 1 (1p) which is shorter in the bustard (Fig. 4A). The arm ratio (q/p) is 2.46 in the chromosome 1 of the Houbara bustard while it is equal to 1.69 for that of the chicken. Furthermore, the comparison of GTG bands showed an inversion of patterns. The difference in the morphology of the chromosome 1 of Houbara bustard and the chicken, associated to the difference in GTG banding pattern in these two species, could be explained by a pericentric inversion that occurred in the chromosome 1 of the chicken, which is close to the ancestral chromosome 1 (Fig. 4A). This result must be confirmed by the use of molecular markers that will confirm this hypothesis and determine the extent of the rearrangement.

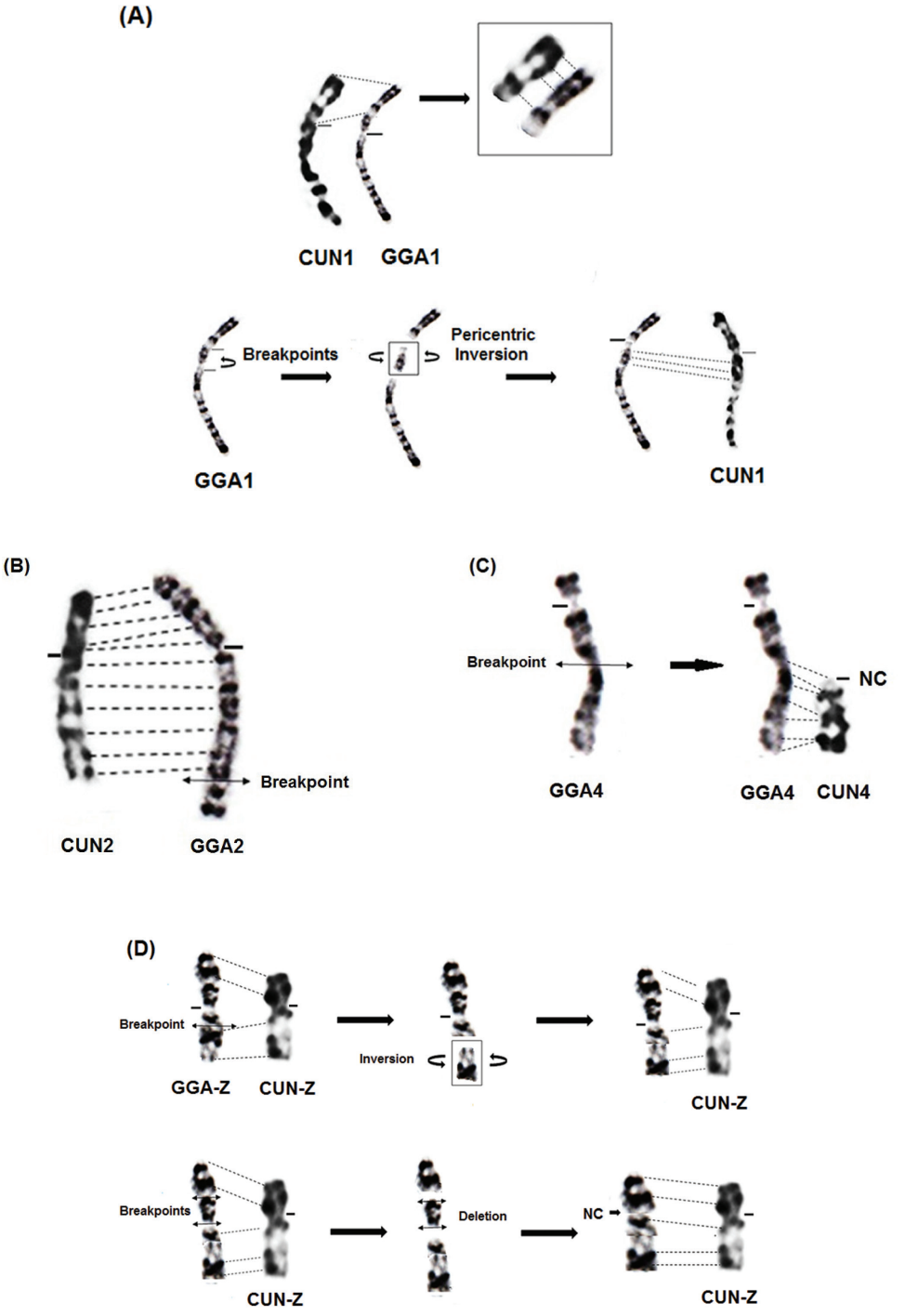


Figure 4. Representation of chromosomal rearrangements that could have occurred during the formation of the chromosome 1 (**A**), the chromosome 2 (**B**), the chromosome 4 (**C**) and the chromosome Z (**D**) of the Houbara bustard.

As for chromosomes 1 of Japanese quail and chicken, the high-resolution mapping using fluorescent *in situ* hybridisation with molecular markers on stretched chromosomes in the lampbrush showed that the difference in the morphology of this chromosome should be explained by de novo centromere formation and the hypothesis of centrometric inversion should be excluded (Zlotina et al. 2012).

Likewise, the long arm (q) of chromosome 2 of the Houbara bustard is shorter than that of the chicken and that would be the consequence of a terminal fission (Fig. 4B), and this distal part lost would eventually be involved in another independent event of chromosomal rearrangement (Furo et al. 2015). Because the bustard is phylogenetically distant from the domestic fowl, the different rearrangements are not visible (Prum et al 2015). The chromosome 3 is acrocentric and is apparently conserved in the two species.

The chromosome 4 of the Houbara bustard is acrocentric while that of the chicken is telocentric. Their arm ratios are equal to 10.98 and 3.86 respectively (Table 2). The comparison of the banding pattern reveals that chromosome 4 of the bustard corresponds to the terminal (q) arm of that of the chicken (Fig. 4C). This would be the consequence of a loss of the short arm (p) and a part of the long arm (q) during evolution. In fact, the hybridisation of chicken macrochromosomes on the metaphases of avian species from Anseriformes, Gruiformes and Passeriformes, revealed the hybridisation of the GGA4 on three different chromosomes in Gruiformes. The large region of GGA4 corresponds to the short arm of the metacentric chromosome of the coot FAT4 (*Fulica atra*, Gruiformes) and the remaining part is found on two other chromosomes (FAT 7 and FAT 13) (Nanda et al. 2011).

The chromosome 5 of the Houbara bustard is acrocentric like that of the chicken but it seems to have lost the terminal part of the long arm. Indeed, chromosome 5 of the Houbara bustard measures 1.29 ± 0.22 µm and that of the chicken 3 ± 0.51 µm. This could be explained by a fission event that would have occurred during evolution. In fact, chromosome 5 of the chicken appears to be distributed on the short arm (p) of chromosome 4 of the coot (FAT4) and on microchromosome 12 (FAT12) (Nanda et al. 2011). The most noticeable is the association between GGA4 / GGA5 in this species of coot (*Fulica atra)*, since chromosomes 4 and 5 of the chicken hybridized on the same FAT4 chromosome. This proves the presence of several fission and fusion events in Gruiformes (Nanda et al. 2011).

In contrast to chromosome 6 of the bustard which appears to be morphologically similar to that of the chicken, the chromosomes 7 and 8 of these two species are different. Houbara bustard chromosomes 7 and 8 are acrocentric whereas they are, respectively, telocentric and submetacentric in chicken (Ladjali-Mohammedi et al. 1999). Their arm ratios are, respectively, 41.89 and 92.52 in the bustard and equal to 3.18 and 1.46 in the chicken (Table 2). The morphological difference of these two chromosomes between the Houbara bustard and the chicken could be explained by the formation of neocentromere, or the occurrence of a pericentric inversion. The different suggestions for chromosomal rearrangements must be confirmed by molecular investigations in order to elucidate the phylogenetic relationship between the Houbara bustard and the Domestic fowl, as has already been done in other species.

Finally, the sex chromosome Z of the Houbara bustard differs from that of the chicken. It is submetacentric in the first species and metacentric in the second. The arm ratio (q/p) is 2.17 for the chromosome Z of the bustard while it is equal to 1.12 for that of the chicken (Table 2). In addition to the position of the centromere that is different in the chromosome Z of the chicken, we noted the loss of the p1.1 → p1.3 region corresponding to the chicken chromosome Z, as well as an inversion in the order of the GTG bands in the distal part of the long arm (Fig. 4D).

Chromosome Z that is metacentric in chicken appears to be submetacentric in many other species of Galliformes (Nanda et al. 2008). Also, a terminal inversion has been reported on chromosome Z of Chukar partridge (Ouchia-Benissad and Ladjali-Mohammedi 2018).

Despite the conservation of this chromosome in its totality during evolution, it appears to be subject to intrachromosomal rearrangements (Griffin et al. 2007, Nanda et al. 2008). This was confirmed by the inverted order of five orthologous genes (DMRT1, GHR, CHRNB3, ALDOB, B4GALT1) located on the chicken Z chromosome and mapped in eight other species (Nanda et al. 2008).

The W chromosome is submetacentric in the Houbara bustard. It appears to be morphologically similar to that of the chicken. Depending on its size, it can be classified between chromosome 6 and 7. The W chromosome in birds and reptiles seems to have degenerated during evolution. It is physically small, with a high proportion of heterochromatin (Ellegren 2011) that it is supposed to come from the accumulation of repetitive sequences and their conservation during evolution (Schartl et al. 2016).

In conclusion, this analysis of the chromosomes of the endangered Houbara bustard provided a precise description of a part of its karyotype in GTG and RBG bands. Chromosomal informations have been obtained for the newly established Otidiformes order. The identification of microchromosomes by fluorescence *in situ* hybridisation of specific BAC clones of chicken chromosomes is conceivable to complete the description of the karyotype of this species. The various rearrangements suggested must be confirmed by molecular studies of BAC clones localisation and chromosome painting for a better knowledge of avian karyotypes evolution.

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SHORT COMMUNICATION

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Two types of highly ordered micro- and macrochromosome arrangement in metaphase plates of butterflies (Lepidoptera)

Vladimir A. Lukhtanov[1,2]

1 *Department of Karyosystematics, Zoological Institute of the Russian Academy of Sciences, Universitetskaya nab. 1, St. Petersburg 199034, Russia* **2** *Department of Entomology, St. Petersburg State University, Universitetskaya nab. 7/9, St. Petersburg 199034, Russia*

Corresponding author: *Vladimir A. Lukhtanov* (lukhtanov@mail.ru)

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Abstract

In karyotype of many organisms, chromosomes form two distinct size groups: macrochromosomes and microchromosomes. During cell divisions, the position of the macro- and microchromosomes is often ordered within metaphase plate. In many reptiles, amphibians, birds, insects of the orthopteran family Tettigoniidae and in some plants, a so called "reptilian" type organization is found, with microchromosomes situated in the center of metaphase plate and with macrochromosomes situated at the periphery. An opposite, "lepidopteran" type is known in butterflies and moths (i.e. in the order Lepidoptera) and is characterized by macrochromosomes situated in the center and by microchromosomes situated at the periphery. The anomalous arrangement found in Lepidoptera was previously explained by holocentric organization of their chromosomes. Here I analyse the structure of meiotic metaphase I plates in ithomiine butterfly, *Forbestra olivencia* (H. Bates, 1862) (Nymphalidae, Danainae, Ithomiini) which has a clear "reptilian" organization, contrary to previous observations in Lepidoptera. In this species large bivalents (i.e. macrochromosomes) form a regular peripheral circle, whereas the minute bivalents (i.e. microchromosomes) occupy the center of this circle. The reasons and possible mechanisms resulting in two drastically different spatial chromosome organization in butterflies are discussed.

Keywords

Asymmetrical karyotype, DNA barcoding, bivalent, COI, holocentric, holokinetic, kinetochore, meiosis, metaphase, spindle, spermatocyte, Lepidoptera, Nymphalidae, Danainae, Ithomiini, Peru

Introduction

The spatial organization of chromosomes and chromosome bivalents may be highly ordered during interphase and cell divisions (White 1973, Cremer et al. 1982, 2017, Solé et al. 2017, Sarrate et al. 2018). For example, a special ("reptilian" according to White 1973) type of the ordered metaphase plate organization was found in taxa with asymmetrical karyotype including groups of micro- and macrochromosomes, e. g. in many reptiles, amphibians, birds, in some insects and in some plants (White 1973, Lewitsky 1976). In these taxa, the microchromosomes occupy position in the center of metaphase rosette inside of the spindle, and the macrochromosomes are situated at the periphery and form a ring around the spindle.

In our previous work we demonstrated that butterflies and moths have inverted spatial karyotype organization at the first male meiotic metaphase, with larger chromosomes situated in the center and smaller chromosomes situated at the periphery (Lukhtanov and Dantchenko 2002). The latter observation has been confirmed in numerous subsequent studies (e.g. Vershinina and Lukhtanov 2010, Przybyłowicz et al. 2014, Vershinina et al. 2015, Lukhtanov 2015).

After our research was published (Lukhtanov and Dantchenko 2002), a study appeared focused on the chromosome evolution in Neotropical Danainae and Ithomiinae (Lepidoptera, Nymphalidae) (Brown et al. 2004). Although the spatial organization of chromosomes was out of the focus of this study and was not discussed at all, the article provided numerous microphotographs that demonstrated the central position of larger bivalents at the male first meiotic metaphase, but also a single figure (fig. 23, *Forbestra proceris* Weymer, 1883) in which this order was inverted. Therefore, during the expedition of St. Petersburg University to Peru in 2013, I paid special attention to collecting representatives of the genus *Forbestra* R. Fox, 1967 as well as other taxa of the tribe Ithomiini. Description of karyotypes and bivalent spatial organization in three species of the Ithomiini is given below.

Material and methods

Samples

Karyotypes were studied in two specimens of *Forbestra olivencia olivencia* (H. Bates, 1862) (form *huallaga* Staudinger, [1884]), four specimens of *Oleria gunilla serdolis* (Haensch, 1909) and two specimens of *Godyris dircenna* (C. Felder & R. Felder, 1865). The information on localities where the specimens were collected is presented in caption to the Figure 1. The samples were identified through their comparison with the butterflies figured at Butterflies of America site (https://www.butterfliesofamerica.com/L/Nymphalidae.htm). The specimens are deposited in the Zoological Institute of the Russian Academy of Sciences, St. Petersburg, Russia.

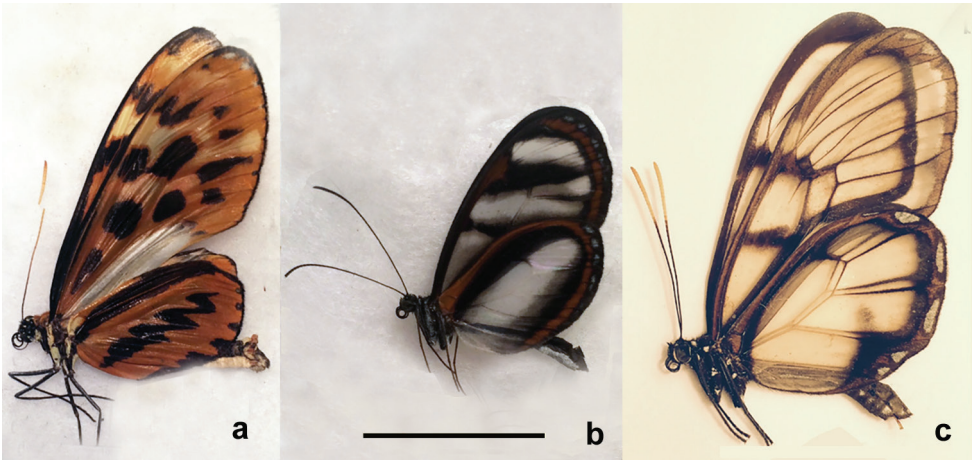

Figure 1. The analyzed samples of Ithomiini **a** *Forbestra olivencia olivencia* (Bates, 1862) (form *hual-laga* Staudinger, [1884]), NOB003-17 (CCDB-23376_A03, 2013_A109), Peru, 50 km S of Ikitos, 04°11'47"S; 73°28'39"W, 114 m, 30 August 2013, V. Lukhtanov leg **b** *Oleria gunilla serdolis* (Haensch, 1909), NOB012-17 (CCDB-23376_A102, 2013_A127), Peru, Tingo Maria, 09°21'02"S; 76°03'21"W, 835 m, 3 September 2013, V.Lukhtanov leg **c** *Godyris dircenna* (C. Felder & R. Felder, 1865), NOB026-17 (CCDB-23376 C02, 2013_A145), 09°29'43"S; 75°58'01"W, 800 m, 6 September 2013, V.Lukhtanov leg. Scale bar: 20 mm in all figures.

Standard *COI* barcodes (658-bp 5' segment of mitochondrial cytochrome oxidase subunit I) were obtained for the sample NOB003-17 (CCDB-23376_A03, 2013_A109) of *F. olivencia*, for the samples NOB008-17 (CCDB-23376_A08, 2013_A121), NOB009-17 (CCDB-23376_A09, 2013_A122), NOB010-17 (CCDB-23376_A10, 2013_A123) and NOB012-17 (CCDB-23376_A102, 2013_A127) of *O. gunilla* and for the sample NOB026-17 (CCDB-23376 C02, 2013_A145) of *G. dircenna*. The barcodes were obtained at the Canadian Centre for DNA Barcoding (CCDB, Biodiversity Institute of Ontario, University of Guelph) using standard protocols (Hajibabaei et al. 2005, Ivanova et al. 2006 and deWaard et al. 2008). These DNA barcodes were used to confirm the species identification (http://boldsystems.org/index.php/IDS_OpenIdEngine).

Chromosomal analysis

Gonads were removed from the abdomen and placed into freshly prepared fixative (3:1; 96% ethanol and glacial acetic acid) directly after capturing the butterfly in the field. Testes were stored in the fixative for 3–36 months at +4 °C. Then the gonads were stained in 2% acetic orcein for 30–60 days at +18–20 °C. Spatial organization of meiotic bivalents was studied in intact (not squashed) spermatocytes using protocol described in Vishnevskaya et al. (2016).

Results and discussion

The meiotic karyotype of *Forbestra olivencia olivencia* was found to include 9 large and 1 medium elements (interpreted as 10 macrobivalents) and 5 very small elements (interpreted as 5 microbivalents) (Fig. 2a). Thus, the karyotype is similar (but not exactly identical) to the previously studied karyotypes of *F. olivencia* and *F. proceris* for which a low basic haploid number (nine) and a variable amount (from one to eight) additional minute chromosome elements were reported (Brown et al. 2004).

In all studied metaphase plates the same picture was observed: the species showed the distinct disk-like structure of the metaphase I plates, having all the bivalents inside the meiotic spindle. The structure of the intact metaphase I plates was simple and stable. Large bivalents (i.e. pairs of macrochromosomes) formed a more or less regular peripheral circle, whereas the minute bivalents (i.e. pairs of microchromosomes) occupied the center of this circle. Thus, *F. olivencia* has typical "reptilian" type (the terminology of White 1973) of the spatial organization of bivalents.

The meiotic karyotype of *Oleria gunilla serdolis* was found to include 11 bivalents (Fig. 2b) confirming results of the previous cytogenetic analysis of this species (Brown et al. 2004). Two bivalents were significantly larger than the other nine ones. These two larger bivalents occupied the position in the center of metaphase plate in accordance with observation on other Lepidoptera (Lukhtanov and Dantchenko 2002). Thus, *O. gunilla serdolis* has the typical "lepidopteran" type of the spatial organization of bivalents.

The meiotic karyotype of *Godyris dircenna* was found to include 36 bivalents (Fig. 2c) confirming results of the previous cytogenetic analysis of this species (Brown et al. 2004). The bivalents had different sizes and shapes. One bivalent was slightly larger than the rest ones and had a tendency to be located in the center of metaphase plate in accordance with observation on other Lepidoptera (Lukhtanov and Dantchenko 2002). Thus, *Godyris dircenna* has the "lepidopteran" type of the spatial organization of bivalents.

The spatial arrangement of the large and small bivalents in *Forbestra olivencia* is fundamentally different from the structure found in this and in previous studies in other butterflies, e.g. in *Polyommatus* (*Agrodiaetus*) *dagestanicus* (Forster, 1960) (Fig. 2d). In the latter species the bivalents show a regular concentric arrangement with the largest bivalent situated in the central part of the rounded metaphase plate. The medium bivalents formed two internal rings and the microelements formed an external, peripheral ring of the metaphase plate.

Previously we hypothesized that the lepidopteran type of the metaphase plate organization in butterflies can be explained by holocentric nature of their chromosomes, which are characterized by kinetic activity distributed along almost the entire chromosome length (Lukhtanov et al. 2018). We suggested that during congregation at the prometaphase stage there was a centripetal movement of bivalents made by a pulling force directed to the centre of the metaphase plate transverse to spindle. The magnitude of this force may be depending on the quantity of microtubules contacted to the chromosome and, correspondingly, on the kinetochore size. Therefore, large bi-

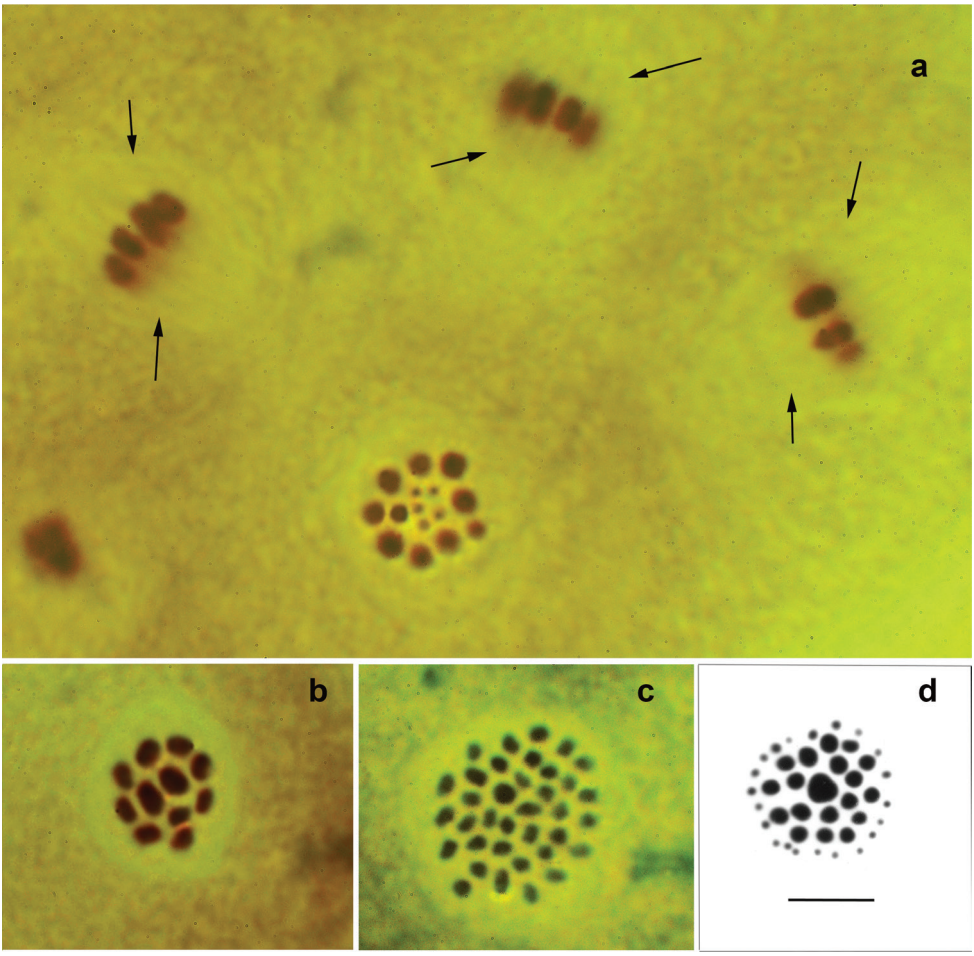

Figure 2. Intact male meiotic metaphase I plates in Ithomiini (**a–c**) and Polyommatini (**d**) butterflies **a** *Forbestra olivencia olivencia* (Bates, 1862), n=15 (10 macrobivalents + 5 microbivalents); three metaphase plates on the top are from the side (=equatorial) view; the plate on the bottom is from pole view; meiotic spindle is indicated by arrows **b** *Oleria gunilla serdolis* (Haensch, 1909), n=11 **c** *Godyris dircenna* (C. Felder et R. Felder, 1865), n=36 **d** *Polyommatus* (*Agrodiaetus*) *dagestanicus* (Forster, 1960), n=40 (19 macrobivalents + 21 microbivalents) (from Lukhtanov and Dantchenko 2002). Scale bar: 10 μ in all figures.

valents having large kinetochores were situated in the central part of metaphase plate (Lukhtanov and Dantchenko 2002). However, the unusual organization of metaphase plate in *F. olivencia* demonstrates that the suggested explanation is not universal and not necessarily true. Recently, McClure et al. (2017) hypothesized that some Ithomiini butterflies had an atypical holocentric chromosomes, and each anaphasic chromosome seemed to be driven by a single microtubule, and not by multiple ones. This hypothesis, if it is true, can explain the unusual structure of metaphase plate in *Forbestra olivencia,* but first this hypothesis itself must be tested.

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RESEARCH ARTICLE

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Constitutive heterochromatin heteromorphism in the Neotropical armored catfish *Hypostomus regani* (Ihering, 1905) (Loricariidae, Hypostominae) from the Paraguay River basin (Mato Grosso do Sul, Brazil)

Greicy Ellen de Brito Ferreira[1], Ligia Magrinelli Barbosa[1], Ana Camila Prizon-Nakajima[1], Suzana de Paiva[1], Margarida Maria de Rossi Vieira[2], Raquel Bozini Gallo[3], Luciana Andreia Borin-Carvalho[1], Renata da Rosa[3], Cláudio Henrique Zawadzki[4], Isabel Cristina Martins dos Santos[1], Ana Luiza de Brito Portela-Castro[1]

1 *Universidade Estadual de Maringá, Departamento de Biotecnologia, Genética e Biologia Celular, 87020-900 Maringá, Paraná, Brazil* **2** *Universidade Estadual do Mato Grosso do Sul, Centro de Ciências Biológicas, 79400-000 Coxim, Mato Grosso do Sul, Brazil* **3** *Universidade Estadual de Londrina, Departamento de Biologia Geral, 86057-970, Londrina, Paraná, Brazil* **4** *Universidade Estadual de Maringá, Departamento de Biologia/Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura (Nupélia), 87020-900 Maringá, Paraná, Brazil*

Corresponding author: *Ana Luiza de Brito Portela-Castro* (albpcastro@nupelia.uem.br)

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Abstract

A cytogenetic analysis based on the integration of a number of different chromosomal methodologies, including chromosome microdissection was carried out to characterize the chromosomally polymorphic *Hypostomus regani* population from the Paraguay River basin, state of Mato Grosso do Sul in Brazil. All specimens had 2n=72 (FN=116) but two distinct karyotype formulas: karyomorph A (12m+14sm+18s+28a) and karyomorph B (13m+14sm+17st+28a). Karyomorph A and B differed only for pair 19 that consisted of two subtelocentrics in karyomorph A and a large metacentric and a subtelocentric in karyomorph B.

This heteromorphism was due to extensive heterochromatinization of the short arm of the large metacentric, as highlighted by C-banding. The microdissection of the large metacentric of pair 19 allowed the production of a probe, named HrV (*Hypostomus regani* Variant), that hybridized to the whole *p* arm of the large metacentric and the pericentromeric region of the short arm of its (subtelocentric) homologue (karyomorph B) and of both homologs of pair 19 in karyomorph A. Additional cytogenetic techniques (FISH with 18S and 5S rDNA probes, CMA_3 and DAPI staining) allowed a finer distinction of the two karyomorphs. These results reinforced the hypothesis that the novel large metacentric of *H. regani* (karyomorph B) was the result of the amplification of heterochromatin segments, which contributed to karyotypic diversification in this species.

Keywords
Chromosome painting, chromosomal polymorphism, chromosome specific probe, FISH

Introduction

Hypostomus Lacépède, 1803 is the most species-rich catfish genus in the Neotropical subfamily Hypostominae (Loricariidae), which comprises around 135 species (Zawadzki et al. 2016). The species-level taxonomy of this genus is complex, being hampered by the considerable morphological variation found in local populations and the presence of numerous cryptic species with major intraspecific variation in morphology and body pigmentation patterns (Dias and Zawadzki 2018).

The genus *Hypostomus* is cytogenetically highly diversified, with a wide range of diploid (2n=64–84) and fundamental (FN = 82–121) numbers as well as, inter- and intra-specific differences in the number and position of 18S and 5S rDNA clusters (Bueno et al. 2014, Lorscheider et al. 2015, Rubert et al. 2016). In fish, the amount and position of the heterochromatic blocks have been related to the occurrence of chromosomal rearrangements or amplifications, especially during the origin and evolution of specific chromosomes, such as sex chromosomes and B chromosomes (Vicari et al. 2010). However, although scarce, available data on the heterochromatin of *Hypostomus* species indicate a great diversity in its amount and constitution (Artoni and Bertollo 1999, Kavalco et al. 2004, Bittencourt et al. 2011a, Traldi et al. 2012, Baumgärtner et al. 2014, Kamei et al. 2017).

One of the first analyses of the genomic distribution of heterochromatin in *Hypostomus* revealed two general distribution patterns: (i) species with a small amount of heterochromatin, located in subterminal and/or centromeric regions, and (ii) species with a large number of heterochromatic regions located in interstitial sites in several acrocentric chromosomes (Artoni and Bertollo 1999, 2001).

Regarding the molecular composition of heterochromatin in *Hypostomus* species, analysis has demonstrated CG- or AT-rich content (Chromomycin A_3 or Mithramycin A and 4′-6-Diamin-2-Phenylindole–CMA_3/DAPI) revealing heterogeneity in these regions, which suggests important implications for the karyotype evolution of this genus (see e.g. Artoni and Bertollo 2001, Kavalco et al. 2004, Rubert et al. 2008, 2011, Milhomem et al. 2010, Maurutto et al. 2013) and other groups of fishes, such as *Gymnotus* Linnaeus, 1758 (Scacchetti et al. 2011), *Bryconamericus* Eigenmann, 1907

(da Silva et al. 2014) and *Ancistrus* Kner, 1854 (Prizon et al. 2016). In addition, analyses with restriction enzymes banding (as AluI, BamHI, HaeIII and DdeI), associated with C-banding technique, revealed heterogeneous heterochromatin patterns in four populations of *Hypostomus* prope *unae* (Steindachner, 1878) (Bittencourt et al. 2011a) and the existence of distinct evolutionary units in allopatric populations of *Hypostomus* prope *wulchereri* (Günther, 1864) (Bittencourt et al. 2011b).

The ichthyofauna of the Paraguay River is still poorly-studied, although 14 *Hypostomus* species are known to occur in this basin (Cardoso et al. 2016). *Hypostomus regani* (Ihering, 1905) was originally described for specimens collected in the Piracicaba River (Upper Paraná River basin), but it has also been reported for the Upper Paraguay basin (Zawadzki et al. 2014). Therefore, the present study was aimed to investigate the chromosomal characteristics of the *Hypostomus regani* population from the Upper Paraguay basin which had a chromosomal polymorphism. Some specimens of this population possessed a chromosome heteromorphism due to constitutive heterochromatin expansion in the *p* arm of one of the homologues of pair 19. Both classical and molecular cytogenetic (including chromosome painting) techniques were applied to investigate this heteromorphism.

Material and methods

Samples and chromosome preparations

Forty-eight *Hypostomus regani* specimens (23 males, 20 females, and 5 specimens of unidentified sex) were collected from Onça Stream (18°32'18"S, 54°33'43"W), a tributary of the Taquari River, which is part of the Paraguay River basin, located in the municipality of Coxim, in Mato Grosso do Sul State, Brazil. Sampling was authorized by SISBIO (the Brazilian Federal Biodiversity Information and Authorization System), under license number 40510-1. Voucher specimens were deposited in Nupélia (Núcleo de Pesquisa em Limnologia, Ictiologia e Aquicultura) ichthyological collection of Maringá State University (NUP 9820).

Mitotic chromosomes were obtained from kidney cells by the "air drying" method described by Bertollo et al. (1978) at UEMS-UCX (Universidade Estadual do Mato Grosso do Sul, Coxim city) Laboratory. Active NORs sites were evidenced by silver nitrate impregnation (Howell and Black 1980) and the constitutive heterochromatin was detected by the C-banding technique (Sumner 1972) with modifications in the coloring, as proposed by Lui et al. (2012). Fluorescence in situ Hybridization (FISH) with 18S and 5S rDNA probes was based on Pinkel et al. (1986) protocol. The 18S rDNA probe was obtained from *Prochilodus argenteus* Spix & Agassiz, 1829 (Hatanaka and Galetti Jr 2004), whereas the 5S rDNA probe was obtained from *Leporinus elongatus* Valenciennes, 1850 (Martins and Galetti Jr 1999). Both probes were labeled by nick translation using commercially available kits and following manufacturers' instructions. Biotin-14-dATP (Bio Nick Labeling System, Gibco, BRL) was used for labeling

18S probe and digoxigenin-11-dUTP (DIG-Nick Translation Mix, Roche) for labeling 5S probe. The hybridization signals were detected using avidin-FITC (fluorescein isothiocyanate) for the 18S rDNA probe and anti-digoxigenin-rhodamine for the 5S rRNA probe. The chromosomes were counterstained with DAPI AntiFade solution (ProLong Gold Antifade Mountant with DAPI, Thermo Fisher).

Metaphases were photographed with an epifluorescence microscope (Axioskop, Zeiss) equipped with a digital camera. The chromosomes were identified based on the modified arm ratio (AR) criteria of Levan et al. (1964), and classified as metacentric (m), submetacentric (sm), subtelocentric (st), and acrocentric (a). The fundamental number (FN) was established considering the metacentric, submetacentric and subtelocentric chromosomes as having two arms, and the acrocentric chromosomes, only one.

Microdissection and amplification

Five heteromorphic chromosomes (the large metacentric of karyomorph B) found in *H. regani* cells were microdissected using an inverted microscope (Olympus IX71) equipped with a mechanical micromanipulator (TH4-100). The microneedles (approximate diameter 0.7 mm) were prepared from glass capillaries using a micropipette puller (Narishige PC-10). The microdissected chromosomes were transferred to 0.5mL microtube and amplified with GenomePlex Single Cell Whole Genomic Amplification WGA4 kit (Sigma). The products of this amplification were reamplified with GenomePlex WGA3 kit (Sigma). In this reamplification reaction with WGA3 kit, the nucleotide digoxigenin 11-dUTP was incorporated with the ratio 7dTTP: 3digoxigenin-11-dUTP to label the chromosome probe. Both procedures with kits (WGA4 and WGA3) were performed according to manufacturers' instructions. The final products of these reactions was named HrV (*Hypostomus regani* Variant) and used as a probe for FISH experiments on both karyomorphs (A and B), following the protocol of Pinkel et al. (1986).

Results

All *Hypostomus regani* specimens had a diploid number of 72 chromosomes (FN=116), but two different karyotypic formulas. The majority (27) of the specimens had a karyotypic formula of 12m+14sm+18st+28a, named karyomorph A, whereas the remaining 21 specimens had a formula of 13m+14sm+17st+28a, named karyomorph B (Figure 1a, b). Karyomorph B was characterized by a chromosome heteromorphism due to the presence of a large metacentric chromosome (the largest of the complement) and a subtelocentric chromosome corresponding to pair 19 (Figure 1b). This heteromorphism was observed in both males and females, and it was present in 43.74% of the analyzed specimens. Regarding pair 19, C-banding revealed that to the whole *p* arm of the large metacentric of karyomorph B was entirely heterochromatic (Figure 2b),

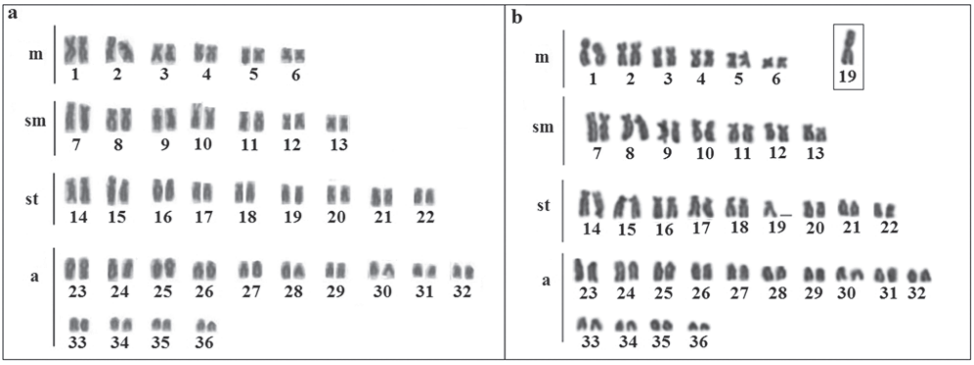

Figure 1. Giemsa stained karyotypes of *Hypostomus regani*: **a** karyomorph A **b** karyomorph B. Scale bar: 10μm.

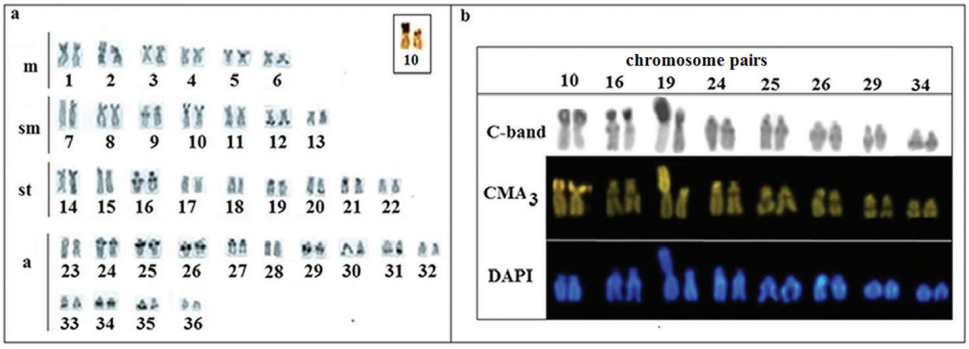

Figure 2. Karyotype of *Hypostomus regani* (karyomorph A) after: **a** C-banding and the NOR-bearing chromosome pair (in box) **b** Some pairs of chromosomes of the karyomorph B showing corresponding bands of C-banding, CMA_3 and DAPI stained. Scale bar: 10μm.

whereas its subtelocentric homologue had heterochromatin in pericentromeric position. This latter pattern also characterized subtelocentric of pair 19 in karyomorph A (Figure 2a). Constitutive heterochromatin was also identified in interstitial positions in pairs 2, 9, 16, 24, 25, 26, 27, 29 and 34 and in subterminal positions in the other chromosomes of both karyomorphs (Figure 2a). C-banding also revealed extensive CMA_3-positive blocks in chromosomal pairs 10 and 19 (Figure 2b). In contrast, the interstitial heterochromatic blocks in pairs 16, 24, 25, 26, 29 and 34 were negative for CMA_3 and positive in DAPI (Figure 2b).

FISH experiments with HrV probe derived from the heteromorphic metacentric chromosome of karyomorph B revealed two equal-sized signals on the short arm of the two subtelocentric pair 19 of karyomorph A (Figure 3b), coinciding with heterochromatic blocks (Figure 2a). For karyomorph B, the HrV probe revealed a larger fluorescent signal throughout the short arm of the heteromorphic metacentric and on the short arm of the subtelocentric chromosome, the homologous of the pair 19

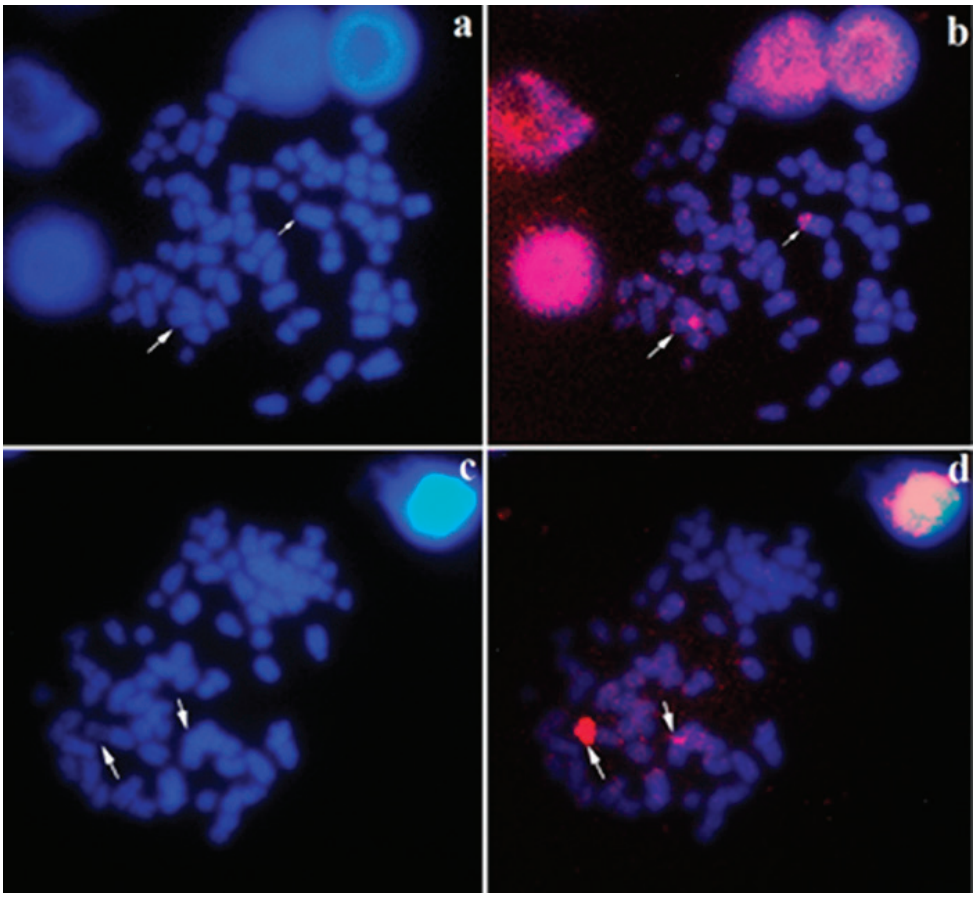

Figure 3. Metaphases of *Hypostomus regani* after FISH with the HrV probe: karyomorph A (**a, b**) and karyomorph B (**c, d**) **a** DAPI stained metaphases of karyomorph A and the arrows indicate pair 19 **b** merged image of metaphase showing intense fluorescent signals positive for HrV probe in the pair 19 (arrows) **c** DAPI stained metaphases of karyomorph B and the arrows indicate heteromorphic pair 19 **d** merged image of metaphase showing intense fluorescent signals positive for HrV probe in the heteromorphic pair 19 (arrows). Scale bar: 10μm.

(Figure 3d). To better visualize the morphology of the pair involved in the heteromorphism, we also showed these metaphases stained in DAPI (Figure 3a, c). Other hybridization fluorescent signals of this probe were observed in several chromosomes of the complement, but they were small and scattered, and did not represent a consistent pattern for the analyzed metaphases (Figure 3b, d).

NORs were located in subterminal position on the short arm of submetacentric pair 10, as revealed by the Ag-NOR (Figure 1a, box) and 18S rDNA-FISH (Figure 4) techniques. The 5S rRNA sites were observed in the pericentromeric region of pairs 4 and 33 (Figure 4). These ribosomal sites were observed in both karyomorphs.

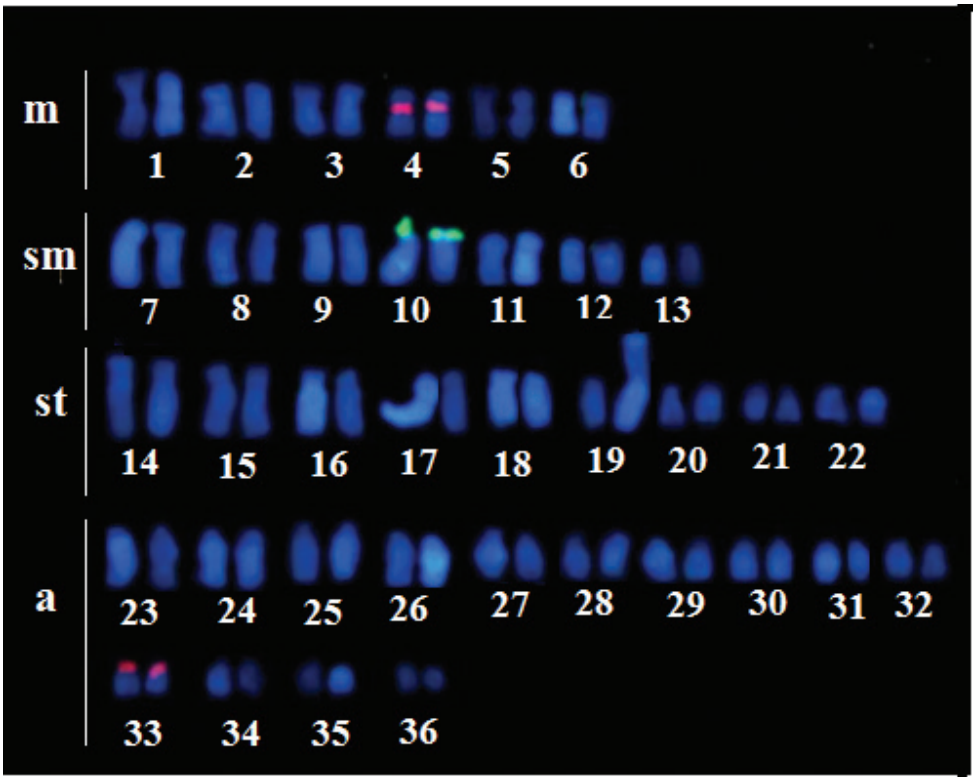

Figure 4. Karyotype of karyomorph B of *Hypostomus regani* after FISH with rDNA probes. Green: 18SrDNA probe; red: 5S rDNA probe. Scale bar: 10μm.

Discussion

The cytogenetic comparison analysis of the *H. regani* population from the Paraguay River with other previously studied populations showed a constant diploid number (2n = 72) for this species. Despite the uniform diploid number in this species, this comparison highlighted different karyotype formulas, including those of the present study, as well as differences in the position and amount of rDNA clusters (18S and 5S). This variability, summarized for *Hypostomus* by Rubert et al. (2016), suggests a cryptic diversity in *H. regani* and the need for a taxonomic revision of this species.

Hypostomus regani karyotypes were characterized by a chromosomal polymorphism involving a structural change in a single chromosome pair, subtelocentric pair 19, which resulted in an asymmetry in the karyotype formulas in the analyzed specimens. A similar polymorphism was found in *Hypostomus strigaticeps* Regan, 1908 (identified as *Hypostomus* sp. B, but subsequently revised by Lorscheider et al. 2015) by Artoni and Bertollo (1999), who reported two distinct karyotypic formulas, 12m+18sm+42st/a and 13m+18sm+41st/a. The formula of the second karyotype differed from the other

by the presence of a large metacentric, the largest of the complement, and a median-sized acrocentric chromosome, which corresponded to pair 21 in the homomorphic condition. Their results obtained by C-banding and mithramycin-staining in metaphases containing the large metacentric indicated heterochromatin amplification in one of the ST/A chromosomes.

In the present study, the extensive heterochromatic blocks in the *p* arm of the heteromorphic metacentric chromosome of *H. regani* (karyomorph B) indicate the amplification of repetitive sequences in this region. The fact of the HrV probe has hybridized to the whole *p* arm of the large metacentric, to the pericentromeric regions of the short arm of its (subtelocentric) homologue of karyomorph B and to both homologs of pair 19 in karyomorph A, reinforces the hypothesis that the novel large metacentric of *H. regani* (karyomorph B) was a result of the amplification of heterochromatin segments. The presence of extensive heterochromatic blocks on only one chromosomal arm is an intriguing trait of the chromosome morphology found in some *Hypostomus* species. Heterochromatinization processes and/or an amplification of this region were suggested as an attempt to explain the heterochromatic chromosomal polymorphism in a population of *Hypostomus iheringii* Regan, 1908 (Traldi et al. 2012), in *H. strigaticeps* (Baumgärtner et al. 2014) and *Hypostomus* prope *plecostomus* Linnaeus, 1758 (Oliveira et al. 2015). Furthermore, the presence of transposable elements (TEs) has been confirmed in the heterochromatic regions of a number of fish species (Ferreira et al. 2011), including two *Hypostomus* species (Pansonato-Alves et al. 2013), which could explain the origin of the heteromorphic metacentric in *H. regani*. Finally, heterochromatic chromosomal heteromorphism has been a recurring process in *Hypostomus*, highlighting the role of the heterochromatin in the differentiation of karyotypes, and the potential contribution to chromosome evolution in this group.

The heterochromatic blocks in both karyomorphs of *H. regani* presented heterogeneous composition. Subterminal blocks tended to be rich in GC (CMA_3^+, pairs 10 and 19), whereas the interstitial blocks are rich in AT (DAPI+ pairs 16, 24, 25, 26, 29 and 34). It is also interesting to point out that while CMA_3 blocks are scarce in most *H. regani* chromosomes, the accumulation of GC sequences (CMA_3^+) was observed on the short arm of the heteromorphic metacentric of karyomorph B. The homology of the GC-rich sequences on the short arm of the subtelocentric pair 19 of karyomorph A, which presumably represents the original form of the heteromorphic pair of karyomorph B, it further reinforces the hypothesis that the novel large metacentric of *H. regani* (karyomorph B) was the result of the amplification of pre-existing heterochromatin segments.

In a panmictic population, the expected frequency of the chromosomal polymorphism in *H. regani* can be estimated based on the observed frequency of the ST (subtelocentric) and M (metacentric) chromosomes, which were *p* (ST) = 0.78 and *q* (M) = 0.22, respectively. Given a sample of 48 specimens, the expected number of each genotype would be 29.28 ST/ST, 16.32 ST/M, and 2.40 M/M, whereas 27 of the specimens were ST/ST, and 21 ST/M. This represents a significant deviation from Hardy Weinberg Equilibrium ($X^2 = 3.92$, d.f. = 1, $p < 0.05$), although the absence of the M/M genotype may be at least partly due to the small sample size. Alternatively,

the M/M genotype may suffer negative selection pressure, determining its absence from the *H. regani* population.

Chromosome mapping data with rDNA sequences are available for few *Hypostomus* species. In this genus, the NORs may be single or multiple, but multiple sites is the most frequent arrangement. This is considered to be a derived trait in Locariids (Bueno et al. 2013, 2014). The mapping of the 18S rDNA gene in other *H. regani* populations has demonstrated multiple sites, located in the terminal position, mostly on the short arms of the st/a chromosomes (Rubert et al. 2016), which contrasts with the findings of the present study, given that the specimens of *H. regani* analyzed here presented single NORs, with active NOR and the 18S rDNA sites located in a terminal position on the short arms of submetacentric pair 10. Thus these chromosomes can be considered markers for the *H. regani* populations from Paraguay River basin.

Chromosomal mapping of 5S rDNA clusters has been carried out for only a few *Hypostomus* species and two patterns have been observed: (i) single 5S-bearing pair has been reported in *Hypostomus iheringii* (Traldi et al. 2012), *H. nigromaculatus* Schubart, 1964 (Traldi et al. 2013) *H. albopunctatus* Regan, 1908 and *H. topavae* Godoy, 1969 (Bueno et al. 2014) and *H.* prope *hermanni* Ihering, 1905 (Kamei et al. 2017); (ii) multiple sites have been observed in *H. ancistroides* Ihering, 1911 (Traldi et al. 2013, Kamei et al. 2017), *H. affinis* Steindachner, 1877 (Kavalco et al. 2004), *H. cochliodon* Kner, 1854, *H. commersoni* Valenciennes, 1836, *H. faveolus* Zawadzki, Birindelli & Lima, 2008 (Bueno et al. 2014) and *H. topavae* (Kamei et al. 2017). The presence of a centromeric/pericentromeric 5S rDNA sites on the short arm of a metacentric or submetacentric pairs is a frequent feature observed in the most species of *Hypostomus* (Bueno et al. 2014), also detected in the present study to *H. regani*, in pair 4, as well as in pericentromeric position in acrocentric pair 33. These findings indicate a shared condition among *Hypostomus* species that may be a primitive trait (Traldi et al. 2013). However, the number of chromosomes bearing 5S rRNA sites varies among *H. regani* populations, ranging from one pair in the population from Piquiri River (Bueno et al. 2014) to nine chromosomes in the one from Piumhi River (Mendes-Neto et al. 2011). The evolutionary dynamics of the ribosomal genes seems to be related to their association with transposable elements, as observed in some fish species, which indicates that these elements may play a role in the dispersion of the 5S rDNA sites (da Silva et al. 2011, Pansonato-Alves et al. 2013, Gouveia et al. 2017).

Conclusion

The chromosomal heteromorphism detected in *H. regani* from Onça Stream, in the Taquari River basin, and investigated by chromosome painting provides an important model for the cytogenetic analysis for other species of the genus, in addition to other fish genera in which the role of the heterochromatin in differentiation and evolution of karyotypes need to be better understood. The divergence in karyotype formulas found among different populations of *H. regani* (Rubert et al. 2016) suggests the existence of cryptic species within this taxon, and emphasizes the need of a thorough revision of

the taxonomy of this group. While the taxonomic complexity of the genus *Hypostomus* is still far from being sorted out, cytogenetic analyses based on high resolution techniques, such as those applied in the present study, should help to reduce the taxonomic uncertainties in this genus.

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REVIEW ARTICLE

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Cytological markers used for identification and transfer of *Aegilops* spp. chromatin carrying valuable genes into cultivated forms of *Triticum*

Michał T. Kwiatek[1], Danuta Kurasiak-Popowska[1], Sylwia Mikołajczyk[1], Janetta Niemann[1], Agnieszka Tomkowiak[1], Dorota Weigt[1], Jerzy Nawracała[1]

1 *Department of Genetics and Plant Breeding, Poznań University of Life Sciences, Dojazd 11, 60-632, Poznań, Poland*

Corresponding author: *Michał T. Kwiatek* (michal.kwiatek@up.poznan.pl)

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Abstract

There are many reports describing chromosome structure, organization and evolution within goatgrasses (*Aegilops* spp.). Chromosome banding and fluorescence *in situ* hybridization techniques are main methods used to identify *Aegilops* Linnaeus, 1753 chromosomes. These data have essential value considering the close genetic and genomic relationship of goatgrasses with wheat (*Triticum aestivum* Linnaeus, 1753) and triticale (× *Triticosecale* Wittmack, 1899). A key question is whether those protocols are useful and effective for tracking *Aegilops* chromosomes or chromosome segments in genetic background of cultivated cereals. This article is a review of scientific reports describing chromosome identification methods, which were applied for development of prebreeding plant material and for transfer of desirable traits into *Triticum* Linnaeus, 1753 cultivated species. Moreover, this paper is a resume of the most efficient cytomolecular markers, which can be used to follow the introgression of *Aegilops* chromatin during the breeding process.

Keywords

Aegilops, chromosome, banding, fluorescence *in situ* hybridization (FISH), genomic *in situ* hybridization (GISH), prebreeding, triticale, wheat

Introduction

There are twenty three species of goatgrasses (*Aegilops* spp.) (Slageren 1994) and several of them are the closest relatives to wheats (*Triticum* spp.) (Kilian et al. 2011). The genomic constitution of goatgrasses is wide and include six genomes (D, S, U, C, N and M), which can be organized as diploids, tetraploids or hexaploids. What is more, most polyploid *Aegilops* Linnaeus, 1753 species are assumed to contain a common (pivotal) subgenome (U or D) while the second - differential genome (or genomes) is (are) much more genetically diversified (Zohary and Feldman 1962; Feldman and Levy 2012; Mirzaghaderi and Mason 2017). The evolution of *Aegilops* species was also intertwined with speciation of *Triticum* Linnaeus, 1753 forms (Goncharov 2011). It is reported that hexaploid wheat (*Triticum aestivum* Linnaeus, 1753; genomes AABBDD) originated through one or more hybridization events between a tetraploid wheat, *T. turgidum* Linnaeus, 1753 (genomes AABB), with the diploid goatgrass *Aegilops tauschii* Cosson, 1849 [genomes DD; syn. *Triticum tauschii* (Cosson,1849) Schmalhausen, 1897; syn. *Aegilops squarrosa* auct. non Linnaeus, 1753, *Patropyrum tauschii* (Cosson, 1849) A. Love, 1984] (Kihara 1924, 1954; McFadden and Sears 1946). More precisely, *Aegilops tauschii* subsp. *strangulata* (Eig, 1929) Tzvelev, 1973, has been accepted to be a donor of D-genome of wheat (Dvořák et al. 1998). Tetraploid wheat originated via hybridization of a species closely related to the extant *Aegilops speltoides* Tausch, 1837 [genomes SS; syn. *Sitopsis speltoides* (Tausch, 1837) Á. Löve, 1984; syn. *Triticum speltoides* (Tausch, 1837) Grenier, 1890], which contributed the wheat B genome (Sarkar and Stebbins 1956; Dvořák et al. 1993; Feldman and Levy 2012; Salse et al. 2008), with diploid wheat (genomes AA). The most likely donor of A-genome of polyploid wheats is *T. urartu* Tumanian ex Gandilyan, 1972 (Konarev et al. 1974; Petersen et al. 2006; Golovnina et al. 2009). Some reports describe both genera jointly, as *Aegilops-Triticum* complex (Li et al. 2015; Ozkan et al. 2003; Zohary and Feldman 1962). A close relationship between the genera *Aegilops* and *Triticum* is widely adopted for introducing new genes by interspecific hybridization into cultivated cereals (Ruban and Badaeva 2018). Such introgression forms are important genetic resources for breeding. These kinds of genetic stocks can be used as an interesting plant material to study the expression of alien traits and for mapping particular loci (genes) onto *Aegilops* chromosomes (Rakszegi et al. 2017).

The ability to distinguish alien chromosomes, which were introduced into a genetic background of an acceptor plant, is the initial step in characterization of introgression lines. The first chromosome identification studies in wheat were done by Sears (1948), who assigned the loci for several agronomic and morphological traits on particular chromosomes and chromosome arms. Later, in 1970s all chromosomes of wheat could be distinguished using the C-banding or N-banding techniques (Gill and Kimber 1974; Iordansky et al. 1978; Endo and Gill 1984; Lukaszewski and Xu 1995). In 1990s, molecular biology protocols were combined with classical cytogenetic techniques to develop the fluorescence *in situ* hybridization (FISH) method. FISH allows the identification of DNA sequences directly on the chromosomes.

The first molecular probes used for FISH purposes on *Aegilops-Triticum* chromosomes contained conserved high-copy sequences, such as telomere sequences or 5S and 45S ribosomal RNA genes (Gerlach and Bedbrook 1979; Gerlach and Dyer 1980). The number and distribution of rDNA loci mapped on chromosomes of species belonging to *Aegilops-Triticum* complex turned out to be invariant. Hence, these probes were often used as markers in evolution and speciation studies, as well as in the evaluation of interspecific divergence (Badaeva et al. 1996a; b; 2002; 2004; 2015). Mukai et al. (1993) used pSc119.2 and pAs1 sequences to identify all 21 chromosome pairs in wheat. Over time a number of cytomolecular markers were developed for the identification of chromosome arms or segments. For example, Cuadrado et al. (2000; 2008) used synthetic oligonucleotides (three base-pair repeats) to detect FISH signals on wheat chromosomes. BAC genomic libraries were also screened to develop FISH chromosome markers (Zhang et al. 2004). Komuro et al. (2013) screened 2000 plasmid wheat clones in order to detect multiple tandem repeated sequences, using *in situ* hybridization, and selected 47 of them, which gave clear signals on wheat chromosomes. Apart from physical mapping of DNA sequences onto chromosomes, the major breakthrough in chromosome identification was the development of an *in situ* hybridization technique utilizing total genomic DNA as a probe (GISH). This variant of *in situ* hybridization appeared to be a powerful tool for characterization of alien introgressions in cereals. The first GISH was carried out on chromosomes of synthetic hybrids of *Hordeum chilense* Roemer & Schultes, 1817 × *Secale africanum* Stapf, 1901 (Schwarzacher et al. 1989) and *Triticum aestivum* (wheat) × *S. cereale* Linnaeus, 1753 (rye) hybrids (Le et al. 1989). This technique is based on the divergence of repetitive DNA (Belyayev and Raskina 1998; Belyayev et al. 2001a; b) and was effectively used for identification of alien chromosomes/chromosome segments in hybrids or translocation lines of cereals (Schwarzacher et al. 1989; 1992; Leitch et al. 1990). GISH in combination with FISH was also used to study the genome constitution of natural and artificial hybrids, or to identify the introgression of alien chromosomes or chromosome segments (Jiang and Gill 2006).

The structure and organization of chromosomes of species belonging to the genera *Aegilops* and *Triticum* are collinear, as chromosomes within each homoeologous group are related by descent from a chromosome of the ancestor of the *Triticum-Aegilops* complex (Akhunov et al. 2003). Hence, large numbers of cytogenetic markers have a similar localization in the same homoeologous group (McCouch 2001). Moreover, this genetic resemblance can hamper the use of GISH in some instances (Majka et al. 2017). The synteny between the homoeologous *Aegilops* and *Triticum* chromosomes may be disturbed because of chromosome rearrangements, which appeared during the evolution process (Devos et al. 1993; Zhang et al. 1998). Moreover, it is known that the level of chromosome synteny decreases the more distant a chromosome region is from the centromere. It is also decreased in regions with increased meiotic recombination rates, also known as hotspots of recombination on chromosome arms (Akhunov et al. 2003). Such changes result in distribution variability of chromosome markers. This review summarizes cyto-

molecular techniques, which differentiate *Aegilops* and *Triticum* chromosomes, and are used most often for effective tracking of *Aegilops* chromosomes (or chromosome segments) in cultivated cereals.

Banding methods for identification of *Aegilops* chromatin introgression

Since the 1970s C-banding and N-banding techniques were used to distinguish the chromosomes of *Aegilops-Triticum* complex (Friebe et al. 1992; Gill and Kimber 1974; Landjeva and Ganeva 2000). C-banding has been employed to study genetic diversity and to create karyotypes of many *Aegilops* species. Giemsa C-banding was one of the first methods which allowed for identification of all 21 chromosome pairs of wheat (Endo 1986; Gill et al. 1991). This method was widely used to identify *Aegilops-Triticum* chromosome addition, substitution and translocation lines (Friebe et al. 1991; 1992; 1995; 1996a; 1996b; 1999; 2000; 2003). The results obtained by means of C-banding chromosome analysis of the majority of goatgrasses were reported in a series of articles describing the most important genomes of *Aegilops* (Badaeva et al. 1996a; 2002; 2004). C-banding analyses allowed the authors to discover that the S-genome of *Ae. speltoides* was most syntenic to B- and G-genomes of *Triticum*, but was different from other species of section *Sitopsis* (Badaeva et al. 1996a). Moreover, those authors observed minor polymorphisms in C-banding patterns of chromosomes of D-genome (Badaeva et al. 2002) and U-genome (Badaeva et al. 2004) belonging to different *Aegilops* species. All those results were later compared and confirmed by means of FISH studies (FISH methods are described in the third section of this review).

Polymorphisms in C-banding patterns were also utilised to distinguish *Aegilops* chromosomes in wheat genetic background. *Ae. speltoides* turned out to be one of the largest sources of valuable genes and was used to develop *Aegilops-Triticum* introgression lines. Friebe et al. (1991) used C-bands to establish the chromosome constitution of wheat streak mosaic virus (WSMV) and greenbug (*Schizaphis graminum* Rondani, 1852) resistant lines, derived from wheat - *Agropyron intermedium* - *Aegilops speltoides* crosses. Three lines carried 7S(7A) chromosome substitution (derived from *Ae. speltoides*). The results indicated that the greenbug resistance gene *Gb5* was located on chromosome 7S. This chromosome was also used to transfer leaf rust (caused by *Puccinia triticina* Eriksson, 1899) resistance gene combined with greenbug resistance gene *Gb5* into wheat genetic background (Dubcovsky et al. 1998). The authors induced a homologous recombination events using *ph1b* wheat mutant and developed Ti7AS-7S#1S-7AS.7AL translocation line conferring resistance to leaf rust and Ti7AS.7AL-7S#1L-7AL line conferring resistance to greenbug. The chromosome segments transferred from *Ae. speltoides* were characterized by means of C-banding and the fact of the translocation was supported by restriction fragment length polymorphisms (RFLP) analysis. Friebe et al. (1996a) applied C-banding analysis to identify T4AS.4AL-7S#2S chromosome translocations in wheat - *Ae. speltoides* lines with *Lr28* leaf rust resistance gene. Moreover, a chromosome translocation (2B.2S) involved in the *Lr35/Sr39*

transfer derived from *Ae. speltoides* was identified using a C-banding method (Friebe et al. 1996a). C-banding technique was also used to determine the introgression carrying *Yr8/Sr34* yellow rust and stem rust resistance genes from *Ae. comosa* Smith, 1806 into wheat. Miller (1988) detected 2AS-2ML.2MS and 2DS-2ML.2MS chromosome translocations. Friebe et al. (1992) adopted the C-banding method and identified complete set of chromosomes of *Ae. caudata* Linnaeus, 1753 in the amphiploid *Triticum aestivum* cv 'Alcedo' - *Ae. caudata*. Furthermore, the authors developed six chromosome addition lines in which the *Ae. caudata* chromosome pairs were called B, C, D, F, E and G. Friebe et al. (1995) established a karyotype of *Ae. umbellulata* Zhukovsky, 1928 using C-banding analysis of ten accessions collected in ten different geographic locations. This approach allowed for the identification of individual alien chromosomes in wheat-*Ae. umbellulata* chromosome monosomic and telosomic addition and wheat - *Ae. umbellulata* translocation lines (Friebe et al. 1995).

One of the most notable applications of the C-banding technique was the identification of radiation-induced translocation lines resistant to leaf rust (*Lr9*) and assignment of *Lr9* loci to 6UL chromosome of *Ae. umbellulata*. The following chromosome translocations were identified by means of C-banding analysis: 6BL.6BS-6UL, T4BL.4BS-6UL, 2DS.2DL-6UL, T6BS.6BL-6UL and 7BL.7BS-6UL (Friebe et al. 1995). C-banding method was also used to identify 3BL.3BS-3S and 3DL.3DS-3S chromosome translocations conferring resistance to powdery mildew (*Pm13* gene), which was transferred from *Ae. longissima* Schweinfurth & Muschler, 1912 into wheat (Ceoloni et al. 1992; Friebe et al. 1996a). Another powdery mildew gene (*Pm32*) was transferred from *Ae. speltoides* into wheat and T1BL-1SS chromosomal translocation was revealed by means of C-banding analysis (Hsam et al. 2003). However, in some cases the C-banding method was not sufficient to discriminate between *Aegilops-Triticum* translocations. For example, C-banding patterns of the translocated 7DL arms from *Aegilops ventricosa* Tausch, 1837, carrying *Pch1* gene (responsible for resistance to eyespot) in cultivars Rendevous and Roazon was impossible to visualize as the patterns identified in 7DL chromosome of Chinese Spring wheat and 7DL of *Ae. ventricosa* were similar (Martin 1991). It was not until more sensitive C-banding protocol was applied that clear differences in the C-banding patterns between 7D of Chinese Spring and 7D of *Ae. ventricosa* were demonstrated by Badaeva et al. (2008). Another difficulty was reported by Apolinarska et al. (2010), who could not unambiguously identify the *Aegilops variabilis* Eig, 1929-rye chromosome translocations by means of C-banding.

The N-banding method was less often used to investigate *Aegilops-Triticum* introgression lines. Landjeva and Ganeva (1996; 2000) reported the N-banded karyotype of *Aegilops ovata* Linnaeus, 1753 (syn. *Ae. geniculata* Roth, 1787) and the chromosomal constitution of its partial amphiploid with bread wheat *Triticum aestivum* cv.'Chinese Spring'. N-banding patterns made it possible to distinguish all *Ae. ovata* and wheat chromosomes. Ganeva et al. (2000) also used this technique, supported by gliadin electrophoresis, to reveal the structural changes in chromosomes 1A, 2A, 4B, 6B, 7B, 1D, and 2D of the *Ae. umbellulata*-wheat amphiploid (2n=6x=42, AABBUU), which showed leaf rust resistance conferred by *Lr9* gene homolog. C- and

N-banding methods are effective techniques to distinguish alien chromatin in a large number of introgression lines. However, the precise identification of translocation breakpoints requires additional supporting technique – in most cases genomic *in situ* hybridization (GISH) would suffice.

Fluorescence *in situ* hybridization methods for identification of *Aegilops* introgressions

A combination of molecular techniques and classical cytology became a breakthrough tool for science and crop breeding, especially for the development and characterization of *Aegilops-Triticum* introgression lines. First reports of adaptation of fluorescence *in situ* hybridization protocol for analyses of wheat chromosomes were published by Rayburn and Gill (1985) and Yamamoto and Mukai (1989). The ideal set of chromosome markers should cover the entire chromosome arms. This is a crucial requirement, which defines the usefulness of cytological landmarks for the identification of chromosome translocations. Hence, the most useful landmarks are DNA repetitive sequences that are richly represented in almost all chromosome regions, and can be used for evaluation of intra- and interspecific or intergeneric chromosome polymorphisms (Table 1).

To date the most popular probe used for identification of *Aegilops-Triticum* chromosomes is a D-genome specific repetitive DNA sequence called pAs1, derived from of *Aegilops squarrosa* Linnaeus, 1753 (syn. *Ae. tauschii* Cosson, 1849; 2n = 14, genome DD) (Nagaki et al. 1995; Rayburn and Gill 1985). This sequence is AT rich (65.2%) and is widely distributed in many species of *Aegilops-Triticum* complex. It is included into *Afa-family* repeated sequences, because the recognition site of *AfaI* restriction enzyme was the most conserved sequence in this unit (Nagaki et al. 1995). Another much-used chromosome marker is a pSc119.2 repetitive sequence, derived from rye (*Secale cereale*) (Bedbrook et al. 1980). FISH landmarks of pSc119.2 and pAs1 are widely distributed in the chromosomes of *Aegilops* and *Triticum* species. A combination of those two probes was the first effective marker set used for chromosome identification of *Triticum* (Mukai et al. 1993) and *Aegilops* (Badaeva et al. 1996a; b; Schneider et al. 2005) species. However this set of markers was insufficient to describe some of *Aegilops* segments transferred into *Triticum* chromosomes. Hence, there was a need to develop more diversified and abundant chromosome landmarks.

Vershinin et al. (1994) identified dpTa1 family of repetitive sequences that are present in subtelomeric and interstitial regions of chromosomes belonging to *Aegilops-Triticum* complex. Salina et al. (2004; 1998; 2009) isolated, characterized and designated repetitive sequence called Spelt-1, which is located in subtelomeric regions of *Ae. speltoides*. Another repetitive sequence, Spelt52, pGC1R-1 belongs to the family of tandem repeats pAesKB52, located at subtelomeric regions of chromosomes *Ae. speltoides*, *Ae. longissima* and *Ae. sharonensis* Eig, 1928 (Anamthawat-Jonsson and Heslop-Harrison 1993; Zhang et al. 2002; Salina et al. 2004). Additionally, Kishii and Tsujimoto (2002) characterized TaiI family of tandem repeats, which are localized to

Table 1. Tandem repeats used as effective FISH markers for identification of *Aegilops* chromatin introgression.

Tandem repeats	Clones/sequences	References
Satellite DNA sequences	pAs1, pSc119.2, pTa-71, pTa-86, pTa-465, pTa-535, pTa-566, pTa-713, pTa-794	Badaeva et al. 1996a; b; 2015; Schneider et al. 2005; Zhao et al. 2016; Kwiatek et al. 2015; 2016a; 2016b; 2017a; 2017b; Goriewa-Duba et al. 2018
Microsatellite DNA sequences (simple sequence repeats - SSR)	AAC, ACG, GAA	Molnar et al. 2005; 2011

the centromeric regions. Moreover, there are some groups of repetitive sequences, originated from related genera such as *Secale* sp. (subtelomeric repeats represented by 350 family pSc200 and pSc250) (Vershinin et al. 1994) and *Hordeum vulgare* Linnaeus, 1753 (HvRT telomere-associated sequences) (Kilian and Kleinhofs 1992), which are also represented in chromosomes of cultivated wheat or triticale. Other repetitive sequences that effectively discriminate between *Aegilops* and *Triticum* chromosomes were derived from BAC libraries of species belonging to Triticeae tribe. Komuro et al. (2013) screened 2000 plasmid wheat clones for signal occurence using FISH. 47 clones showed distinct signals on wheat chromosomes, and clones pTa-86 and pTa-535 were related to pSc119.2 and pAs1, respectively (Komuro et al. 2013). Kwiatek et al. (2017a; 2017b) used pTa-86, pTa-103, pTa-k374, pTa-465, pTa-535, pTa-k566, and pTa-713 to discriminate between the chromosomes of *Aegilops biuncialis* de Visiani, 1851, *Ae. ovata*, respectively and *Ae. kotschyi* Boissier, 1846 (unpublished, Figure 1) which were transferred into a triticale genetic background. This set of chromosome markers allowed for the identification of 1BS-1BL.5ML, 5MS-5ML.1BL, 7US.6BS-6BL, 6BS.7US-7UL, 1BS-1BL.5ML and 5MS-5ML.6BL chromosome translocations (Kwiatek et al. 2017a). Zhao et al. (2016) combined pSc119.2, pTa71 and pTa-713 and identified each of the 14 pairs of *Ae. variabilis* chromosomes.

Apart from the use of long repetitive sequences, one of the most effective ways to saturate chromosome regions with markers as much as possible is to apply microsatellite sequences as cytomolecular probes. Such trinucleotide sequences (i.e. AAC, GAA, ACG) were used to distinguish between chromosomes of wheat (Cuadrado et al. 2000) and *Aegilops* (Molnar et al. 2011). Furthermore, GISH effectively complemented FISH analysis so as to locate and identify the *Aegilops-Triticum* chromosome translocation breakpoints (Friebe et al. 1992; Kwiatek et al. 2017a). A combination of banding techniques and FISH/GISH methods were used for precise *Aegilops* chromosome identification in a *Triticum* background during the development of introgression lines with valuable traits. Friebe et al. (1995) combined C-banding and GISH using total genomic DNA of *Ae. umbellulata* to identify the chromosome breakpoints in radiation-induced *Triticum-Aegilops* translocation lines resistant to leaf rust (*Lr9*), which involved 4B and 6B chromosomes of wheat and 4U chromosome of *Ae. umbellulata*. In addition, Friebe et al. (2003) used C-banding and FISH to identify *Ae. sharonensis* chromosomes car-

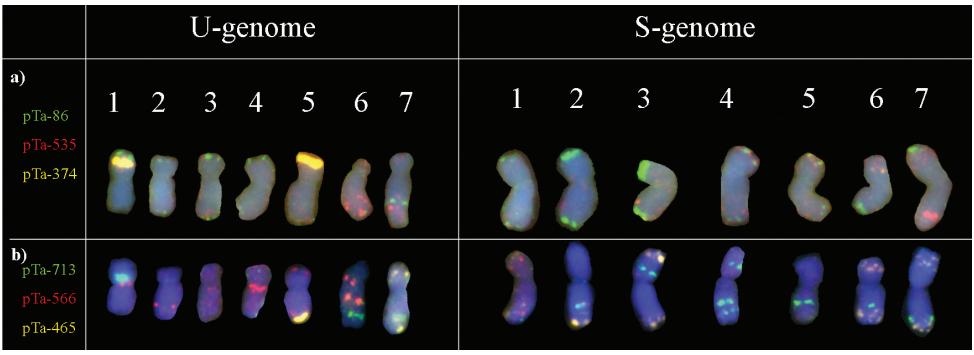

Figure 1. Karyograms of *Aegilops kotschyi* 2n=4x=28 chromosomes; UUSS) showing U- and S-genome chromosomes after two rounds of FISH with: **a** pTa-86 (green; Atto-488 fluorochrome; Jena Bioscience), pTa-535 (red; Atto-550 fluorochrome; Jena Bioscience), pTa-374 (25S rDNA; yellow; Atto-647 fluorochrome; Jena Bioscience) and **b** pTa-713 (green; Atto-488 fluorochrome; Jena Bioscience), pTa-k566 (red; Atto-550 fluorochrome; Jena Bioscience) and pTa-465 (yellow; Atto-647 fluorochrome; Jena Bioscience) probes (Kwiatek, unpublished)

rying gametocidal genes in a wheat genetic background. A 4BS.4BL-4S chromosome translocation was identified using clone pGclR-1, which is a 258 bp fragment of a tandem repetitive element and hybridizes to telomeric and subtelomeric regions of *Ae. speltoides*, *Ae. sharonensis*, and *Ae. longissima* chromosomes (Friebe et al. 2000).

A combination of C-banding and GISH methods was also used for development of wheat introgression lines with resistance genes against one of the most virulent races of stem rust (*Puccinia graminis* var. *tritici* Persoon, 1794), namely Ug99. Liu et al. (2011a) used this combination of cytomolecular methods, supported by SSR marker analysis, to identify three Robertsonian translocations (T3AL·3S^{s}S, T3BL·3S^{s}S and T3DL·3S^{s}S) and one recombinant (T3DS-3S^{s}S·3S^{s}L) line with stem rust resistance as a common feature of the analysed forms. Faris et al. (2008) examined a durum wheat-*Aegilops speltoides* chromosome translocation line (DAS15), which was resistant to Ug99 and six other races of stem rust. GISH methods made it possible to identify 2BL-2SL.2SS translocation, which harbours stem rust resistance. GISH was also used to identify the 5DL-5M^{g}L·5M^{g}S chromosome translocation, which introduced resistance to stem rust races RKQQC and TTKSK (Ug99) into wheat (Liu et al. 2011b). Chromosome 5M^{g} of *Ae. geniculata* is also a source of leaf and yellow rust resistance genes (*Lr57* and *Yr40*, respectively). Kuraparthy et al. (2007) identified wheat-*Ae. geniculata* translocation lines (5DL·5DS-5M^{g}S) using GISH. Molnar et al. (2005) combined GAA sequence probe with GISH to discriminate between the 1U, 2U, 4U and 5U chromosomes of *Ae. biuncialis* in wheat introgression lines, which showed limited tolerance to drought stress. Furthermore, Schneider et al. (2005) combined GISH and FISH using three repetitive DNA clones (pSc119.2, pAs1, and pTa71) to identify 2M, 3M, 7M, 3U, and 5U chromosome pairs in those lines. FISH/GISH methods, using pSc119.2, pAs1, 5S and 35S rDNA (from pTa71) sequence FISH probes together with GISH probes were also used to identify 2D^{t} and 3D^{t} chromosomes, carrying

Lr39 and *Lr32* genes, respectively in *Ae. tauschii*-triticale introgression lines (Kwiatek et al. 2015). The same set of FISH markers was used together with GISH to discriminate between 2S and 3S chromosomes of *Ae. variabilis*, which were transferred into triticale with intent to introduce the powdery mildew resistance gene *Pm13* (Kwiatek et al. 2016a). Mirzaghaderi et al. (2014) observed FISH patterns of the U^t- and C^t -genome chromosomes of *Ae. triuncialis* Linnaeus, 1753 and *Ae. cylindrica* (Host, 1802) in wheat background. The following probes: pSc119.2-1, pTa535-1, pAs1-1, $(CTT)_{10}$ and the 45S rDNA clone from wheat (pTa71), supported by GISH, were sufficient to discriminate between three different non-reciprocal homologous or heterologous translocations involving C^c and D^c chromosomes of *Ae. cylindrica*.

Modifications and changes of FISH protocols for identification of *Aegilops* introgressions

In order to screen large populations of *Aegilops-Triticum* introgression forms, the methods for cytomolecular marker analysis should be easy to handle and cost-efficient. FISH protocols require fluorescent DNA probes, heat treatment and are labour and time consuming. There are reports describing modifications and changes to the protocols used to conduct repetitive sequence preparation for FISH. One of such techniques, primed *in situ* labeling (PRINS), combines polymerase chain reaction (PCR) with FISH to visualize sequences on chromosomes (Koch et al. 1989). This technique is based on the annealing of short, sequence-specific unlabelled DNA to denatured chromosomes (Kubalakova et al. 2001). Tang et al. (2014) designed oligonucleotides to replace the repetitive sequences pAs1, pSc119.2, pTa-535, pTa71, CCS1, and pAWRC.1 for *Aegilops-Triticum* chromosome identification. Kwiatek et al. (2016b) and Goriewa-Duba et al. (2018) developed specific primers to amplify some of the repetitive sequences reported by Komuro et al. (2013) from wheat genomic DNA. This approach reduces the time and the costs of BAC library maintenance. The modifications of FISH protocols also facilitate the chromosome identification. Cuadrado and Jouve (2010) investigated telomeres of barley (*Hordeum vulgare* L.) using non-denaturing FISH (ND-FISH). This method was used to study chromosomes of *Triticum* (Fu et al. 2015). The analytical potential of this technique was demonstrated by Tang et al. (2018), who developed new oligo probes that make possible the identification of particular chromosomal segments, i.e.: the intercalary regions of 4AL and 2DL chromosome arms, and the pericentromeric regions of 3DL and 6DS arms of wheat chromosomes.

Another way to saturate the chromosome arms with markers is the use of cDNA probes. Danilova et al. (2014) carried out FISH experiment with more than 60 full length wheat cDNAs, which were selected using BLAST against mapped EST markers (expressed sequence tags). FISH analysis revealed 1U-6U chromosome translocation in *Aegilops umbellulata* and showed synteny between chromosome A of *Ae. caudata* and group-1 wheat chromosomes. There are certain reports, showing technical modifications of FISH procedures, which reduce the time and costs of experiments. For exam-

ple, Kwiatek et al. (2016b) used four different fluorescence labels (Atto488, Atto550, Atto647 and DAPI) that made possible the examination of three different probes at the same time. Of course, this approach requires investing in excitation wavelength specific filter cubes, which are cost-consuming. When there is a need to examine hundreds of plants resulting from genetic crosses, in some cases the time and labour consuming cytological methods could be substituted. For example, Rey and Prieto (2017) used dot-blot genomic hybridization experiments instead of microscopy to detect alien genetic introgressions to bread wheat.

Closing remarks: large scale selection of *Aegilops-Triticum* introgressions, perspectives for the future

Cytogenetic methods seem to be essential to verify genomic constitution in interspecific hybrids. The main problems are: limited sensitivity and spatial resolution, laborious and expensive protocols, which seriously limit the application of cytogenetic markers for large scale selection of *Aegilops-Triticum* introgressions. High-resolution and high-throughput methods are being progressively developed for identification of micro-introgressions, chromosome breakpoints and spatial localization of alien chromatin in donor nuclei. These require the use of new DNA markers, sequencing and new combinations of cytomolecular techniques. For example, three dimension FISH (3D-FISH) was applied to track the spatial organization of rye chromatin in wheat host genome (Burešová 2018). However, the main aim for development of *Aegilops-Triticum* introgressions is the transfer of desirable genes. Hence, there is a need to improve the cytogenetic methods for single gene physical mapping. Danilova et al. (2014) used single copy gene FISH with probes developed from cDNA of cytosolic acetyl-CoA carboxylase (ACCase) gene (Acc-2) and mapped them onto chromosomes of wheat. Another promising tool can be the combination of CRISPR (clustered regularly interspaced short palindromic repeats) with FISH. Deng et al. (2015) used a bacterial protein, CRISPR, combined with RNA sequences as probes to find the genes of interest. This method is comparably rapid and allows for keeping natural organization of the nucleus. What is more, CRISPR-FISH enables the analysis of spatial relationships between the genetic elements that are significant for gene expression. Apart from identification of *Aegilops-Triticum* introgressions, newly developed cytogenetic markers and methods could shed some light on the behaviour of chromatin, incorporated into the wheat genome, and show the results of the interaction between wheat genome and expression of introduced alien genes.

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Comparative analysis of C-heterochromatin, ribosomal and telomeric DNA markers in chromosomes of Pamphagidae grasshoppers from Morocco

Olesya G. Buleu[1,2], Ilyas Y. Jetybayev[2,3], Dragan P. Chobanov[4], Alexander G. Bugrov[1,2]

1 *Novosibirsk State University, Pirogova Str. 2, Novosibirsk 630090, Russia* **2** *Institute of Systematics and Ecology of Animals, Russian Academy of Sciences, Siberian Branch, Frunze str. 11, 630091 Novosibirsk, Russia* **3** *Institute of Cytology and Genetics, Russian Academy of Sciences, Siberian Branch, Pr. Lavrentjeva 10, 630090 Novosibirsk, Russia* **4** *Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, Tsar Osvobodotel Boul. 1, Sofia 1000, Bulgaria*

Corresponding author: *Olesya G. Buleu* (buleu.olesya@mail.ru)

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Abstract

The karyotypes and the localization of C-bands, clusters of ribosomal DNA and telomeric repeats of 10 species of the family Pamphagidae from Morocco are described for the first time. The species studied belong to the subfamilies *Pamphaginae* and *Thrinchinae*. All species have karyotypes consisting of 19 and 20 acrocentric chromosomes and X0/XX sex chromosome system in males and females, respectively (2n♂=19, NF=19; 2n♀=20, NF=20). Despite the karyotype conservatism, we revealed differences in the location and size of C-heterochromatin blocks and ribosomal DNA clusters. A comparative analysis of these differences shows that karyotype divergences in this group is connected not to structural chromosome rearrangements, but to the evolution of repetitive DNA.

Keywords

Pamphagidae grasshoppers, karyotypes, C-banding, FISH, telomeric repeats $(TTAGG)_n$, ribosomal DNA repeats

Introduction

The family Pamphagidae Burmeister, 1840 is a comparatively small group in Acridoidea grasshoppers, which includes 96 genera and 561 species and subspecies (Ünal 2016). The species of this family inhabit the desert, semidesert, and mountainous landscapes of the Palaearctic and Afrotropical Regions (Uvarov 1966, Massa 2013, Ünal 2016). More than 90 species were described in North Africa, most of which occur in Morocco (Massa 2013, Ünal 2016). To date, the Pamphagidae remain one of the least cytogenetically studied groups among the grasshoppers. The early studies of karyotypes of some species from North and South Africa, South-West Europe and East Asia revealed the exceptional karyotype conservatism of this family (Chen 1937, White 1973, Hewitt 1979, Camacho et al. 1981, Santos et al. 1983, Cabrero et al. 1985, Fossey 1985, Fu Peng 1989, Mansueto and Vitturi 1989, Vitturi et al. 1993, Warchałowska-Śliwa et al. 1994). In these studies the diploid sets of chromosomes of Pamphagidae species consisted of 19 (♂) and 20 (♀) acrocentric chromosomes with X0♂/XX♀ sex determination mechanism. It allowed drawing a conclusion about the exceptional karyotype conservatism of this family. Further karyotyping of some Pamphagidae species from Central Asia, Bulgaria and Western and Central Anatolia, however, led to a revision of the notion of a uniform karyotype structure within the family. The vast majority of Pamphagidae species from these regions have a karyotype consisting of 16 acrocentric autosomes and a neo-XY sex chromosome system (2n♂♀=18, neo-XX♀/neo-XY♂) (Bugrov 1996, Bugrov and Warchałowska-Śliwa 1997, Bugrov and Grozeva 1998, Bugrov and Warchałowska-Śliwa 2016, Jetybayev et al. 2017). These sex chromosomes arose due to the centric fusion of the original X chromosome with an autosome. In addition, there are karyotypes in which several pairs of chromosomes (*Melanotmethis fuscipennis* (Redtenbacher, 1889)) (Bugrov and Warchałowska-Śliwa 1997) or even all of them (*Eremopeza festiva* (Saussure, 1884)) have short second arms (Bugrov et al. 2016). These indicate that not all Pamphagidae have a conserved chromosomal set, making this group a good model for understanding the karyotype evolution.

The majority of Pamphagidae species that possess a derived karyotype are distributed in Western and Central Asia, which led to assumption that the evolutionary events resulted in karyotypic changes occurred most likely within these territories (Bugrov 1996, Bugrov and Warchałowska-Śliwa 1997, Bugrov and Grozeva 1998, Bugrov et al. 2016, Jetybayev et al. 2017). Unfortunately, scarce cytogenetic data on Pamphagidae from other centers of biodiversity of this family does not allow us to confirm whether such a karyotype derived is characteristic only of the indicated geographical region. For this reason, the study of the species of this family from other areas is indispensable.

In this work, we provide new data on the comparative cytogenetic analysis of some Pamphagidae species from Morocco. To obtain additional information on linear differentiation of chromosomes, we used C-banding and fluorescence *in situ* hybridization (FISH) with $(TTAGG)_n$ telomeric and ribosomal DNA probes. The data on the distribution of the C-bands and the clusters of ribosomal DNA and telomeric repeats is available for various insect species (Sahara et al. 1999, Cabrero and Camacho 2008, Grozeva et al. 2011, Jetybayev et al. 2012, Bugrov et al. 2016, Kuznetsova et al. 2017), which provides the base for comparative cytogenetic studies.

Table 1. List of species, collection places and number of specimens of the studied Pamphagidae species.

Taxa	Species	Location	Number of specimens
Pamphaginae Pamphagini	*Paracinipe alticola* (Werner, 1932)	Morocco (pass N of Taroudant) 30°51.53'N, 8° 22.66'W	2
	Paracinipe crassicornis (Bolívar, 1907)	Morocco (Oum Rbia valley) 32°45.40'N, 7°58.33'W	1
	Paracinipe dolichocera (Bolívar, 1907)	Morocco (El Kebab) 32°45.37'N, 5°38.72'W	2
	Paracinipe theryi (Werner, 1931)	Morocco (pass in Antiatlas to Tafraout) 29°49.87'N, 9°2.25'W	2
	Pseudoglauia tarudantica (Bolívar, 1914)	Morocco (Antiatlas – pass to Tafraout) 29°49.87'N, 9°2.25'W	1
	Acinipe hesperica lepineyi Chopard, 1943	Morocco (Achahaoud towards road Marakesh-Ouarzazad) 31°15.67'N, 7°23.32'W	1
Pamphaginae Euryparyphini	*Euryparyphes rungsi* Massa, 2013	Morocco (Col du Zad N of Midelt) 33°2.12'N, 5°4.32'W	2
	Eunapiodes granosus (Stål, 1876)	Morocco (NW Ouaourioud) 32°20.41'N 5°43.18'W	2
	Paraeumigus parvulus (Bolívar, 1907)	Morocco (pass N of Taroudant) 30°51.98'N, 8°21.48'W	3
Thrinchinae Thrinchini	*Tmethis cisti* (Fabricius, 1787)	Morocco(near Beni Ayadet) 33°41.25'N, 3°40.82'W	2

Material and methods

Material collection

Ten species of Pamphagidae from the High and Middle Atlas in Morocco were collected during May-June 2013. Nine species belong to the subfamily *Pamphaginae* and one to the subfamily *Thrinchinae*. Table 1 describes the material analyzed and the collection localities, as well as the number of individuals examined.

Methods

Fixation, C-banding and Fluorescence *in situ* hybridization (FISH)

The testes were dissected from adult males and placed into 0.9% solution of sodium citrate for 20 min. The testes were fixed in 3:1 ethanol:glacial acetic acid for 15 min. The fixed material was then rinsed and kept in 70% ethanol. Air-dried chromosome preparations were made by squashing testis follicles in 45 % acetic acid and then freezing them in dry ice.

C-banding of the chromosome preparations was performed according to Sumner (1972) with minor modifications. Chromosome preparations were treated with 0.2 N HCl for 15–20 min, rinsed in distilled water, incubated in a saturated solution of $Ba(OH)_2$ at 61 °C for 3–5 min, rinsed in tap water, and then incubated in 2×SSC at 61 °C for 60 min. After being rinsed in distilled water, the slides were stained with 2% Giemsa.

Fluorescence *in situ* hybridization (FISH) with $(TTAGG)_n$ telomeric and ribosomal DNA probes on meiotic chromosomes was carried out according to the protocol by Pinkel (1986) with modifications (Rubtsov et al. 2000, 2002). In brief, the slides were treated with 0.1 μg/ml solution of RNAse (Sigma-Aldrich, USA) in 2×SSC for one hour, washed three times in 2×SSC and then dehydrated in 70%, 80% and 96% ethanol for two minutes. After dehydration, the slides were treated with 0.04% pepsin solution (activity ≥ 400 U/mg, Sigma-Aldrich, USA) in 0.01M HCl for 8 minutes at 37 °C, and washed in PBS for 5 minutes, in PBS with 0.1M MgCl for 5 minutes, in 0.1% formaldehyde in PBS with 0.1M MgCl for 10 minutes, and then again in PBS for 5 minutes, and lastly dehydrated in the ethanol series as described above. The 10 μl of the hybridisation solution of 30 ng of each DNA probe and 1 μg of sonicated salmon sperm DNA in hybridisation buffer (50% deionized formamide, 10% dextran sulfate, 2×SSC, 0.01% Tween 20) was applied under cover glass and denaturated on the hotplate for 5 minutes at 75 °C and then hybrised in the humid chamber overnight at 37 °C. The three washing steps were carried out in 50% formamide solution in 2XSSC at 45 °C for 5 minutes, three times in 2XSSC at 45 °C for 5 minutes, three times in 0,2XSSC at 45 °C for 5 minutes and three final times in 0,1XSSC at 65 °C for 5 minutes.

Unlabeled rDNA probe was generated by the polymerase chain reaction (PCR) of six fragments of 18S and 28S rRNA genes using specific primers according to Jetybayev et al. (2017) and Buleu et al. (2017). Because 18S rRNA and 28S rRNA genes are parts of a single 45S rRNA gene, they both were used to detect rDNA cluster. The fragments of the genes were labeled in additional PCR cycles with Fluorescein-12-dUTP (Biosan, Novosibirsk, Russia) and mixed together into a single rDNA probe. Telomeric repeats $(TTAGG)_n$ were generated by the non-template PCR method with 5'-TAACCTAAC-CTAACCTAACC-3' and 5'-TTAGGTTAGGTTAGGTTAGG-3' primers. Further labeling with Tamra-dUTP (Biosan, Novosibirsk, Russia) was performed in additional 33 cycles of PCR as described previously (Sahara et al. 1999).

For the description of chromosomes, karyotypes and C-bands, the nomenclature previously proposed for grasshoppers was used (King and John 1980, Santos et al. 1983, Cabrero and Camacho 1986).

Microscopic analysis was performed at the Centre for Microscopy of Biological Objects (Institute of Cytology and Genetics, Novosibirsk, Russia). Chromosomes were studied with an AxioImager M1 (Zeiss, Germany) fluorescence microscope equipped with filter sets #49, #46HE, #43HE, and a ProgRes MF (MetaSystems GmbH, Germany) CCD camera. The ISIS5 software package was used for image capture and analysis.

Results

Karyotype

The diploid sets (2n) of chromosomes all the studied species consist of nine pairs of acrocentric autosomes and one unpaired acrocentric X-chromosome in males and two

X chromosomes in females (X0/XX sex determination system). The karyotype structure is represented by of four large (L1–L4), three medium (M5–M7) and two small (S8–S9) pairs of autosomes, and the medium sized X chromosome.

C-banding

The C-banding of the chromosomes in the studied species reveals three different localizations of the C-blocks: pericentromeric, interstitial and telomeric. The pericentromeric C-bands appear in every species analyzed.

Pericentromeric C-bands

The size of the pericentromeric C-bands differs in various chromosomes within the karyotypes of the species studied: most of the chromosomes have small-sized pericentric C-positive blocks, but in some chromosomes medium-sized blocks have been also observed. *Acinipe hesperica lepineyi* is the only species with medium-sized pericentric C-block in all its chromosomes (Fig. 1k). The medium-sized pericentric C-blocks are also detected in some chromosomes in the following species: *Paracinipe alticola* (L4, M5, S8, S9) (Fig. 1a); *P. crassicornis* (L1 – L4, M5, M6) (Fig. 1c); *P. dolichocera* (L3, L4, M6, S8) (Fig. 1e); *P. tarudantica* (L1 – L4, S8) (Fig. 1i); *Euryparyphes rungsi* (L1 – L4) (Fig. 1m); *Eunapiodes granosus* (L1, L2, L4) (Fig. 1o); *Paraeumigus parvulus* (L1) (Fig. 1q). In *Paracinipe theryi* (Fig. 1g) and *Tmethis cisti* (Fig. 1s), the pericentric C-blocks are of small size in all of the chromosomes.

The X chromosome in all the examined species possesses a small C-heterochromatic block, except for the X chromosome of *P. alticola*, *P. dolichocera* and *A. hesperica lepineyi*, which has a medium-sized pericentric C-block (Fig. 1a, e, k).

Interstitial C-bands

The interstitial C-bands are found in five of the studied species: *P. alticola*, *P. crassicornis*, *P. dolichocera*, *P. theryi* and *A. hesperica lepineyi* (Fig. 1a, c, e, g, k). These blocks are of small or medium-sized and are generally localized in the proximal part of the autosomes. In *P. alticola*, medium-sized interstitial C-bands are observed in three large (L1, L2, L4) and one medium (M6) pair of autosomes. In the fourth largest (L4) pair of autosomes, the interstitial C-bands are detected only in one of the homologues. In the middle-sized sixth pair (M6), the interstitial block is dispersed (Fig. 1a). In *P. crassicornis*, a small interstitial block is located in the second largest pair of autosomes (L2) (Fig. 1c). In *P. dolichocera*, the medium interstitial C-bands are identified in a middle (M5) and small (S8) pair of autosomes. The small autosome pair (S8) has a complex of interstitial heterochromatic bands consisting of two or three small blocks. In a large (L3) and medium (M7) autosome pairs, the small-sized interstitial C-bands are detected only in one of the homologues

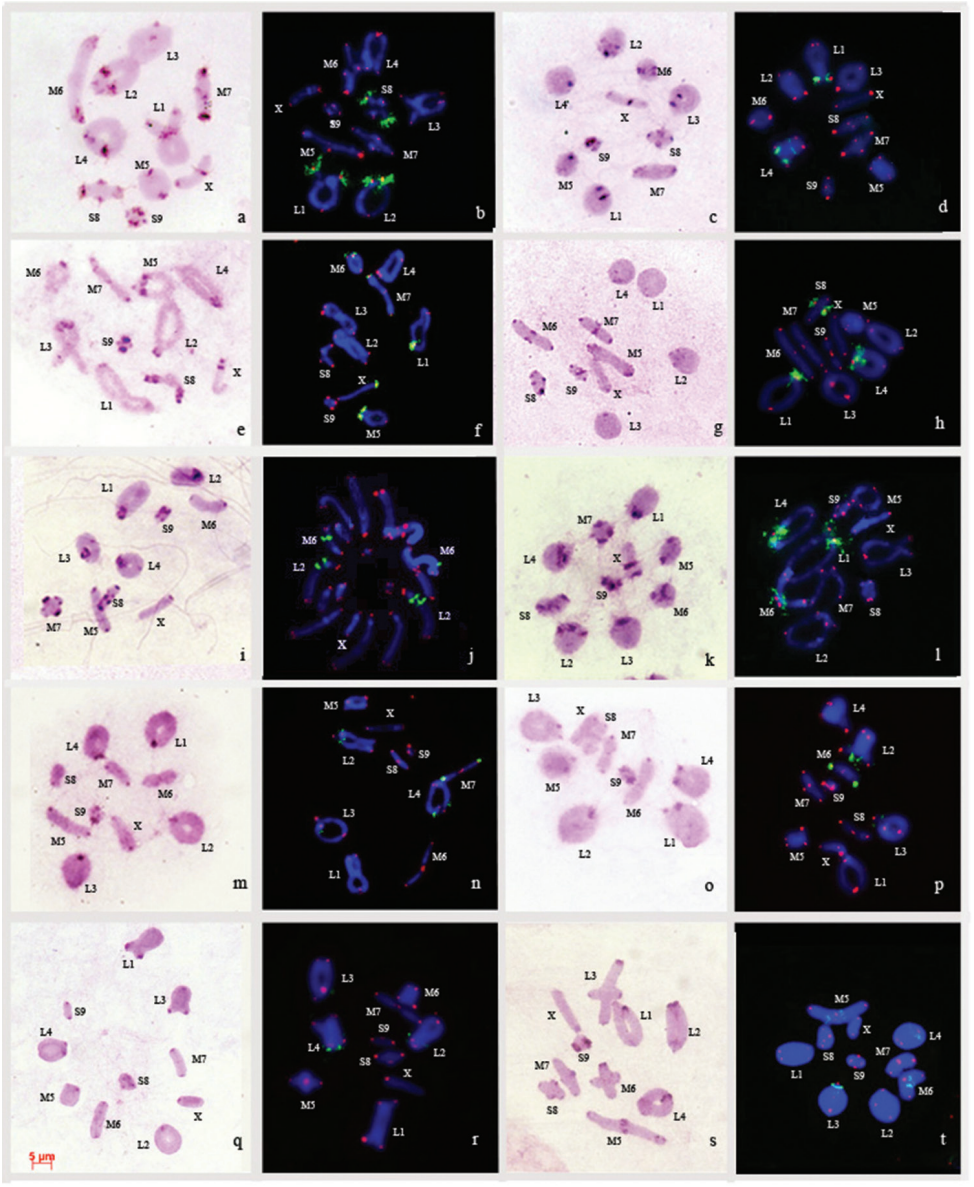

Figure 1. C-banding (**a, c, e, g, i, k, m, o, q, s**) and FISH of rDNA (green) and $(TTAGG)_n$ (red) probes (**b, d, f, h, j, l, n, p, r, t**) in meiotic chromosomes of species studied. *Paracinipe alticola* (**a, b** diakinesis); *Paracinipe crassicornis* (**c, d** metaphase I); *Paracinipe dolichocera* (**e, f** diakinesis); *Paracinipe theryi* (**g, h** diakinesis); *Pseudoglauia tarudantica* (**i** metaphase I **j** spermatogonial metaphase); *Acinipe hesperica lepineyi* (**k** metaphase I **l** diakinesis); *Euryparyphes rungsi* (**m** metaphase I **n** diakinesis); *Eunapiodes granosus* (**o, p** metaphase I); *Paraeumigus parvulus* (**q, r** metaphase I); *Tmethis cisti* (**s, t** metaphase I). Scale bar: 5 µm.

(Fig. 1e). In *P. theryi*, tiny interstitial C-bands are detected in the middle (M5) and large (L2) autosome pairs (Fig. 1g). In *A. hesperica lepineyi*, medium-sized interstitial C-bands on two large (L2, L4) and the sixth middle (M6) autosome pairs are found. The third large pair of autosomes (L3) has very small interstitial C-bands (Fig. 1k).

The X chromosome has a medium-sized interstitial C-band in *P. alticola*, *P. crassicornis*, *P. dolichocera* and *A. hesperica lepineyi*. The location of interstitial C-bands on the X chromosome differs in these species. In *P. alticola* and *P. crassicornis*, this band is located in the proximal part (Fig. 1a, c), while in *P. dolichocera* and *A. hesperica lepineyi* was observed in the distal part of the X chromosome (Fig. 1e, k).

Telomeric C-bands

Telomeric C-blocks of large, medium and small-sized is revealed in all of the studied species. When telomeric C-bands are present, they are usually located either in medium or small chromosomes.

In *P. alticola*, *P. crassicornis*, *P. dolichocera* and *P. theryi*, telomeric blocks are detected on medium-sized autosomes: two autosome pairs in *P. alticola* (M5, M7) and *P. theryi* (M6, M7) (Fig. 1a, g), and on one chromosome pair in *P. crassicornis* (M6) and *P. dolichocera* (M7) (Fig. 1c, e). The S8 chromosome pair carries telomeric blocks of different sizes in all studied species except for *P. dolichocera*, *E. rungsi*, *E. granosus*, and *P. parvulus* (Fig. 1e, m, o, q). Another autosome pair that has a large telomeric block in all the species studied is the S9 autosome pair. In *P. crassicornis* and *P. tarudantica*, telomeric C-band is located only in one homologue in the S9 autosome pair (Fig. 1c, i). In *T. cisti*, the autosome pairs L1–L4, M5 and S9 have medium-sized telomeric C-bands whereas the M6, M7, and S8 pairs exhibit small-sized telomeric C-blocks (Fig. 1s). Very thin telomeric blocks are detected in M6 and M7 pairs of the *E. granosus* karyotype, and also in L3 and M7 pairs of the *P. parvulus* chromosome set (Fig. 1o, q). The X chromosome has very small-sized telomeric blocks only in *E. rungsi* (Fig. 1m).

Fluorescence in situ hybridization

FISH of telomeric $(TTAGG)_n$ DNA probe

FISH experiments with telomeric DNA-probe reveal fluorescent hybridization signals at the ends of all autosome bivalents (Fig. 1b, d, f, h, j, l, n, p, r, t). The telomeric signals are revealed only in the X chromosome of *P. parvulus* in one of its terminal regions (Fig. 1r). The hybridization signals of the telomeric DNA-probe show variation in intensity between chromosomes in the karyotype and among chromosome sets of the studied species (Fig.1).

FISH with the rDNA probe

The cluster of rRNA genes consists of many copies of 45S rRNA gene that are interlaced with non transcribed spacer (Srivastava and Schlessinger 1991). At the same time, 45S rRNA gene contains both 18S and 28S rRNA genes. The FISH experiments of these two DNA probes showed complete colocalization in meiotic chromosomes of all species analysed. The using both DNA probes labeled with same fluorophore as a single rDNA probe allow to show higher intensity of the hybridization signal. Therefore, this combined probe is used to reveal rDNA clusters and to map their distribution and location in further studies.

The clusters of rDNA repeats localize on two (*P. crassicornis*, *P. tarudantica*, *P. parvulus*) (Fig. 1d, j, r), three (*P. alticola*, *P. theryi*, *A. hesperica lepineyi*, *E. rungsi* (Fig. 1b, h, l, n) or four (*P. dolichocera*, *E. rungsi*, *T. cisti* (Fig. 1f, n, t) autosome bivalents, and on the X chromosome (*P. dolichocera* (Fig. 1f).

The rDNA repeats are found only in pericentromeric and interstitial regions of chromosomes. The pericentromeric rDNA clusters are detected in karyotypes of all analyzed species except *P. tarudantica* (Fig. 1j). The rDNA is observed in pericentromeric region of the L1 bivalents in all *Paracinipe* species and in *A. hesperica lepineyi* (Fig. 1b, d, f, h, l). The L2 bivalent bears rDNA genes at pericentromeric region only in *P. alticola* (Fig. 1b). The pericentromeric region of the L3 bivalents have a single cluster of rRNA genes in *E. granosus* and *T. cisti* (Fig. 1p, t). Hybridization signals are found in L4 bivalents of *P. crassicornis*, *P. theryi*, *A. hesperica lepineyi* and *P. parvulus* (Fig. 1d, h, l, r). A single cluster of signals are found in the pericentromeric region of medium autosome bivalents of four species: *P. dolichocera* (M5–M7); *A. hesperica lepineyi* (M6) and *E. rungsi* (M7) (Fig. 1f, l, n). In *P. alticola* and *P. theryi*, the probe hybridizes to the pericentromeric region of S8 bivalents (Fig. 1b, h).

Interstitial clusters of rRNA genes are revealed in seven species. The hybridization signals are found in the large autosome bivalents of *P. crassicornis* (L4), *P. tarudantica* (L2), *E. rungsi* (L2, L4), *E. granosus* (L2), *P. parvulus* (L2), and *T. cisti* (L4) (Fig. 1d, j, n, p, r, t). In *P. tarudantica*, the L2 autosome bivalent has two adjacent interstitial rDNA clusters (Fig. 1j). In *T. cisti*, interstitial rDNA clusters are also found in two of the medium bivalents (M5, M6) (Fig. 1t). In the four species *P. tarudantica*, *E. rungsi*, *E. granosus*, *P. parvulus* and *T. cisti*, the rDNA clusters are located in the proximal region of the bivalents (Fig. 1j, n, p, r, t), whereas in *P. crassicornis* (L4) and *P. tarudantica* (M6), the signals were placed in the distal part of the autosome bivalents (Fig. 1d, j). The probe identifies one rDNA cluster only in the X chromosome of *P. dolichocera* at the pericentromeric region (Fig. 1f).

Discussion

The cytogenetic analysis of the Pamphagidae grasshoppers from Morocco confirmed that the species of Pamphaginae and Thrinchinae subfamilies from the Western Mediterranean region have an exceptionally conservative karyotype consisting of 19 (♂) and

20 (♀) acrocentric chromosomes with a X0♂/XX♀ sex chromosome system. Previously, we described five species of Pamphagidae (*Eunapiodes atlantis* (Chopard, 1943), *Paraeumigus fortius* (Bolivar, 1907), *Euryparyphes flexuosus* Uvarov, 1927, *Acinipe tubericollis* Werner, 1932, and *Pseudoglauia terrea* (Bolivar, 1912)) from the same region and showed that these species had a male and female diploid chromosome number of 19 and 20, respectively with standard type of sex chromosome system (X0♂/XX♀) (Buleu et al. 2015). These results indicate that only the Nocarodeini tribe of Pamphaginae subfamily which prevails in Western Asia, the Caucasus and Transcaucasia, has a 2n=18, XY♂/XX♀ karyotype (Bugrov et al. 2016, Jetybayev et al. 2017).

In general, the collected data on the distribution of C-heterochromatin in chromosomes of the species studied agrees with the results of certain Pamphagidae species from Spain (Camacho et al. 1981, Santos et al. 1983, Cabrero et al. 1985). The difference in size and localization of C-positive blocks in several species allow proposing that the repetitive DNA sequences would be the responsible of the existing diversity of karyotypes in this group, and not the structural rearrangements of chromosomes. The analysis of the C-banding revealed three different chromosomal positions of the C-positive blocks: pericentromeric, interstitial and telomeric. The pericentromeric and telomeric C-heterochromatic blocks were detected in all species analysed, whereas interstitial C-positive blocks were observed only in four species of the genus *Paracinipe* and in *Acinipe hesperica lepineyi*. In most of the species of the present work, the pericentromeric block was small-sized. However, in some of the species, large, medium or small bivalents had a medium-sized pericentromeric block. In previously species studied from the same region (*E. atlantis*, *P. forties*, *A. tubericollis*, *P. terrea*), we revealed similar sized pericentromeric blocks in large pairs of autosomes (Buleu et al. 2015). Occasionally small- or medium-sized interstitial blocks were placed in the proximal part of medium (M5 in *P. theryi*; M6 in *P. alticola*) and small (S8 in *P. dolichocera*) bivalents. These dispersed interstitial blocks were located in close proximity to the near pericentromeric region. Similar interstitial C-blocks were previously observed on medium and small autosomes pairs in *Pseudoglauia terrea* (Buleu et al. 2015). It was suggested that findings of such blocks support the hypothesis that the differences in the size of C-blocks may be caused by addition (or loss) of heterochromatin (Camacho et al. 1981).

Besides, the X chromosome of four species, *P. alticola*, *P.* cf. *crassicornis*, *P. dolichocera*, and *A. hesperica lepineyi*, had an interstitial C-positive block. The interstitial block in the X chromosome of *P. alticola* and *P.* cf. *crassicornis* was located in its proximal part, whereas in *P. dolichocera* and *A. hesperica lepineyi* it was in the distal part of the X chromosome. It is possible that the relocation of this block was caused by inversions (Jetybayev et al. 2012).

Telomeric C-heterochromatin blocks were detected in all of the studied species in one or two medium or small autosome bivalents. Only in *E. rungsi*, the X chromosome had a very small size telomeric block. The small bivalent (S9) had large or medium-sized telomeric C-blocks in all studied species. The presence of large telomeric C-blocks in these small bivalents have already been observed in Pamphagidae (Camacho et al. 1981, Bugrov and Warchałowska-Śliwa 1997, Bugrov et al. 2016, Jetybayev et al. 2017).

Furthermore, in L4 of *P. alticola*, and in L4 and M7 of *P. dolichocera*, the interstitial C-bands were detected only in one of the homologues of these bivalents (Fig. 1a, e). This observation may indicate the presence of a polymorphism in the populations of these species. However, the study of a small number of specimens of the same species does not allow drawing conclusions about C-band polymorphisms, as found in *Asiotmethis heptapotamicus* (Bugrov et al. 2016).

The FISH analysis with $(TTAGG)_n$ probe revealed that this DNA motif is a component of the telomeres in all chromosomes of species herein studied. This motif is widespread through different lineages of insects and other arthropods, and it is considered as the ancestral sequence of telomeres in chromosomes of arthropods (Vítková et al. 2005, Traut et al. 2007). FISH with the telomere DNA probe revealed a variation in the intensity of hybridization signals among chromosomes in the karyotype and among chromosome sets of the species studied. These variations may be associated with the peculiarities of the labeled probe penetration through the cell cytoplasm during the FISH, or with the quantity of telomeric DNA repeats. The presence of interstitial telomeric sequences (ITSs) was not revealed. This fact may indicate the absence of structural rearrangements involving terminal regions in Pamphaginae karyotype evolution. Such rearrangements were previously detected in Acrididae grasshoppers (Jetybayev et al. 2012).

In previous studies, rDNA genes was mapped using plasmid containing complete 45S rDNA (Cabrero and Camacho 2008), or 18S rDNA (Jetybayev et al 2012), or 28S rDNA (Buleu et al. 2017). In current study, we used the 18S and 28S DNA probes and observed complete colocalization of the signals from 18S and 28S rDNA. These results confirm that 18S and 28S rRNA genes are in fact parts of single 45S rRNA cluster.

In this study, rDNA-FISH revealed an interspecific variation in the localization of ribosomal genes. In most of the species, the rDNA cluster were located at the pericentric region in the large bivalents and in the fifth, sixth and seventh medium ones. In *P. alticola* and *P. theryi*, the rDNA clusters were also mapped at the pericentric region of small chromosomes (S8). The ribosomal clusters at interstitial regions of large bivalents (L2, L4) were revealed in *P. crassicornis*, *E. rungsi*, *E. granosus*, *P. parvulus* and *T. cisti*. In *P. tarudantica* and *T. cisti*, interstitial rDNA genes was detected in the medium autosome bivalents M5 and M6. Usually these bivalents have a single interstitial rDNA cluster. However, two interstitial rDNA clusters were located in one large chromosome pair (L2) of *P. tarudantica*. Multiple localization of rDNA clusters in a single chromosome was previously reported for *Pamphagus ortolaniae* (Vitturi et al. 2008), *Pseudoglauia terrea* (Buleu et al. 2015) and some Pamphagidae species from Armenia (Bugrov et al. 2016) and Turkey (Jetybayev et al. 2017). It should be emphasized, that the multiple localization of rDNA clusters in a single chromosome among Acridoid grasshoppers has been detected so far in species of the Pamphagidae family exclusively (Jetybayev et al. 2017, Buleu et al. 2017). In the cytogenetically well-studied family Acrididae, the distribution of rDNA clusters was limited mainly to the one or two pair of chromosomes per karyotype (Cabrero and Camacho 2008, Jetybayev et al. 2012, Palacios-Gimenez et al. 2013). The multiple rDNA clusters in Pamphagidae may sup-

port the hypothesis of mutual translocations of two pairs of autosomes in the 19-chromosome karyotype of Pamphagidae from the basal 23-chromosome karyotype of Acridoidea (White 1973). Probably, two ancestral pair of chromosomes carried rDNA clusters and they formed a chromosome pair with two clusters of rDNA after the fusion. This hypothesis is based on that rDNA clusters usually tend to localize in pericentric or proximal regions (Cabrero and Camacho 2008, Loreto et al. 2008, Cabral-de-Mello et al. 2011, Jetybayev, 2012, Palacios-Gimenez et al. 2013). Therefore, if the initial fusion were centric, two rDNA clusters would lie very close to each other or even fuse into one cluster. In the case under consideration, however, all the observed double rDNA clusters were clearly distinct, thus the fusion would be a tandem one and not centric.

A pericentromeric rDNA cluster in the X chromosome were only found in one species (*P. dolichocera*). Conversely, it is worth noting that the presence of ribosomal genes in the X chromosome was reported for many grasshoppers (Cabrero and Camacho 2008, Cabrero et al. 2009, Veltsos et al. 2009). In Pamphagidae species with an X0/XX sex system, the rDNA loci was found in the X chromosome of only two species, namely *Asiotmethis muricatus* (Pallas, 1771) and *A. tauricus* (Tarbinsky, 1930) (unpublished data). Among the Pamphagidae species that have neoXY/neoXX sex chromosome system, the neo-X often carries rDNA cluster and they are usually located at interstitial region (Jetybayev et al. 2017). This observation may indicate evolutionary changes that have occurred in the X chromosomes. Possible mechanisms explaining changes in rDNA cluster location could be paracentric inversion, or insertion of DNA fragments containing rDNA into the chromosome, with subsequent rDNA amplification and elimination of the old rDNA cluster, or transposition of the NOR region (i.e. interchromosomal mobility of NOR regions) (Arnheim et al. 1980, Schubert and Wobus 1985, Dubcovsky et al. 1995, Roy et al. 2005, Cabrero and Camacho 2008).

In conclusion, in spite of the karyotypic conservatism of the Pamphagidae species studied, cytogenetic differences in the location of chromosome markers (C-heterochromatin blocks, telomere sequences and ribosomal genes) were found in both closely related species of one genus and between different genera. The differences in localization of these cytogenetic markers in closely related species appear to be associated with chromosomal rearrangements known to play a fundamental role in speciation (White 1968). Since many Pamphagids have a standard set of chromosomes, these changes need to be taken into account to explain the speciation processes within and between genera.

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RESEARCH ARTICLE

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Analysis of chromosome karyotype and genome size in echiuran *Urechis unicinctus* Drasche, 1880 (Polychaeta, Urechidae)

Zhenkui Qin[1,*], Xueyu Li[1,*], Danwen Liu[1], Qing Wang[1], Li Lu[1], Zhifeng Zhang[1]

1 *Ministry of Education Key Laboratory of Marine Genetics and Breeding, College of Marine Life Sciences, Ocean University of China, Qingdao 266003, China*

Corresponding author: *Zhifeng Zhang* (zzfp107@ouc.edu.cn)

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Abstract

Karyotype and genome size are two primary cytogenetic characteristics of species, which are of great significance to the study of cytogenetics, taxonomy, phylogenesis, evolution as well as molecular biology. However, this basic cytogenetic information in echiurans is lacking. Therefore, we analyzed characteristics of karyotype and genome size in the echiuran worm *Urechis unicinctus* Drasche, 1880. In this study, coelomic cells of *U. unicinctus* were used for analyzing the genome size by a flow cytometry with chicken erythrocytes as DNA standard, and the 2C DNA content was determined to be 1.85 pg, which was corresponded to the genome size of 904.58 Mbp approximately. Furthermore, trochophores of *U. unicinctus* were dissociated and cells were utilized for preparing the chromosomes stained with DAPI, and the karyotype was determined as 2n = 30 (10m + 6sm + 6st + 8t), FN=52. Our data provided the basic cytogenetic information of *U. unicinctus*, which could be utilized in taxonomic study and whole-genome sequencing in future.

Keywords

Urechis unicinctus, karyotype, genome size, flow cytometry

* These authors contribute equally to this work.

Introduction

Echiurans (spoon worms) are a group of marine worms which are unsegmented, coelomate, bilaterally symmetrical and soft-bodied (Goto 2016). Traditionally, echiurans have been excluded from Annelida because of their non-segmented characteristics (Fisher and MacGinitie 1928). Recently, based on molecular phylogenetic data (Struck et al. 2007, Wu et al. 2009, Struck et al. 2011, Weigert et al. 2014, Andrade et al. 2015, Goto 2016;)the species in Echiura have often been considered as a group of derived annelids that secondarily lost segmentation. Thus, a controversial issue of whether echiurans belong to Annelida or a separate Echiura phylum has emerged and this needs to be elucidated from different research scopes. Chromosome karyotype and genome size are two important cytogenetic characteristics and have been applied widely in taxonomic, phylogenetic and evolutionary studies (Ipucha et al. 2007, Kashmenskaya and Polyakov 2008, Leitão et al. 2010, Palomina et al. 2017). Regrettably, only very few studies about chromosomes in the echiuran worms are available (Griffin 1899, Lefevre 1907, Singhal and Dattagupta 1980), which were reported several decades ago, and no report was related to their genome size. Therefore, these basic cytogenetic characteristics of echiurans need to be revealed urgently.

Karyotype, including chromosome number and composition, could reflect the taxonomic relationship between species and be used as a tool to explore biological diversity (Dobigny et al. 2004, Leitão et al. 2010, Cioffi et al. 2012). Gallardo-Escárate and Del Río-Portilla (2007) analyzed the cytogenetical relationships of three abalone species *Haliotis corrugata* W. Wood, 1828, *H. fulgens* Philippi, 1845, and *H. rufescens* Swainson, 1822 based on their chromosomal morphology, and proposed that *H. rufescens* and *H. corrugata* are cytogenetically more similar to each other than to *H. fulgens*. Ipucha et al. (2007) discussed the phylogenetic relationship by analyzing the karyotypes of seven species from Nereididae, and suggested the karyotypes are relatively similar and stable in nereidid species at the family level, while the main mechanism of chromosomal evolution could be pericentric inversions.

Genome size is the total DNA content within a single copy genome and is also referred to C-value, which is specific in every species and ranges from 0.02 pg (*Pratylenchus coffeae* Zimmermann, 1898, a plantparasitic nematode) to 132.83 pg (*Protopterus aethiopicus* Heckel, 1851, a marbled lungfish) in animals (Gregory 2018). C-value estimation is important for genomic sequencing and analysis (Gregory 2005). Nevertheless, variation of genome size in different species is rarely used as a direct or single factor in evolution analysis due to the C-value paradox (the phenomenon that C-value is inconsistent with the complexity of biological structure or composition), which means the genome size among organisms were diverse and possessed no relationship to organismal complexity (Gregory 2010).

Urechis unicinctus, a commercial echiuran worm inhabiting the U-shaped burrows in the coastal mud flats, has unique roles in animal evolution, coastal sediment im-

provement and marine drug development (Liu et al. 2015). In this study, chromosome counting and composition analysis of *U. unicinctus* were carried out in the well-developed trochophore for the first time, and the genome size of *U. unicinctus* coelomic cells was also determined using flow cytometry. We aim to reveal the basic cytogenetic characteristics of *U. unicinctus*, which provide useful information for taxonomic and genomic studies.

Material and methods

Animals

U. unicinctus adults with 9.96 ± 0.42 cm in body length were purchased from an aquatic product market, which were collected from a coastal intertidal flat in Yantai, China.

Chromosome preparation and karyotype analysis

Sampling

The mature sperms and oocytes were obtained by dissecting the nephridia of the healthy *U. unicinctus*, respectively. Artificial fertilization was conducted by mixing sperms and oocytes at a ratio of 10:1 in filter seawater (FSW), and then these fertilized eggs were cultivated until hatched in FSW (19.7 ± 0.3 °C, salinity 29 PSU, pH 8.29 ± 0.02). The hatched trochophores were collected using a 500 mesh sieve.

Chromosome preparation and karyotype analysis

Chromosomes of *U. unicinctus* trochophores were prepared as described by Earley (1975) with some modifications. The larvae were incubated in FSW containing 0.02% colchicine for 2.5 h at room temperature, and then transferred into Ca^{2+}/Mg^{2+}-free Dulbecco's phosphate buffered saline (CMF-DPBS, 137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4{\times}7H_2O$, 1.1 mM KH_2PO_4) for 30 min with continuous mild shaking to obtain the dissociated larval cells. Successively, the cells were treated with 0.075 mol/L KCl for 30 min, collected by centrifuging at 1200 g for 5 min, and then fixed three times with cold Carnoy's fixative (3 ethanol: 1 glacial acetic acid) for 15 min each. After centrifugation, the cells were re-suspended in 50% glacial acetic acid aqueous solution, and then dropped onto preheated clean glass slides at 56 °C, and air-dried. Finally, these samples were stained with 10 μg/ml 4', 6-diamidino-2-phenylindole (DAPI) (Solarbio, China) for 15 min, and were examined and photographed under a Nikon Eclipse 80i fluorescence microscope (Nikon, Tokyo, Japan).

The chromosome lengths, chromosome relative lengths and arm ratios from well-formed chromosomes in metaphase were measured and calculated using MICROMEASURE 3.3 software. Data were presented as mean ± SEM (n = 5). Chromosomes were classified according to the description of Levan et al. (1964), and the homologous chromosome was assigned based on the similarities in length and centromere position using PHOTOSHOP CS6 software. The idiogram was constructed according to the arm ratio and relative length of the chromosomes. The karyotype was classified as described by Stebbins (1971).

Estimation of DNA content

Preparation of single cell samples

Coelomic fluids from 11 healthy worms were collected by syringe puncturing *U. unicinctus* body wall, respectively, and three duplicate samples were obtained from each individual. The coelomic cells were pelleted by centrifugation at 1000 g (4 °C) for 5 min, washed three times with PBS (pH 7.2), and then resuspended with PBS (pH 7.2). The suspension was added dropwise to the precooled 70% ethanol and fixed overnight at 4 °C. The next day, cell samples were collected through a 50 μm nylon mesh filtration, adjusted to 5×10^5 cells/ml, digested with 20 μg/μl RNase A for 10 min and then stained with 1 μg/μl propidium iodide (PI) for 30 min in the dark at room temperature.

Chicken erythrocytes (2C = 25 pg DNA) were used as an internal standard (Darzynkiewicz and Juan 1997). Fresh blood was acquired by puncturing the heart and mixed with 5% sodium citrate to prevent coagulation, and the subsequential processing procedures were as described in above.

Flow cytometric analysis

Twelve samples, including a chicken erythrocyte, a *U. unicinctus* coelomic cell, and ten mixed samples containing 500 μl erythrocytes and 500 μl coelomic cells, were analyzed using a Coulter Cytomics FC500-MPL flow cytometer (Beckman, California, USA) equipped with a 488 nm laser source to detect the DNA content, and the output was processed in the software FLOWJO 7.6.1. Coefficients of variation (CV) were adjusted below 5% to ensure the reliability. The DNA content of *U. unicinctus* was then calculated according to Doležel et al. (2007) using the equation: $Y = N/M \times X$ (Y means the 2C DNA content of *U. unicinctus*, N means the fluorescence mean values of *U. unicinctus* samples, M means the fluorescence mean values of internal standard (chicken erythrocytes), and X means the 2C DNA content of internal standard). The genome size of *U. unicinctus* was calculated using the equation: genome size (bp) = $(0.978 \times 10^9) \times$ DNA content (pg).

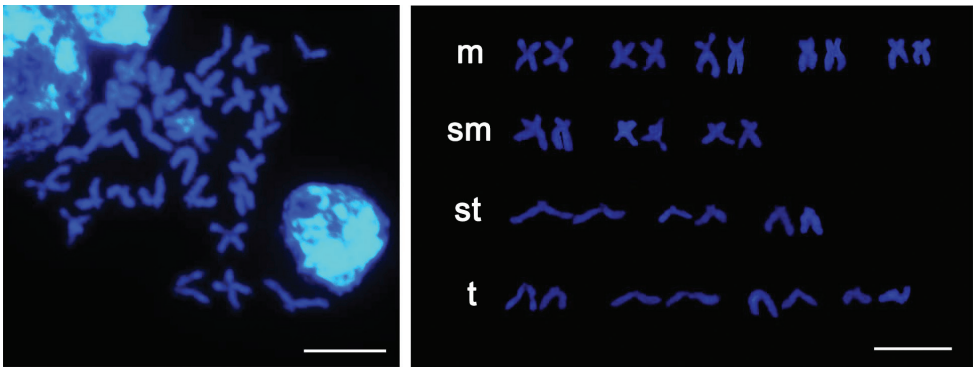

Figure 1. Metaphase chromosome and karyotype of *U. unicinctus* (2n = 30) stained with DAPI. m, metacentric chromosome; sm, submetacentric chromosome; st, subtelocentric chromosome; t, telocentric chromosome. Scale bar: 10 μm.

Results

Chromosome characteristics of *U. unicinctus*

The well dispersed metaphase chromosomes from *U. unicinctus* trochophore cells are shown in Fig. 1. The diploid chromosome number of *U. unicinctus* was 30, and fundamental number (FN) was 52. Fifteen pairs of the homologous chromosomes were matched, including 5 pairs of metacentric (m), 3 pairs of submetacentric (sm), 3 pairs of subtelocentric (st) and 4 pairs of telocentric chromosomes (t). No secondary constriction or satellite was found here. According to the measurement data (Table 1), the karyotype formula was deduced as 2n = 30 (10m + 6sm + 6st + 8t) (Fig. 1). Moreover, the idiogram was drawn based on the results above (Fig. 2). The index of karyotypic asymmetry (AsK) was 74.4%, which was the ratio of total length of long arms to that of all chromosomes. The chromosome size was 2.36–5.93 μm, with the longest to shortest chromosome length ratio (L/S) 2.51, and the percentage of chromosomes with arm ratio greater than 2:1 was 66.7%. Therefore, the karyotype of *U. unicinctus* was classified as 3B.

Genome size of *U. unicinctus*

The frequency histograms of DNA content from chicken erythrocytes and *U. unicinctus* coelomic cells were presented based on the flow cytometric analyses (Fig. 3). No overlap between the two peaks (Fig. 3C) indicated that chicken erythrocytes as the internal standard was suitable for DNA content determination of *U. unicinctus* coelomic cells.

The 2C mean values of chicken erythrocytes (M) and *U. unicinctus* coelomic cells (N) in ten mixed samples and their ratios were presented in Table 2. The results showed

Table 1. Karyotypic parameters of *U. unicinctus* (2n = 30).

Chr	p (μm)	q (μm)	Total (μm)	RL (%)	AR	Type
1	1.97 ± 0.01	2.20 ± 0.02	4.17 ± 0.03	7.09 ±0.04	1.12 ± 0.01	m
2	1.82 ± 0.05	2.15 ± 0.06	3.97 ± 0.09	6.75 ±0.11	1.18 ± 0.03	m
3	2.45 ± 0.04	3.48 ± 0.01	5.93 ± 0.01	10.1 ±0.01	1.42 ± 0.01	m
4	1.42 ± 0.04	2.21 ± 0.02	3.63 ± 0.04	6.18 ±0.05	1.56 ± 0.05	m
5	1.43 ± 0.01	2.40 ± 0.02	3.82 ± 0.03	6.50 ±0.04	1.68 ± 0.01	m
6	1.42 ± 0.02	2.94 ± 0.05	4.36 ± 0.07	7.42 ±0.08	2.08 ± 0.02	sm
7	1.24 ± 0.02	2.94 ± 0.05	4.17 ± 0.06	7.09 ±0.07	2.37 ± 0.04	sm
8	1.29 ± 0.03	3.29 ± 0.09	4.58 ± 0.08	7.79 ±0.10	2.55 ± 0.10	sm
9	0.92 ± 0.02	3.08 ± 0.07	4.01 ± 0.09	6.82 ±0.11	3.35 ± 0.04	st
10	0.59 ± 0.02	2.59 ± 0.04	3.18 ± 0.06	5.41 ±0.07	4.40 ± 0.08	st
11	0.53 ± 0.01	3.29 ± 0.07	3.82 ± 0.08	6.50 ±0.10	6.27 ± 0.09	st
12	–	3.74 ± 0.08	3.74 ± 0.08	6.36 ±0.10	∞	t
13	–	3.95 ± 0.07	3.95 ± 0.07	6.72 ±0.08	∞	t
14	–	3.09 ± 0.02	3.09 ± 0.02	5.26 ±0.02	∞	t
15	–	2.36 ± 0.02	2.36 ± 0.02	4.01 ±0.02	∞	t

p, length of short arm; q, length of long arm; Total, total length of chromosome; RL, relative length of chromosome; AR, arm ratio of long arm to short arm from metaphase chromosomes. m, metacentric chromosome; sm, submetacentric chromosome; st, subtelocentric chromosome; t, telocentric chromosome.

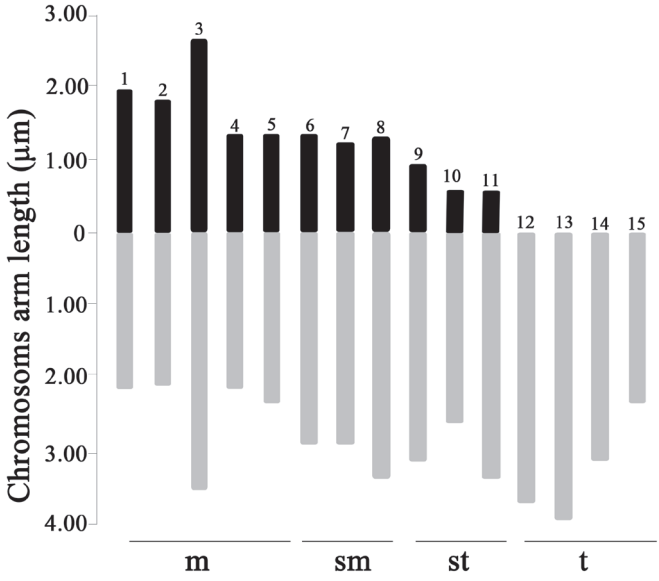

Figure 2. Chromosome idiograms of *U. unicinctus*. The dark regions showing short arms and the gray regions showing long arms.

that the average ratio of *U. unicinctus* coelomic cells to chicken erythrocytes was 0.74, therefore the 2C DNA content of *U. unicinctus* was calculated to be 1.85 pg, and its genome size was 904.58 Mb.

Table 2. Summary of DNA content and genome size of *U. unicinctus* estimated using flow cytometry.

Sample	M	N	N/M	DNA content (pg)	Genome size (Mb)
1	14.9	10.6	0.71	1.78	869.70
2	14.6	11.2	0.77	1.92	937.81
3	15.4	11.0	0.71	1.79	873.21
4	15.5	10.5	0.68	1.69	828.15
5	16.9	13.2	0.78	1.95	954.85
6	15.2	10.4	0.68	1.71	836.45
7	14.6	11.0	0.75	1.88	921.06
8	12.8	9.5	0.74	1.85	905.41
9	12.3	10.5	0.85	2.13	1043.60
10	14.8	10.6	0.72	1.79	875.57
Mean	14.7 ± 0.4	10.8 ± 0.3	0.74 ± 0.02	1.85 ± 0.04	904.58 20.21

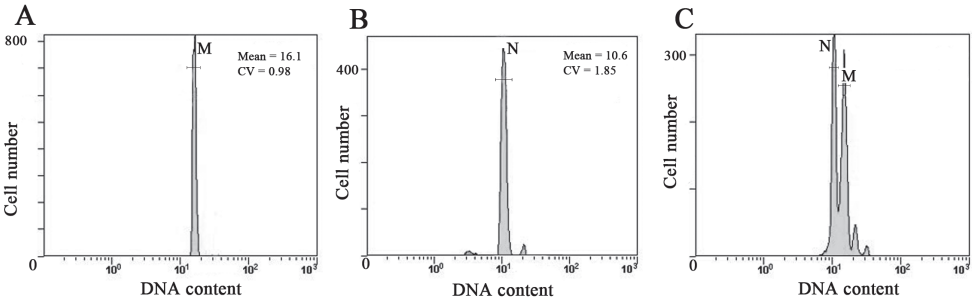

Figure 3. Estimation of nuclear DNA contents in *U. unicinctus* using flow cytometer. **A** Chicken erythrocytes **B** *U. unicinctus* coelomic cells **C** one mixture sample of both. M, the 2C peak of chicken erythrocytes; N, the 2C peak *U. unicinctus* coelomic cells.

Discussion

Studies on the chromosomes in echiurans are very limited, and all of them were conducted several decades ago (Griffin 1899, Lefevre 1907, Singhal and Dattagupta 1980). The only one study of the karyotype was performed in a Bonellidae species, *Achaetobonellia maculata* Fisher, 1953, (Singhal and Dattagupta 1980), which has 2n = 20 (10m), whereas others were focusing on the status and motion of chromosomes during cell division. Until now, no more karyotypic information of echiurans has been investigated. In this study, we obtained the clear karyotype of *U. unicinctus* using fluorescent staining technique and estimated its genome size as well. The karyotype of *U. unicinctus* was 2n = 30 (10m + 6sm + 6st + 8t), the 2C DNA content was 1.85 pg, and the genome size was 904.58 Mb approximately. This is the first study conducted in an Urechidae animal.

Karyotypic information could be utilized to study the taxonomic relationships of species and biological diversity (Dobigny et al. 2004, Ipucha et al. 2007, Leitão et al. 2010, Cioffi et al. 2012). In this study, we collected the karyotypic data of multiple

Table 3. The karyotypes of several echiurans, sipunculids and annelids species.

Species	Category	Karyotype	L/S[1]	AR[2]	Karyotype classification	Reference
Urechis unicinctus Drasche, 1880	Echiura	2n = 30 (10m + 6sm + 6st + 8t); FN = 52	2.51	2.54	3B	This study
Achaetobonellia maculata Fisher, 1953	Echiura	2n = 20 (20m); FN = 40	3.96	1.03	1A	Singhal and Dattagupta 1980
Sipunculus nudus Linnaeus, 1766	Sipuncula	2n = 34 (26m + 8sm); FN = 68	1.68	1.56	2A	Wang et al. 2008
Phasolosma esculenta Chen & Yeh, 1958	Sipuncula	2n = 20 (4m + 10sm + 6st); FN = 40	1.66	2.48	3A	Shi et al. 2013
Nereis oligohalina Rioja, 1946	Annelida: polychaeta	2n = 28 (14m + 2sm + 6st + 6t); FN = 50	2.54	2.01	4B	Ipucha et al. 2007
Perinereis anderssoni Kinberg, 1866	Annelida: polychaeta	2n = 38 (20m + 8sm); FN = 56	2.77	1.64	2B	Ipucha et al. 2007
Hediste diversicolor O.F. Müller, 1776	Annelida: polychaeta	2n = 28 (16m + 4sm + 8st); FN = 56	2.61	2.12	2B	Leitão et al. 2010
Drawida ghilarovi Gates, 1969	Annelida: oligochaeta	2n = 20 (6m + 8sm + 6st); FN = 48	2.46	2.39	3B	Anisimov et al. 2015
Eisenia balatonica Pop, 1943	Annelida: oligochaeta	2n = 36 (10m + 20sm + 6st); FN =72	2.75	2.38	3B	Kashmenskaya and Polyakov 2008
Aporrectodea caliginosa Savigny, 1826	Annelida: oligochaeta	2n = 36 (12m + 18sm + 6st); FN =72	2.38	2.05	2B	Kashmenskaya and Polyakov 2008

[1] L/S: The ratio of longest to shortest chromosome length.
[2] AR: The average arm ratio.

echiurans, sipunculids and annelids, and made some comparisons. It appeared that *U. unicinctus* possesses similar number and morphology of chromosomes with annelids (Table 3), and its karyotypic asymmetry was closely concentrated with annelids (Fig. 4). However, more data and analysis were required to determine the phylogenetic relationship between Echiurans and Annelids in future.

In general, genetic information of higher organisms is more complex than that of lower organisms, so the genomic size of higher organisms is relatively greater. However, there is no inevitable correlation between genome size and organismal complexity, because genome often contains a large number of highly repetitive DNA sequences, resulting in the conflict of DNA content and its evolutionary level. Gregory and Hebert estimated genome sizes from 12 species of freshwater oligochaetes ranging from 0.8 to 7.6 pg, and 15 species of earthworms varied from 0.4 to 1.2 pg (Gregory and Hebert 2002), suggesting that there is such a wide variation in the DNA content even between related species. Variation in genome size of polychaete taxa is not evenly distributed, as species inhabiting interstitial environments have smaller size (0.06–1.1 pg), whereas macrobenthic species are larger (0.4–7.2 pg), and the difference has been considered to adaptation of different environments (Soldi et al. 1994, Gambi et al. 1997, Gregory 2018). In addition, the DNA content among different species was also found to be independent of chromosome numbers, which was also concluded by EI-Shehawi and Elseehy (2017) that no correlation between genome size and chromosome number after the comparison of more than 6000 records. In echiurans, there has been no report of the nuclear genome size up to now. In the present study, the genome size of *U. unicinctus*

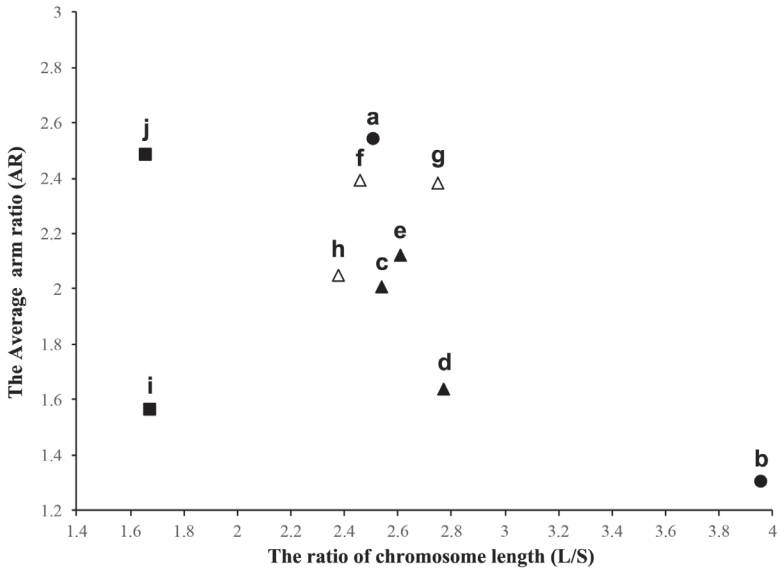

Figure 4. The karyotype asymmetry plot of echiurans, sipunculids and several annelids. **a** echiuran, *U. unicinctus* **b** echiuran, *A. maculata* **c** annelid, *N. oligohalina* **d** annelid, *P. anderssoni* **e** annelid, *H. diversicolor* **f** annelid, *D. ghilarovi* **g** annelid, *E. balatonica* **h** annelid, *A. caliginosa* **i** sipunculid, *S. nudus* **j** sipunculids, *P. esculenta*. symbols: circle, echiurans; square, sipunculids; triangle, polychaeta; hollow triangle, oligochaeta.

was estimated as 1.85 pg, which is relatively small and could also be resulted from the adaptation to the hash and variable intertidal environment. The determination of *U. unicinctus* genome size maybe of little significance for the study of the evolutionary status of Echiura, but it could provide effective data support for large-scale whole-genome sequencing of *U. unicinctus* in the near future.

Conclusion

In the present study, the karyotype of an Urechidae animal, *U. unicinctus*, was discovered for the first time as 2n = 30 (10m + 6sm + 6st + 8t), FN=52. Meanwhile, the 2C DNA content was detected to be 1.85 pg and its genome size was estimated as 904.58 Mb. Our study provided effective cytogenetic information for taxonomic study and whole-genome sequencing of *U. unicinctus*.

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RESEARCH ARTICLE

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Karyotypes of three species of *Hyperophora* Brunner von Wattenwyl, 1878 (Tettigoniidae, Phaneropterinae) enable morphologically similar species to be distinguished

Bruno Cansanção Silva[1], Lucas Henrique Bonfim Souza[1], Juliana Chamorro-Rengifo[1], Douglas Araujo[2]

1 *Programa de Pós-Graduação em Biologia Animal, Instituto de Biociências, Universidade Federal de Mato Grosso do Sul, UFMS, Cidade Universitária, 79070-900, Campo Grande, Mato Grosso do Sul, Brazil* **2** *Setor de Biologia Geral, Instituto de Biociências, Universidade Federal de Mato Grosso do Sul, UFMS, Cidade Universitária, 79070-900, Campo Grande, Mato Grosso do Sul, Brazil*

Corresponding author: *Lucas Henrique Bonfim Souza* (lucashenriquebs18@gmail.com)

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Abstract

Phaneropterinae is the largest subfamily of Tettigoniidae, distributed across the globe. There are few cytogenetic studies regarding this group, as in the case of the genus group Aniarae, which represents only two karyotyped species. The current study aims to analyze cytogenetically three species of *Hyperophora* Brunner von Wattenwyl, 1878 from Brazil. The male diploid number of *Hyperophora minor* Brunner von Wattenwyl, 1891 and *Hyperophora major* Brunner von Wattenwyl, 1878 is 2n♂= 31, whereas *Hyperophora brasiliensis* Brunner von Wattenwyl, 1878 has shown 2n♂= 29. These three species possess an X0 sex chromosome system and telo/acrocentric chromosome morphology. The only species found in the Pantanal biome, *H. brasiliensis*, can be chromosomally distinguished from the Cerrado biome species *H. major* and *H. minor*, due to the difference in chromosome number (2n♂= 29 and 2n♂= 31, respectively).

Keywords

Aniarae, fluorescent *in situ* hybridization, meiosis, Pantanal

Introduction

Tettigoniidae comprise 7598 species distributed worldwide, 2634 of them belonging to Phaneropterinae, the largest subfamily of the group. The genus *Hyperophora* Brunner von Wattenwyl, 1878 includes 16 South American species and belongs to Aniarae, along with other six genera (*Aniarella* Bolívar, 1906, *Burgilis* Stål, *Corymeta* Brunner von Wattenwyl, 1878, *Coryphoda* Brunner von Wattenwyl, 1878, *Pseudoburgilis* Brunner von Wattenwyl, 1878 and *Tetana* Brunner von Wattenwyl, 1878) (Eades et al. 2018).

For some *Hyperophora* species there is a paucity of descriptive information regarding the intraspecific morphological variations. Rehn (1907) described a male of the species *Hyperophora major* Brunner von Wattenwyl, 1878, commenting that the individual is smaller than those described by Brunner von Wattenwyl (1878) and published a schematic of the male cercus along with a sketch of *Hyperophora brasiliensis* Brunner von Wattenwyl, 1878 cerci apparently based on individuals different from the type material. The drawings of the cerci of *H. brasiliensis* and *H. major* are slightly similar, raising doubts as to whether they are simply morphological variations, since as previously reported there is no complete description that presents other robust characteristics that allow an accurate identification.

Cytogenetic data regarding Tettigoniidae are scarce (Warchałowska-Śliwa 1998). Within Phaneropterinae, at least 160 species were karyotyped (Warchałowska-Śliwa et al. 2011) and the most studied taxa belong to the tribe Barbistini, with more than 50 analyzed species (Warchałowska-Śliwa et al. 2013). Karyological studies in Phaneropterinae showed that the diploid number ranged from 16 to 33 in males, predominantly with the ♂X0/♀XX Sex Chromosome System (SCS) and telo/acrocentric chromosomes. Despite this variation, the most common diploid number for the subfamily is 2n♂= 31 and therefore it is likely to be the Phaneropterinae ancestor karyotype (White 1973, Warchałowska-Śliwa 1998, Hemp et al. 2014).

Aniarella ferraciui Piza, 1977 and *Hyperophora angustipennis* Brunner von Wattenwyl, 1891 are the only species of the whole Aniarae group that were chromosomally analyzed, presenting 2n♂= 21, X0 and 2n♂= 31, X0, respectively (Ferreira 1976, Ferreira and Mesa 2007).

In this work, we describe the karyotype of *Hyperophora brasiliensis* Brunner von Wattenwyl, 1878, *Hyperophora major* and *Hyperophora minor* Brunner von Wattenwyl, 1891, to discuss the chromosomal evolution and the cytotaxonomy of the group.

Material and methods

The specimens were collected at two localities in the state of Mato Grosso do Sul (MS), Brazil, from November 2015 to February 2017 and were deposited in the Coleção Zoológica de Referência da UFMS (ZUFMS) with the exception of one male specimen of *H. major*, which was used in the work of Serrão et al. (2018) (Table 1).

Table 1. Collection data. Site, number and sex of specimens, voucher numbers, and number of analyzed cells of the *Hyperophora* species cytogenetically examined in this study.

Species	Collection site	Specimens	Voucher (ZUFMS)	Number of cells
Hyperophora brasiliensis Brunner von Wattenwyl, 1878	Base de Estudos do Pantanal (BEP), municipality of Corumbá [19°34'37"S, 57°00'42"W]	2♂/1♀	ZUFMS-ORTO710; ZUFMS-ORTO711; ZUFMS-ORTO712	67
Hyperophora major Brunner von Wattenwyl, 1878	Estância Sossego, municipality of Campo Grande [20°29'19.09"S, 54°39'39.06"W]	3♂/1♀	ZUFMS-ORT00713; ZUFMS-ORT00715; ZUFMS-ORT00716	61
Hyperophora minor Brunner von Wattenwyl, 1891	Estância Sossego, municipality of Campo Grande [20°29'19.09"S, 54°39'39.06"W]	1♂/2♀	ZUFMS-ORT00714; ZUFMS-ORT00717; ZUFMS-ORT00718	100

The individuals were anesthetized in ether, dissected and fixed in 70% ethanol, with the exception of the gonads, which were used for chromosomal preparations and Giemsa staining following the procedures of Araujo et al. (2008). Slides from all three species were submitted to Fluorescence *in situ* hybridization (FISH) using the telomeric probe. This process employs a peptidic nucleic acid (PNA) $(AATCC)_3$ probe (PNA Bio, Inc) that is complementary to the typical $(TTAGG)_n$ telomeric repeats of Orthoptera, labeled with Alexa fluor 488 (ThermoFisher Scientific). It was followed the method of Genet et al. (2013), with a hybridization time of four hours at 37 °C, without heat denaturing, and mounted with ProLong Diamond antifade containing DAPI (ThermoFisher Scientific).

All cells were photographed using a Zeiss Axioimager D2 microscope with a monochromatic AxioCam 503 camera, employing the ZEN Pro software. Chromosome morphology was determined using the free software IMAGEJ (Rasband 1997–2018) and the LEVAN plugin (Sakamoto and Zacaro 2009), according to Levan et al. (1964) and Green and Sessions (1991), using respectively ten, nine and 18 mitotic metaphases of *H. brasiliensis*, *H. major* and *H. minor*.

Results

Hyperophora brasiliensis showed 2n♂= 29 and 2n♀= 30 (Fig. 1a). Spermatocytes I in diplotene exhibit 14 autosomal bivalents and one positively heteropycnotic sex univalent (Fig. 2a). Both *H. major* and *H. minor* presented 2n♂= 31 and 2n♀= 32 (Fig. 1b and c), however, only *H. minor* possess an interstitial heteropycnotic negative region in one telo/acrocentric chromosome of medium size (not visible in all cells due to chromosome condensation degree) (Fig. 1c). Male diplotene cells of these species exhibit 15 autosomal bivalents and one positively heteropycnotic sex univalent (Fig. 2b, c).

All three species possess the SCS of the type ♂X0/♀XX and showed exclusively telo/acrocentric chromosomes (Fig. 1), with the exception of one specimen of *H. minor*, which exhibited one submetacentric chromosome in all of the nine analyzed cells. (Fig. 1c).

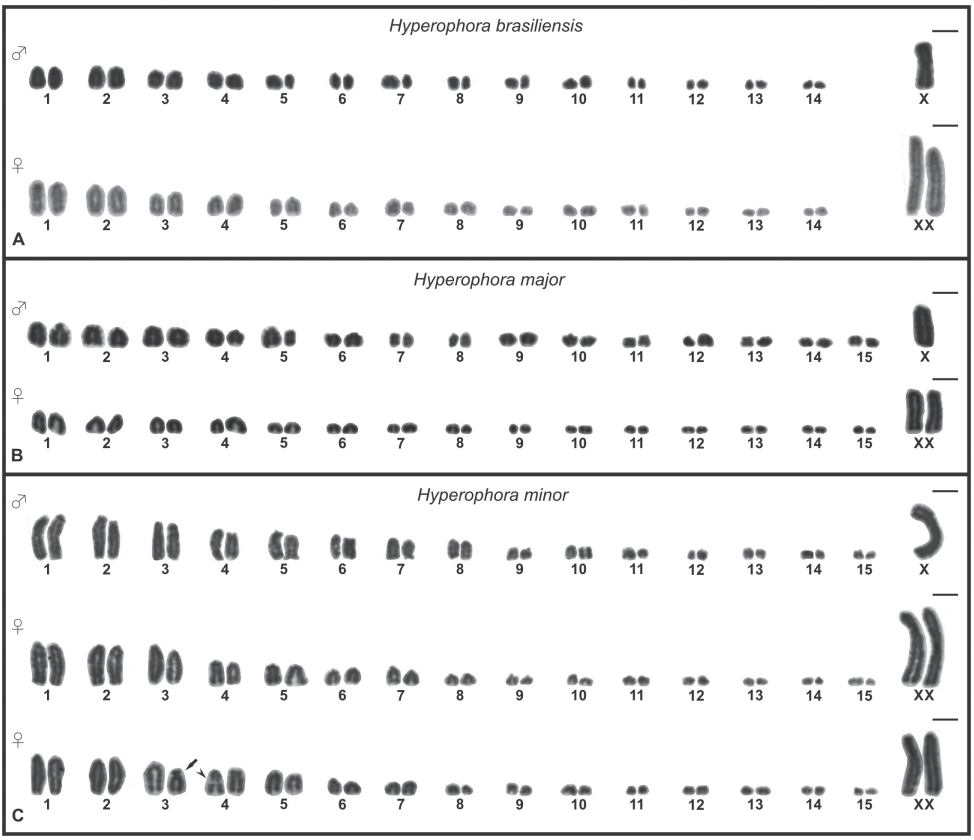

Figure 1. Karyotype of three *Hyperophora* species. **A** *Hyperophora brasiliensis* showing 2n♂=29 and 2n♀=30 **B** *Hyperophora major* with 2n♂=31 and 2n♀=32 **C** *Hyperophora minor* exhibit 2n♂=31 and 2n♀=32. Arrow = heteromorphic chromosome. Arrowhead = heteropycnotic negative region. Scale bars: 5 μm.

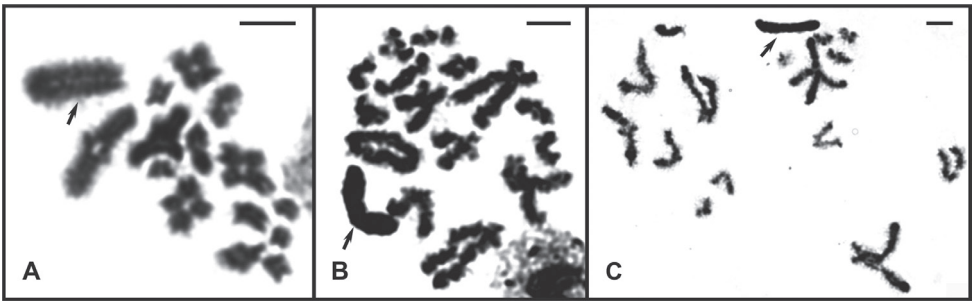

Figure 2. Male diplotenes of three *Hyperophora* species. **A** *Hyperophora brasiliensis* with 14II+X **B** *Hyperophora major* showing 15II+X **C** *Hyperophora minor* exhibit 15II+X. Arrows = X chromosome. Scale bars: 5 μm.

Only the telomeric regions of all chromosomes were hybridized in the three species analyzed (Fig. 3). No interstitial telomeric sites (ITS) were observed in any of the cells submitted to telomeric FISH.

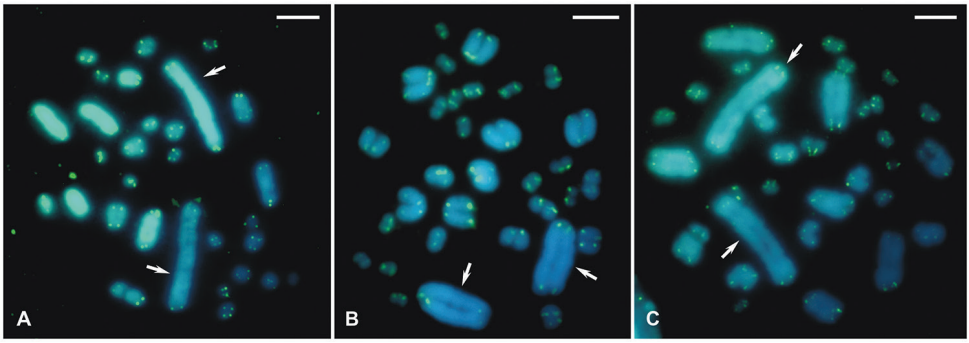

Figure 3. Female metaphases of three *Hyperophora* species with telomeric fluorescent *in situ* hybridization. **A** *Hyperophora brasiliensis* exhibit 2n♀=30 **B** *Hyperophora major* with 2n♀=32 **C** *Hyperophora minor* showed 2n♀=32. Arrows = X chromosome. Scale bars: 5 μm.

Discussion

The karyotype composed of 2n♂= 31 and ♂X0/♀XX SCS, presenting only telo/acrocentric chromosomes found in *Hyperophora major* and *Hyperophora minor* is the most common for katydids (Ferreira 1976, Warchałowska-Śliwa et al. 2011) and it was the same karyotype configuration presented in a congeneric species, *H. angustipennis*, the only *Hyperophora* species citogenetically analyzed up to now (Ferreira 1976). The heteromorphic pair found in one female of *H. minor* is the first recorded in Phaneropterinae, likely a result of one pericentric inversion.

Hyperophora brasiliensis showed 2n♂= 29, that is, one autosomal pair less when compared to the other *Hyperophora* species and the most common pattern in Tettigoniidae (2n♂= 31). In Phaneropterinae, karyotypes with 2n♂= 29 and 2n♂= 31 within the same genus were found in *Holochlora* Stål, 1873, *Phaneroptera* Serville, 1831, and *Scuderia* Stål, 1873 (Warchałowska-Śliwa 1998). Thus, a reduction of one chromosomal pair, probably due to *in tandem* fusion, appears to occur independently several times within Phaneropterinae (Warchałowska-Śliwa 1998, Hemp et al. 2010). Despite the suggested chromosome fusion, no interstitial telomeric sites (ITS) were detected, which can reflect an ancient fusion event, that the telomeric region of the fused element was lost during the rearrangement, or that it is below the limit of FISH technique.

Interestingly, both species which showed 2n♂= 31 are sympatric in the Cerrado of Campo Grande, while *H. brasiliensis* (2n♂= 29), which was not registered in Campo Grande, was collected in the Pantanal of Corumbá (~ distance 270 Km). The cerci of *H. minor* differ enormously from the cerci of *H. brasiliensis* and *H. major*, thus permitting a rapid and accurate morphological identification of *H. minor*. In this study, it was determined that despite the morphological similarity of the cerci of *H. brasiliensis* and *H. major* (Brunner von Wattenwyl 1878), the karyotypes of *H. minor* and *H. major* are more similar to each other than those of *H. brasiliensis*, helping to distinguish these species.

Regarding the Aniarae group, all four *Hyperophora* species karyotyped (Ferreira 1976, present study) exhibited higher diploid numbers (2n♂= 31 or 2n♂= 29) than

the only *Aniarella* species karyotyped up to now (2n♂= 21) (Ferreira and Mesa 2007). Differences of 10 or more chromosomes within karyotypes of closely related genera are uncommon among Phaneropterinae groups. However, the genus group Phyllopterae, *Itarissa* sp. presented 2n♂= 17, while *Phylloptera fosteri* Caudell, 1906 (cited as *Phylloptera modesta* Piza, 1961) and *Phylloptera* sp. evidenced 2n♂= 31 (Ferreira 1977, Ferreira and Mesa 2007). In both cases, the species belong to a "genus group", not a tribe. Genus groups are unreliable since there are not strict systematic studies supporting them. The clusters are allocated due to morphological similarities that could indicate the lack of a close kinship between the genera.

Conclusion

The diploid number was useful in order to distinguish on chromosome level the species of *Hyperophora* from the Pantanal of those from other localities. The external morphological appearance is not directly related to similarity in the chromosome number for *Hyperophora*. Further research of other species of the Aniarae group is fundamental for assessing karyotype patterns within the clade, however, it is possible to affirm that the reduction from 2n♂=31 to 2n♂=29 is a recurrent event in Phaneropterinae.

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