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Banding cytogenetics of chimeric hybrids *Coturnix coturnix* × *Coturnix japonica* and comparative analysis with the domestic fowl

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Abstract

The Common quail *Coturnix coturnix* Linnaeus, 1758 is a wild migratory bird which is distributed in Eurasia and North Africa, everywhere with an accelerating decline in population size. This species is protected by the Bonn and Berne conventions (1979) and by annex II/1 of the Birds Directive (2009). In Algeria, its breeding took place at the hunting centre in the west of the country. Breeding errors caused uncontrolled crosses between the Common quail and Japanese quail *Coturnix japonica* Temminck & Schlegel, 1849. In order to help to preserve the natural genetic heritage of the Common quail and to lift the ambiguity among the populations of quail raised in Algeria, it seemed essential to begin to describe the chromosomes of this species in the country since no cytogenetic study has been reported to date. Fibroblast cultures from embryo and adult animal were initiated. Double synchronization with excess thymidine allowed us to obtain high resolution chromosomes blocked at prometaphase stage. The karyotype and the idiogram in GTG morphological banding (G-bands obtained with trypsin and Giemsa) corresponding to larger chromosomes 1–12 and ZW pair were thus established. The diploid set of chromosomes was estimated as 2N=78. Cytogenetic analysis of expected hybrid animals revealed the presence of a genetic introgression and cellular chimerism. This technique is effective in distinguishing the two quail taxa. Furthermore, the comparative chromosomal analysis of the two quails and domestic chicken *Gallus gallus domesticus* Linnaeus, 1758 has been conducted. Differences in morphology and/or GTG band motifs were observed on 1, 2, 4, 7, 8 and W chromosomes. Neocentromere occurrence was suggested for Common quail chromo-

some 1 and Chicken chromosomes 4 and W. Double pericentric inversion was observed on the Common quail chromosome 2 while pericentric inversion hypothesis was proposed for Chicken chromosome 8. A deletion on the short arm of the Common quail chromosome 7 was also found. These results suggest that Common quail would be a chromosomally intermediate species between Chicken and Japanese quail. The appearance of only a few intrachromosomal rearrangements that occurred during evolution suggests that the organization of the genome is highly conserved between these three galliform species.

Keywords

Avian cytogenetics, cell culture, chimeric hybrids, *Coturnix coturnix* × *Coturnix japonica,* GTG-banding, intrachromosomal rearrangements.

Introduction

Birds represent a class of tetrapod vertebrates which contains a vast diversified variety of species (Jarvis et al. 2014). Extensive studies regarding birds are undertaken by researchers in Developmental Biology and Animal Cytogenetics, with over 1000 avian karyotypes published. However, few of them were deeply and accurately analyzed by using the chromosome banding. This results from difficulty of analysis in cell culture and establishment of chromosome issues (Christidis 1990).

The avian genome is characterized by very high chromosome number, with an average of 2N=76 - 80 (Werner 1927, Bed'Hom et al. 2003). The sex determination is of type ZZ for the homogametic male (equivalent to human XX), and ZW for the heterogametic female (equivalent to human XY). Besides the macrochromosomes which are easily identifiable, the microchromosomes are almost indistinguishable one from another (Masabanda et al. 2004, Griffin et al. 2007, Griffin and Burt 2014, Graves 2014). That is why mostly bird karyotypes are analyzed partially and limited to the few macrochromosomes (Shibusawa et al. 2004). Despite their small physical size, microchromosomes encode 50% of genes and are characterized by high CpG islands content and an early replicating pattern (Dutrillaux 1986, McQueen et al. 1996, Rodionov 1996, Burt 2002, Skinner et al. 2009, Hansmann et al. 2009).

Taxonomically, the majority of avian karyotypes are exceptionally stable and present conserved synteny regions (Shetty et al. 1999, Derjusheva et al. 2004, Shibusawa et al. 2004, Nie et al. 2009, Nanda et al. 2011, Ishishita et al. 2014). Birds have experienced fast series of speciation events during millions of years (Nadachowska-Brzyska et al. 2015, Griffin et al. 2015). Although intra-chromosomal rearrangements occur widely, inter-chromosomal ones are rare events estimated as 1.25 per million years (Zhao and Bourque 2009, Zhang et al. 2014, Hooper and Price 2017, Kretschmer et al. 2018). These reshufflings could be the cause or consequence of speciation, or a result of adaptation (Nishida et al. 2008, Völker et al. 2010, Romanov et al. 2014).

Like the domestic fowl *Gallus gallus domesticus* Linnaeus, 1758, the Common quail *Coturnix coturnix* Linnaeus, 1758 and the Japanese quail *Coturnix japonica* Temminck & Schlegel, 1849 are the representative species of the ancestral order Galliformes. The Japanese quail originates from the eastern Palearctic (Siberia, Mongolia, Korea, Northeastern China and Japan) but has lost migratory behavior, normal in its wild type (Del

Hoyo et al. 1994). The Japanese quail is reared in Europe as a farm animal for meat and eggs (Minvielle 1998, 2004). On the other hand, the Common quail is a wild migratory bird which is hunted for its scrumptious meat and eggs. It is also called the European quail given its characteristic distribution area. It breeds widely in Central and Southern Europe, as well as in Western Asia and North Africa (Johnsgard 1988). The Common quail shows very important annual fluctuations and it is listed under 'Least Concern' in the International Union of Conservation of Nature Red List. Nevertheless, it is protected by several conventions (Bonn and Bern in 1979, appendix II/1 of the birds Directive (2009/147/CE) of the European Parliament) (BirdLife International 2004, Hennache and Ottaviani 2011, Puigcerver et al. 2012).

In Algeria, a global strategy of preservation of the Common quail was organized thanks to collaborations between National research stations and Hunting Centre. The breeding of this species was kept in the form of reduced numbers at the Tlemcen Hunting Centre in the west of the country. The strong phenotypic resemblance between the European and the Japanese quail originated from errors committed during the breeding stage brought about as a result of uncontrolled crossings between these species and the appearance of hybrids (information supplied by the Tlemcen Hunting Centre).

Indeed, the Japanese quail is different from the European quail although they were considered for a long time as two subspecies (Austin and Kuroda 1953, Vaurie 1965, Minvielle 1998). Phylogenetic studies based on the analysis of nucleotide sequences of mitochondrial genes showed that the Japanese quail is of more recent appearance (Nishibori et al. 2001, Huang and Ke 2014).

However, the hybridization has negative consequences on the evolution of the genetic heritage of the species concerned and their preservation (Arnold 1997, Barton 2001). It can be a direct consequence of human activities (Arnold 2004). So, the genetic introgression is a frequent event in closely related animal species (Rhymer and Simberloff 1996, Arnold 1997, Allendorf et al. 2001). Indeed, both taxa of quails are known for their capacity to cross in captivity (Lepori 1964, Dérégnaucourt et al. 2001). During the breeding season, the natural ranges of common and Japanese quail overlap only in the Lake Baikal area (Russia) and in the Kentei region (Mongolia) (Barilani et al. 2005). However, no extensive natural hybridization has been reported (Del Hoyo et al. 1994, Guyomarc'h et al. 1998).

Thus, the introgressive hybridization caused by the uncontrolled release of Japanese quails seems to induce a very worrying genetic shift. In fact, a more or less complete loss of the migratory ability of hybrid subjects has been noted with the appearance of a hybrid song and the assignment of morphological and behavioral characters (Guyomarc'h et al. 1998, Dérégnaucourt and Guyomarc'h 2003, Dérégnaucourt et al. 2005).

Interspecific chimeras can be also met with at an early development stage, resulting from a crossing between closely related species especially in birds (Basrur and Yamashiro 1972). Chimeras are animal bodies stemming from a double fertilization, from an oocyte and from a polar globule, each by a different sperm cell creating two zygotes which would merge in a single embryo. The final result remains the creation of an unprecedented living creature within which different cells, from a genetic point of view, live together (Wolinsky 2007). Indeed, hybrids stemming from related species are often fertile indi-

viduals (Asmundson and Lorenz 1957, Makos and Smyth 1970). It is the case of mice *Mus musculus* Linnaeus, 1758 and *Mus caroli* Bonhote, 1902, chicken-quail hybrid and pheasant-turkeys hybrids (Bammi et al. 1966, Benirschke 1967, Rossant et al. 1983). Hybrids stemming from more distant species have reduced fertility or are even sterile as in the crossings mouse - rat and sheep - goat (Polzin et al. 1987, MacLaren et al. 1993).

Although high resolution molecular techniques are well advanced, chromosome banding remains an effective method for delineating chromosome homologies between phylogenetically related species. Indeed, banding colorations allow participation, in an important way, in the studies of taxonomy and phylogenetics and reveal the ancestral chromosome rearrangements of vertebrates (Rumpler and Dutrillaux 1976, Yunis et al. 1982, Bouayed 2004, Muffato 2010, Ouchia-Benissad and Ladjali-Mohammedi 2018).

The purpose of this study is to establish the karyotype of the Common quail *Coturnix coturnix* at high resolution level with morphological banding techniques. So far, no study of the chromosomes of this species has been reported. Also, considering the possibility of an introgressive hybridization between the Common quail and the Japanese quail, it was necessary to analyze the individuals expected to be the hybrid animals (*Coturnix coturnix* × *Coturnix japonica*) in order to remove the ambiguity within the quail populations bred in Algeria. Comparative chromosome analysis by GTG banding of both species of quails and the domestic fowl *Gallus gallus domesticus* has been conducted to detect certain rearrangements that would have occurred during speciation and to estimate the degree of conservation between these species.

Material and methods

Embryos and Adults

Common quail *Coturnix coturnix*: Five fertile eggs and an adult, 6-month-old male brought during the reproduction period from the Tlemcen Hunting Centre, Algeria (34°53'24"N, 1°19'12"W) have been analyzed in the present study.

Japanese quail *Coturnix japonica*: Five fertile eggs resulting from animals raised in the Hunting Centre of Zeralda, Algeria (36°42'06"N, 2°51'47"E) were also cultivated.

Hybrid animals: The Tlemcen Hunting Centre us to analyze eggs resulting from animals expected to be hybrid and resulting of an uncontrolled crossing between the Common quail and the Japanese quail. So, seven fertile eggs obtained at the 15th generation have been cultured.

Cell cultures

The age of all the embryos put in cultures in the present study varies between 8 and 12 days. The eggs were incubated in a ventilated incubator where the conditions of hygrometry (55%) and temperature (39.5 °C) are maintained. For the embryos and

the adult animal, the cellular cultures were carried out under sterile conditions in a chamber of cellular culture equipped with a vertical laminary flow hood (Polaris72 N°19311). The fibroblast primary cultures were carried out after samples were taken from fragments of various body parts (lung, heart, liver, kidneys and muscles). The cells were put in suspension in medium of RPMI 1640 supplemented by 20mm of HEPES, 1% of L-Glutamine (Gibco ref.: 22409-015, batch: 695608), 10% of foetal calf serum (FCS, Gibco ref.: 10270-106, batch: 41Q4074K), Penicillin-Streptomycin 1% and 1% of Fungizone (Gibco ref.: 15160-047, Batch: S25016D). The cells in culture were incubated at 41 °C (Ladjali 1994, Ladjali et al. 1995).

Chromosomal preparations

Cultures were synchronized with a double thymidine block (10mg /ml, Sigma) during S phase in order to increase the yield of metaphase and early metaphase cells as described by Ladjali et al. (1995). The half-cycle was estimated at 6–7 hours. Cells were treated during 3 to 5 min with colchicine (Sigma: 4µg/ml). Then, cells cultures were harvested by treating them with trypsin (Gibco: 25300-054; 0,05%). After centrifugation, cells were suspended for 13 min at 37 °C in hypotonic solution 1:5 (FCS- Distilled water) supplemented with EDTA or Sodium Citrate. Then, they were fixed in 3:1 (Ethanol:Acetic Acid). Chromosome preparations were dropped onto clean slides, wet with a film of distilled water and air dried. Twelve double synchronizations were performed for each species. We spread about 15 slides per synchronization. Approximately 20 metaphases were analyzed from each individual (embryos, animal).

Chromosome staining and banding

The method of Seabright (1971) modified by Ladjali et al. (1995) was used to induce GTG bands (G-bands obtained with Trypsin and Giemsa). Chromosome preparations aged from 3 to 10 days were incubated for 12 seconds in a trypsin solution (0.25%) prepared extemporaneously. The preparations were rinsed in phosphates buffer (pH = 7) then colored during 10 to 15min with 6% Giemsa, pH= 6.8 (Batch: BCBF9150; Ref: 48900-1-L-F).

Microscopy

Chromosome preparations were screened under a photonic microscope (Zeiss Scope A1, Axio) equipped with a digital black-and-white camera (Cool cube 1). Images have been captured by metasystem processing software. Photos were treated by ADOBE PHOTOSHOP 7.0 software.

Karyotypes

The establishment of karyotypes is based upon nomenclature taking into account the morphology and size of chromosomes according to the International System for Standardized Avian Karyotypes (ISSAK) (Ladjali-Mohammedi et al. 1999).

Chromosomes measurements

The IMAGE J software was used to integrate the scale bar on the photos (Rueden et al. 2017), and the KARYOTYPE 2.0 software allowed us to measure the relative lengths of chromosomes (Altınordu et al. 2016).

Results

Cell Cultures and synchronization

The fibroblast cultures derived from wild quail proved to be very sensitive to the various treatments (thymidine, BrdU and FdU). Indeed, cell culture follow-up showed fibroblast set up after two to five days, but after trypsination (0,05%) and synchronization, most cells died both in the embryos and adult. However, in Japanese quails and hybrids, the cells showed good viability after incorporation of different treatments. The cells from embryos provide a higher mitotic index and a greater potential for cell division compared to adult animal. The mitotic index observed in wild quail averaged one to two metaphases per a field (G×10). On the other hand, in Japanese quails and expected hybrids, the mitotic index was approximately 10 metaphases.

The control of the cell cycle by synchronization seems to be the best and most suitable procedure for blocking the so-called high-resolution chromosomes. The duration of the cell half cycle was estimated at 6 hours for both quail species. The majority of cells, dividing the two quail species, obtained in this experiment were at the metaphase and prometaphase stages.

Diploid number and GTG-banded karyotype of the Common quail *Coturnix coturnix*

Forty-five metaphases which showed well-distributed chromosomes were selected to count the diploid number of the Common quail, thus estimated at 2N=78 and represented by 38 pairs of autosomes and one pair of sex chromosomes (Figure 1A).

The GTG staining technique revealed clear G-banding patterns in all macrochromosomes and microchromosomes to size number 12 at least. Only the first 12 pairs and ZW sex chromosomes of the Common quail were described in this study (Figure 2A).

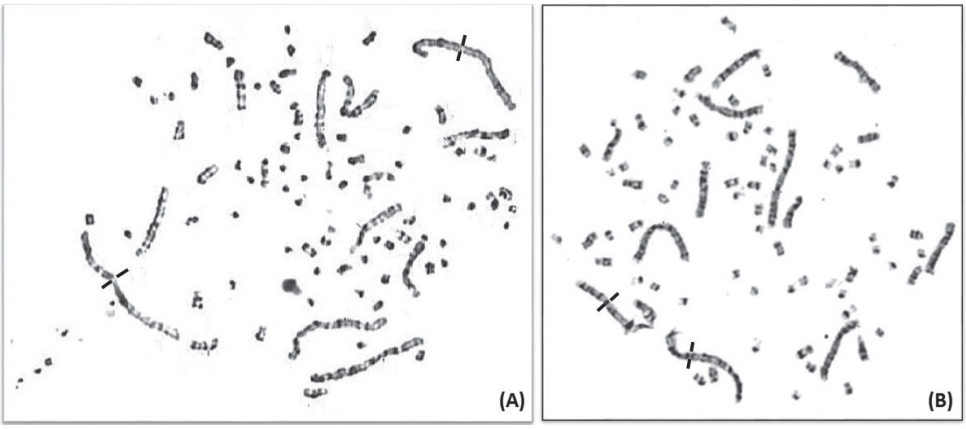

Figure 1. Prometaphase spreads following the GTG-banded chromosomes of **A** the Common quail *Coturnix coturnix* **B** Japanese quail *Coturnix japonica* (Black bars indicate the centromere positions of the chromosomes 1).

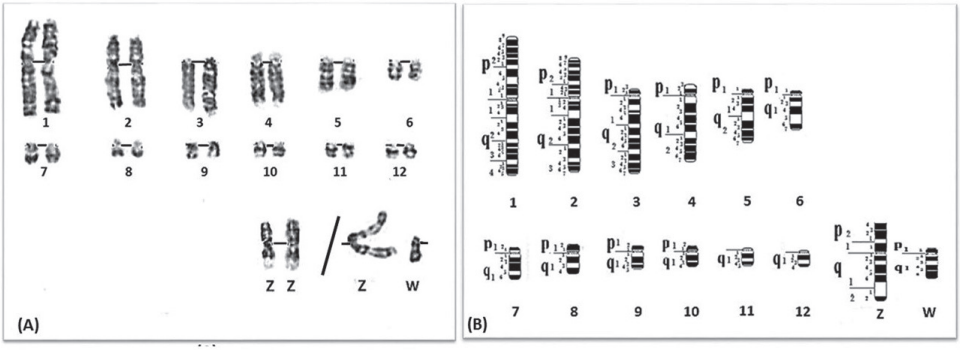

Figure 2. A GTG-banded karyotype for pairs 1 to 12 and sex chromosomes of the Common quail *Coturnix coturnix*. **B** Idiogram corresponding to **A**.

The ISSAK (1999) will be the basis for chromosome nomenclature. The results of measurements of the relative lengths were also presented (Table 1). The corresponding idiogram was proposed on the basis of the mean of 25 metaphases analyzed. It represents the largest pairs 1–10 (arms p, q) and chromosomes of the lesser size (arm q) of pairs 11–12 (Figure 2B).

Chromosomes 1 and 2 are submetacentric. Their arm ratios are quite similar (q/p = 1,32) (Table 1). The p arm of the chromosome 1 has two regions and 15 G-bands. The landmark band 21 divides the p arm into two regions. 19 G-bands are observed on the q arm; three prominent negative bands (21, 31 and 41) divide the arm into four regions. Chromosome 2 has 13 G-bands, one central band (21) which separates the p arm into two regions. Three regions are observed on the q arm with 16 G-bands. Chromosome 3 is acrocentric, it has one region and 3 G-bands on the p arm. The q arm has three regions and 19-G bands. The first region is marked by a prominent negative band (13).

Table 1. Size of the mitotic chromosomes of the Common quail *Coturnix coturnix* (n=14) p: short arm, q: long arm, p+q: relative length, CI: Centromeric index=p/(p+q) × 100.

Chromosomes	p (µm)	q(µm)	q/p	p+q(µm)	CI%
1	1.71	2.29	1.32	4	42
2	1.25	1.66	1.32	2.91	42.95
3	0.12	2.15	17.91	2.27	5.28
4	0.30	1.85	6.16	2.15	13.95
5	0.10	1.40	8.25	1.50	6.7
6	0.11	0.9	8.18	1.01	10.89
7	0.18	0.79	4.38	0.97	18.55
8	0.26	0.51	1.96	0.77	33.76
9	0.1	0.65	6.5	0.75	13.33
10	0.08	0.58	7.25	0.66	12.12
11	0.1	0.55	5.5	0.65	15.38
12	0.08	0.48	6	0.56	14.28
Z	1.06	1.08	0.49	2.14	49.53
W	0.16	0.8	5	0.96	16.66

Table 2. Morphometry of the first twelve macrochromosomes and gonosomes of the Japanese quail *Coturnix japonica* (n=16) p: short arm. q: long arm. p+q: relative length. CI: centromeric index=p/(p+q) × 100.

Chromosomes	p(µm)	q(µm)	q/p	p+q(µm)	CI%
1	1.3	2.8	2.15	4.1	31.70
2	1.25	1.66	1.32	2.91	42.95
3	0.14	2	14.28	2.14	8.18
4	0.32	1.7	5.31	2.02	15.84
5	0.15	1.11	7.4	1.26	11.90
6	0.08	0.76	9.5	0.84	9.52
7	0.1	0.66	6.6	0.76	13.16
8	0.24	0.47	1.95	0.71	33.80
9	0.07	0.56	8.56	0.63	11.11
10	0.09	0.49	5.44	0.58	15.51
11	0.08	0.46	5.75	0.54	14.81
12	0.05	0.48	9.6	0.53	9.43
Z	0.96	1.05	1.09	2.01	47.76
W	0.18	0.92	5.11	1.1	16.36

Chromosome 4 is subtelocentric. The p arm of the chromosome 4 has one region and 3 G-bands. The q arm has two regions. 11 G-bands; region 2 is marked by a subcentral negative band (21). Chromosomes 5 and 6 are acrocentric. Chromosome 5 has a p arm with one region and 1 G-band; a subcentromeric positive band (11). Two regions are observed on q arm with 11 G-bands. On chromosome 6, one region and one narrow subcentromeric positive G-band (11) are observed on the p arm. The q arm has one region. 7 G-bands, band 17 is a positive in the telomere region, which is not always visible. Chromosome 7 is telocentric. It has one region on the p arm, 2 G-bands. One region on the q arm, 6 G-bands. Chromosome 8 is submetacentric. It has one region

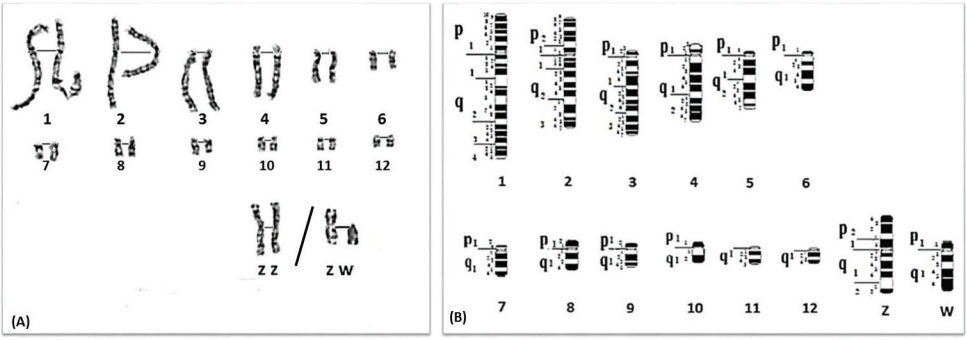

Figure 3. A GTG-banded karyotype for pairs 1 to 12 and sex chromosomes of the Japanese quail *Coturnix japonica*. **B** Idiogram corresponding to **A**.

and 2 G-bands on the p arm. One region and 4 G-bands are observed on the q arm. On the p arm of the chromosome 9, one region and 2 G-bands, a subcentromeric negative band (11) followed by a positive band (12). The q arm has one region, 5 G-bands. Chromosome 10 has one region observed on the p arm with 2 G-bands. The q arm has one region, 4 G-bands. The q arm of the chromosome 11 has one region and 5 G-bands. Chromosome 12 has one region on the q arm. 4 G-bands are observed, a subcentromeric negative band (11) followed by two prominent positive bands (12 and 14) separated by a large negative (13). Sex chromosomes Z and W are respectively, metacentric and subtelocentric. Chromosome Z has two regions on the p arm. 5 G-bands, region 1 has a large subcentromeric positive band (11). The q arm has two regions. 8G-bands, region 1 has a subcentromeric negative band (11). Band 21 is the characteristic large heterochromatic region. One region and one G-band are observed on the p arm of the chromosome W. The q arm has one region and 6 G-bands.

Morphometry of the Japanese quail *Coturnix japonica* chromosomes and GTG-band karyotype

In this study, we confirmed the diploid number of chromosomes of the Japanese quail, 2N=78 (Figure 1B). In general, the karyotype of this species is arranged similarly to that of the previous species. The largest twelve pairs range in size from 4,1µm to 0,53µm (Table 2). These measurements show that chromosomes of Common quail (Table 1) were slightly more decondensed than those of Japanese quail (certainly due to the success of double synchronization).

The GTG-banded karyotype and corresponding idiogram of the Japanese quail are illustrated in Figures 3 (A and B). Chromosome 1 is submetacentric and characterized by a centromere bordered by two narrow positive bands (11p and 11q). The short arm of submetacentric chromosome 8 has a region with 2 G bands, a negative narrow subcentromeric band (11) followed by a wide positive band (12). The q arm has only one region. 4

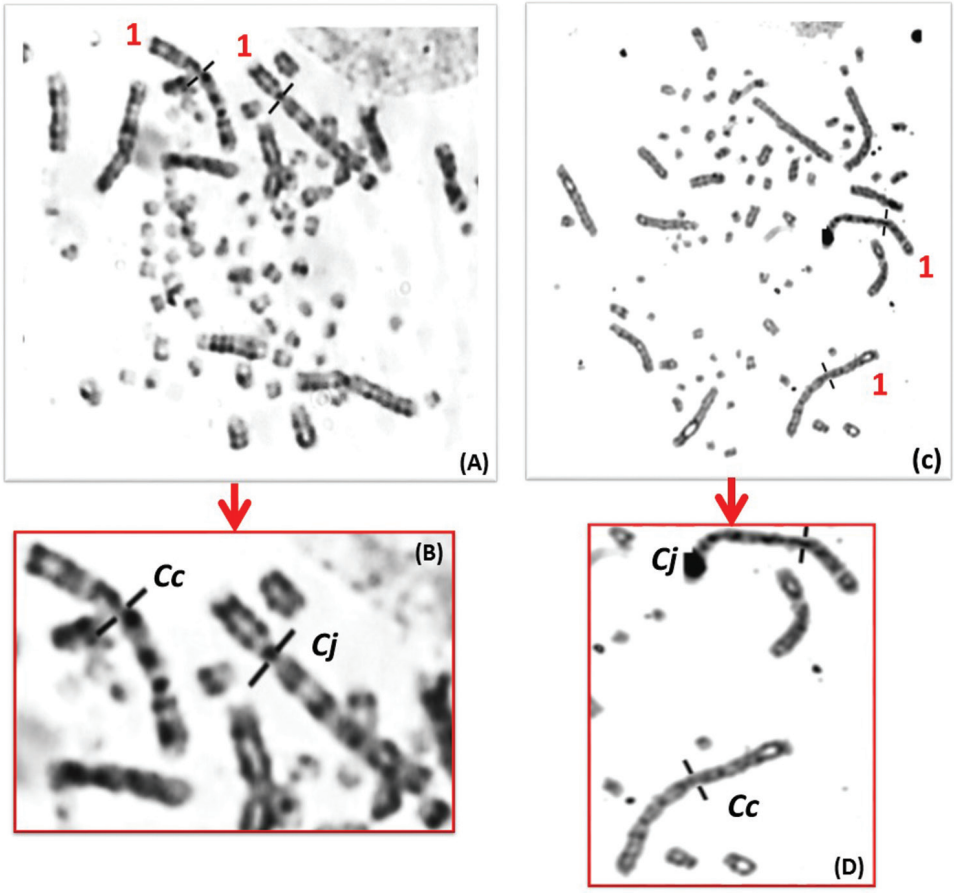

Figure 4. A, C Prometaphase spreads following the GTG-banded chromosomes of hybrid quail **B, D** Black traits indicate the centromere positions of the homologous chromosomes 1 which are morphologically different.

G-bands are observed, one subcentromeric negative band (11) followed by two prominent positive bands (12 and 14) separated by a large negative band (13). The W chromosome is subtelocentric and is ranked at the fifth position. The patterns of the GTG bands show that the p arm has a region with a narrow subcentromeric positive band. The q arm has one region and 6 bands, a subcentromeric negative band (11) followed by three positive bands (12, 14 and 16) separated by two negative bands, a large (13) and a narrow (15).

GTG-banding patterns of chimeric hybrids and gynandromorphism

Of the seven expected hybrid quails cultivated in this project, only two cell cultures have succeeded. These hybrids were analyzed in the 15th generation, were all viable and derived from fertile parents. The homologous chromosomes of the same pair were des-

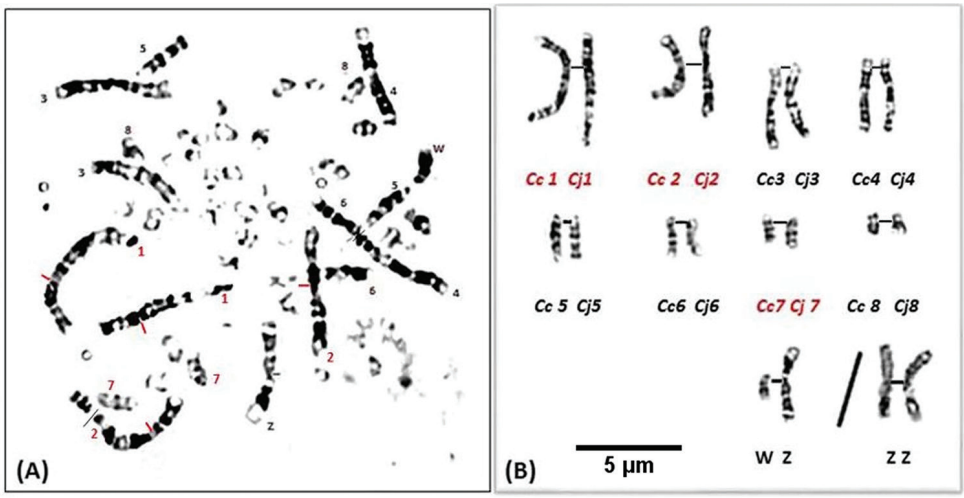

Figure 5. A Prometaphase spread following the GTG-banded chromosomes of hybrid quail **B** patterns of pairs 1 to 8 and sex chromosomes corresponding to **A** showing the differences on chromosomes 1, 2 and 7 of both species. Scale bar: 5µm.

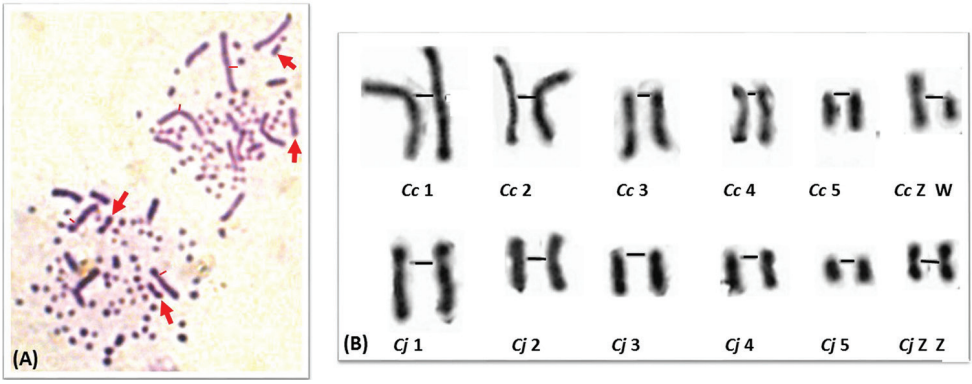

Figure 6. Chimera embryo showing **A** the cohabitation of the Common and Japanese quail cells **B** gynandromorphism corresponding karyotypes to **A** with ZZ/ZW chromosomes indicated by the arrows.

ignated "*Cc*" for Common quail and "*Cj*" for Japanese quail (Figure 4). The karyotype in GTG bands is shown in Figure 5 (A and B). Chromosome analysis of hybrid quails revealed morphological differences only on chromosomes 1, 2 and 7. The W chromosomes of both species are morphologically similar but a difference in size was observed (the W*Cj* is bigger than the W*Cc*) (Table 1 and 2).

The two analyzed hybrid embryos showed a coexistence of three cell types that we have identified as chimeric hybrids (Figure 6A). In fact, a predominance of Japanese quail cells was observed (90%) whereas the cells of Common quails and hybrids were rarer (4% and 11% respectively). It is supposed to be a micro-chimerism.

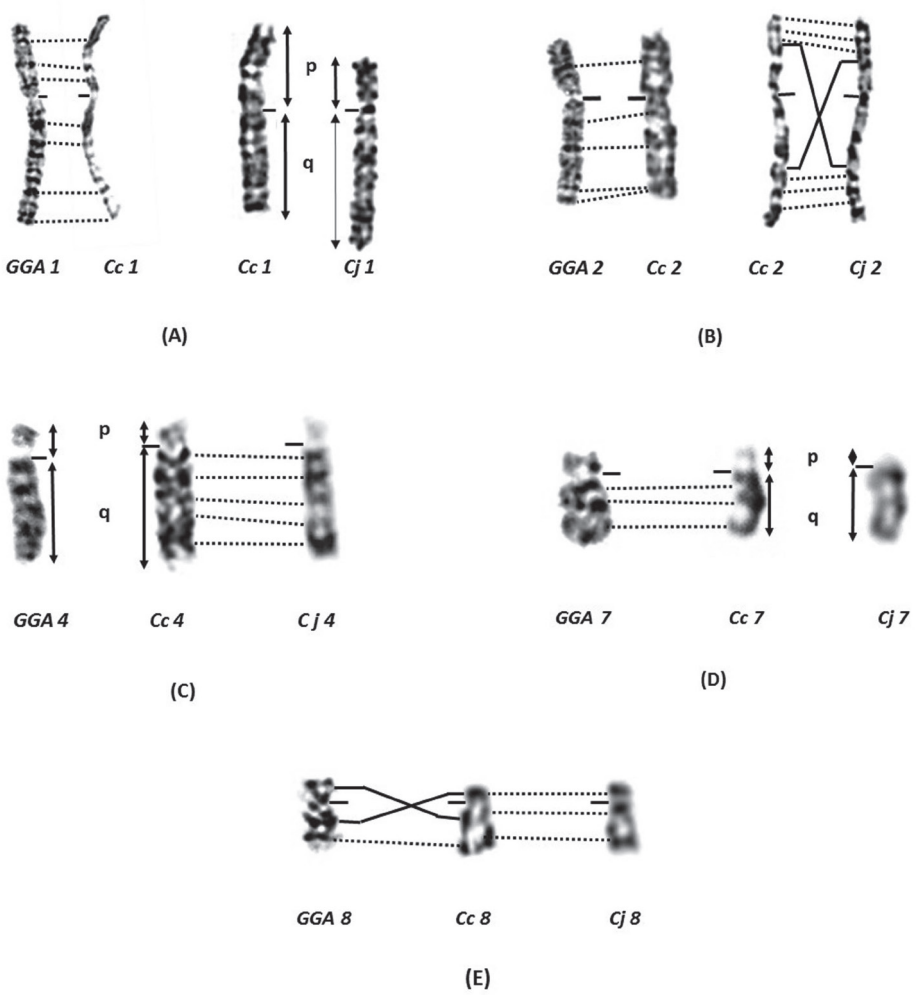

Figure 7. Comparison of chromosomes 1 **A** 2 **B** 4 **C** 7 **D** and 8 **E** of domestic chicken *GGA* (left), Common quail *Cc* (in the middle) and Japanese quail *Cj* (right) with the GTG bands.

Another anomaly has also been detected concerning sex chromosomes, which is a kind of chimerism called gynandromorphism. Thus, one of the two chimeric preparations corresponded to a gynandromorphic individual that corresponds to the presence of two distinct cell populations at a same time: male and female (Figure 6B). This embryo showed ZZ Japanese quail cells in addition to ZZ and ZW Common quail cells and hybrid cells with different sexual formulas. This embryo could be the result of a double fertilization of a female hybrid quail type (Z *Cj* / W *Cc*) by two males, Japanese and hybrid quail. The second embryo analyzed exhibited Common quail cells, in addition to Japanese and hybrid. They are all female cells, which could be the result of a cross between a female hybrid quail (Z *Cc* / W *Cj*) with two hybrid quail males.

Comparative cytogenetic data from the Common quail, Japanese quail and domestic fowl

The chromosome comparison by GTG banding analysis of three species (Common quail, Japanese quail and Chicken *Gallus gallus domesticus* "GGA") confirms the presence of chromosomal rearrangements already described for Japanese quail and Chicken (Sasaki 1981, Stock and Bunch 1982, Shibusawa et al. 2001, 2004, Zlotina et al. 2012). Indeed, similarities between the three species have been observed on most macrochromosomes (3, 5, 6, 9, 10, 11 and 12). In our materials, the presence of chromosomal rearrangements on chromosomes 1, 2, 4, 7, 8 and W was noticeable. The Z chromosomes are morphologically similar in all three species (metacentric) and for which no inversion has been detected.

Important homology was observed on chromosome 1 of the Common quail compared to its homologs in domestic chicken, while a perfect correspondence of the GTG band profiles is observed on chromosomes 1 of the two quail species, a difference in the ratio q/p was found (Figure 7A). Chromosome 2 of the Common quail and its Chicken homolog are very conserved. Some GTG band patterns of chromosomes 2 are completely reversed between the two quail species (Figure 7B). The ratio q/p of chromosomes 4 of the Common quail and Chicken is different (Figure 7C). A short p-arm visible and measurable on chromosome 7 of the Common quail looks more similar to its Chicken homolog than to the Japanese quail (Figure 7D, Table 2). The chromosome 8 of the Common and Japanese quails is morphologically similar. On the contrary, banding patterns differences in homologue chromosome 8 of chicken were observed (Figure 7E). The W chromosomes of the Common and Japanese quails exhibit strong homology (Figure 2 and 3), unlike the Chicken chromosome W (submetacentric). However, the size of the Common quail chromosome W is close to chromosome 7 whereas that of Japanese quail is close to chromosome 5 (Table 1 and 2).

Discussion

Even though the cells of birds remain among the most difficult species to maintain in culture, the prometaphase cells are particularly suitable for bird analysis because the chromosomes are thin and elongate, making the structure of the smaller elements more distinct (Owen 1965, Ladjali et al. 1995, Ladjali-Mohammedi et al. 1999).

The high sensibility observed in cells cultures of wild quail corroborate with the vulnerability of this species in breeding areas unlike the Japanese quail because of its easy practical prolificacy in captivity (Caballero de la Calle et Peña Montañés 1997, personal communication of the Tlemcen Hunting Centre). This is the case for the Barbary and Chukar partridges (Ouchia-Benissad and Ladjali-Mohammedi 2018).

The diploid number of 2N=78 estimated in Common quail and then in Japanese quail, emphasizes the exceptional conservation of karyotypes in the order of Galliformes (Ohno et al. 1964, Takagi and Sasaki 1974, Stock and Bunch 1982, Arruga et

al. 1996, Shibusawa et al. 2004, Ishishita et al. 2014, Ouchia-Benissad and Ladjali-Mohammedi 2018). This is the case for the domestic fowl *Gallus gallus domesticus*, too (Ladjali 1994).

The karyotypes of Common and Japanese quail show 8–10 pairs of macrochromosomes and 30–28 pairs of microchromosomes which are very difficult to distinguish. This is quite similar to that in most Galliformes (Stock and Bunch 1982, Shibusawa et al. 2004).

While the GTG band karyotype of the Japanese quail *Coturnix japonica* was described up to the eighth chromosome pair only (Talluri and Vegnil 1965, Turpin et al. 1974, Ryttman and Tegelström 1981, Sasaki 1981, Stock and Bunch 1982, Shibusawa et al. 2001), in this study we have managed to describe up to the first 12 pairs and sex chromosomes. However, we have detected the presence of some ambiguities on the idiograms of chromosomes 1, 8 and W already proposed (Stock and Bunch 1982, Shibusawa et al. 2001). Indeed, the centromeric region of chromosome 1 is bounded by two positive G-bands that are characteristic of chromosome 1 of the Japanese quail (Figure 3 A, B). The result of chromosome 8 obtained in this work (Table 2) is supported by a previous studies (Talluri and Vegnil 1965, Stock and Bunch 1982), while other authors have described it as acrocentric (Schmid et al. 1989, Shibusawa et al. 2001). The description of the W chromosome corroborates with that of Hartung and Stahl (1974) and Schmid et al. (1989). Chromosomes W and 5 can be confused by size (Talluri and Vegnil 1965).

Comparative chromosomal analysis of both quails with domestic chicken allowed us to discover high conservation as well as differences in the karyotypes. The Common quail karyotype shares more similarities with chicken chromosomes than that of Japanese quail with Chicken. However, the Chicken karyotype is considered as the most similar to the putative ancestral bird karyotype (Griffin et al. 2007). The results obtained in this study suggest that, during speciation, Common quail would make an intermediate species between Chicken and Japanese quail.

In fact, the high conservation of GTG banding patterns of chromosomes 1 in these three Galliformes species is observed, whereas difference in the q/p arm ratio is detected on chromosomes 1 of the two quails. This result could be explained by a formation of an evolutionary new centromere (ENC) (Figure 8). The pericentric inversion hypothesis is therefore not verified in this work (Sasaki 1981, Stock and Bunch 1982, Kayang et al. 2006). This formation of a neocentromere would be more plausible and would correspond to the work of comparative mapping on Japanese quail meiotic chromosomes (Galkina et al. 2006, Zlotina et al. 2010, 2012).

Double pericentric inversion is demonstrated in some G-band motifs when chromosome 2 of Common quail and Japanese quail are compared as was reported in previous studies (Figure 9) (Shibusawa et al. 2001, Schmid et al. 2005, Kayang et al. 2006, Zlotina et al. 2012). The breakpoints on chromosome 2 of the Common quail would be located in the region between the band p 2.3 → q 3.1 and the band p 1.3 → q 1.4 (Figure 2A).

In the present work, we observed perfect conservation patterns in chromosome 4 of the three species. Furthermore, a morphological difference was noted between Chicken and the both quails. This result could suggest repositioning of the centromere

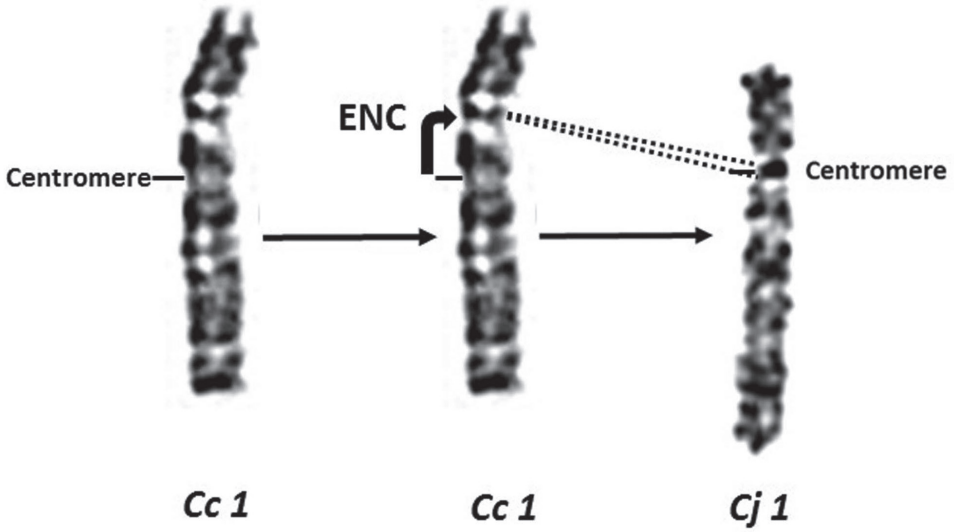

Figure 8. Evolutionary new centromere (ENC) formation on chromosome 1 of the Common and the Japanese quails.

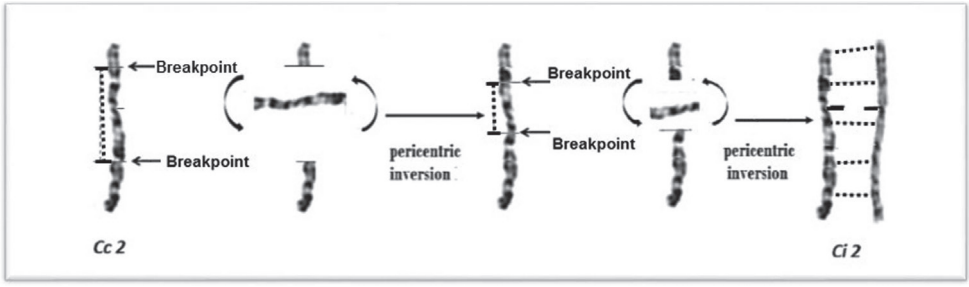

Figure 9. Double inversion that could have occurred during evolution on chromosome 2 between the Common and the Japanese quails.

during the speciation event (Figure 7C). This was already reported by Galkina et al. (2006) showing a perfect conservation of Chicken BAC clones order on Japanese quail chromosome 4. In fact, centromeres appear to be formed *de novo* during the evolution of Galliformes karyotypes (Kasai et al. 2003, Galkina et al. 2006, Skinner et al. 2009, Ouchia-Benissad and Ladjali-Mohammedi 2018).

However, the fourth avian chromosome pair is quite complex in the history of bird evolution (Chowdhary and Raudsepp, 2000). Multiple hypotheses were proposed to explain the differences in chromosome 4 of Japanese quail and domestic Chicken (Fillon et al. 2003, Schmid et al. 2005, Shibusawa et al. 2001, Galkina et al. 2006). Nevertheless, Chicken chromosome 4 is suggested to have arisen from a fusion of ancestral acrocentric chromosome 4 and ancestral microchromosome 10 (Schmid et al. 2000, Shibusawa et al. 2004, Griffin et al. 2007).

Comparative chromosome 7 mapping of Common quail highlighted a large conservation with domestic fowl (Figure 7D). However, deletion of the short arm p would have occurred in the common ancestor of Common and Japanese quail during evolution. We plan to locate molecular markers (chicken-specific BAC clones) that flank the centromere of chromosome 7 to confirm or reverse this type of rearrangement (Shibusawa et al. 2001, Fillon et al. 2003).

In both quails, the 8 chromosomes were highly similar but differences in the disposition of the GTG bands were observed comparing them to Chicken (Figure 7E). This would probably be the result of a pericentric inversion involving the region of the band p 1.1 and the band q 1.2 (Figure 2 B). These results confirm what has already been reported in Japanese quail (Shibusawa et al. 2001, Fillon et al. 2003, Sasazaki et al. 2006).

We observed high conservation between the Z chromosomes of the Chicken and the two quails. This result suggests no presence of pericentric inversion in the common ancestor of the three species, as previously described by Suzuki et al. (1999).

However, W chromosomes of both quails presented similarities. They have a small short arm, unlike the longer one in the Chicken. This morphological difference could be the result of formation of neocentromere (ENC) during the evolution. Moreover, the difference in size observed in the two species of quails could be explained by the fact that the ZW sex chromosomes would undergo unequal condensation/decondensation of the chromosomal arms (Solovei et al. 1993, Saifitdinova et al. 2003).

The observed differences between the Common and Japanese quail chromosomes dealt with chromosomes 1, 2 and 7. All of the rearrangements described probably occurred in the evolutionary process before the separation of the two quail species. The important chromosomal similarity between these two species could allow to obtain a fertile and highly prolific progeny (Asmundson and Lorenz 1957, Hidas 1993). Also, sterility was shown to be related to the presence of large blocks of heterochromatin in the hybrids chromosomes (Wójcik and Smalec 2017). It was not observed on chromosomes of the hybrid embryos that we analyzed.

The presence of different cell types (*Cc* and *Cj*) within the same hybrid individual may be due to double fertilization of the ovule and its polar globule from sperms of different origins. Indeed, surviving spermatozoa from anterior mating can be preserved in the female genital tract at the infundibulum and could then be released into the oviduct lumen (Grigg 1957). The results we obtained can also support the theory of parthenogenesis which is an asexual reproduction without fertilization, exclusive to females (Servella 1974).

Chimerism is an extremely rare abnormality in animals. The proportions of the three cell types obtained represented a micro-chimerism which is defined by the number of cells affected. It is when a genetically foreign population represents less than 5% of the nucleated cells of an individual or organ (Nelson 2010). Similar observations were made over chicken-pheasant hybrids (Basrur and Yamashiro 1972). It has already been observed that females of Japanese quail breed with Japanese wild or hybrid quail males (Guyomarc'h et al. 1998, Collins and Goldsmith 1998). While Common quail females mate mainly with males of the same species (Guyomarc'h 2003, Domjan et al.

2003, Dérégnaucourt et al. 2005, Sanchez-Donoso et al. 2012, Puigcerver et al. 2014). These data corroborate with those obtained in this study.

Gynandromorphism is an anomaly that is not very well answered in birds (Gilgenkrantz 1987). Only one gynandromorph individual was analyzed. Some cases were observed in a red cardinal and chickens (Peer et al. 2014). Gynandromorphism would be the result of a genetic mutation occurring during the early division of the oocyte after fertilization (Hollander 1975). Fusion of two eggs that should have given a male and a female would give birth to individuals with both cells of different sexes (Zhao-Xian et al. 2010, Clinton et al. 2012).

Conclusion

The analysis of hybrid animals bred in western Algeria showed us that introgressive hybridization affected the genetic heritage of the Common quail *Coturnix coturnix* and would be a threat to its preservation. Although the wild quail and Japanese quail are phylogenetically very close, the chromosome banding method allowed us to propose the karyotype of the Common quail and to distinguish these two taxa. The comparative cytogenetic study allowed us to detect ancestral intrachromosomic rearrangements that could have accompanied the speciation and evolution of the karyotypes of the three species of Galliformes. Common quail would be an intermediate species between the Chicken and Japanese quail, which would be more recent in appearance. As a result, cytogenetics is a very important element in taxonomy and phylogeny studies.

In addition, for better knowledge of the Common quail genome, Fluorescence *In Situ* Hybridization (FISH) will be performed for individual microchromosome identification (Lithgow et al. 2014, McPherson et al. 2014). Though specific FISH probes of GGA11-28 chicken lampbrush microchromosomes can be used for the 10 smallest chicken microchromosomes, GGA29-38, no individual molecular tags have been established to date (Galkina et al. 2017, Kretschmer et al. 2018). Also, the characterization of the nuclear genetic markers (microsatellites) allowed to distinguish both taxa of the quail and their hybrids, and to estimate the genetic introgression (Boecklen and Howard 1997, Puigcerver et al. 2000, Rodríguez-Teijeiro et al. 2003, Barilani et al. 2005, Vähä and Primmer 2006, Chazara et al. 2006, 2010). Finally, microdissection of chromosomes or large-scale sequencing could enable us to refine the knowledge of specific microchromosomal regions (Fillon 1998, Masabanda et al. 2004).

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SHORT COMMUNICATION

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Karyotypic description of the stingless bee *Melipona quinquefasciata* Lepeletier, 1836 (Hymenoptera, Meliponini) with emphasis on the presence of B chromosomes

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Abstract

Stingless bees are distributed widely in the tropics, where they are major pollinators of several plant species. In this study, the karyotype of *Melipona quinquefasciata* Lepeletier, 1836 was analysed, with emphasis on the presence of B chromosomes. Post-defecating larvae were analysed using Giemsa staining, the C-banding technique, sequential staining with fluorochromes, and FISH. The chromosome number ranged from 2n = 18 to 22 (females) and from n = 9 to 13 (males) due to the presence of 0–4 B chromosomes. This result demonstrates that *M. quinquefasciata* has the same chromosomal number as other *Melipona* Illiger, 1806 species. Considering the A complement, heterochromatin was located only in the pericentromeric region of pair 1. Staining with chromomycin A_3 (CMA_3) and labelling with rDNA probe, indicated that this region corresponded to the nucleolus organising region. The B chromosomes of *M. quinquefasciata* could be found in individuals from different localities, they were completely heterochromatic (C-banding) and uniformly stained by 4',6-diamidino-2-phenylindole (DAPI). Variations in the number of B chromosomes were detected between cells of the same individual, between individuals of the same colony, and between colonies from different localities.

Keywords

Cytogenetics, heterochromatin, karyotype, fluorochromes, FISH

Introduction

Classical or molecular cytogenetic analysis can be used to determine chromosome number and morphology, the location and quantity of AT or CG rich regions, nucleolus organizing regions, rRNA clusters and repetitive sequences in the genome. This information allows species characterization, identification of cryptic species and the mechanisms involved in their speciation, analysis of population variability, and studies on karyotype evolution, phylogeny and taxonomy of different groups of species (Rocha and Pompolo 1998, Lachowska et al. 2009, Mendes-Neto et al. 2010, Panzera et al. 2012, Mandrioli et al. 2014, Golub et al. 2016).

Such analysis can also identify intra-specific or numerical variations within a population due to the presence of B or extra chromosomes (Brito et al. 1997, Tosta et al. 2004, Martins et al. 2014). These chromosomes are usually heterochromatic, smaller than the normal complement chromosomes, and show a non-Mendelian segregation pattern. They have already been described in many animal and plant species, allowing for studies on their origin, stability and maintenance (Camacho 2005, Houben et al. 2014, Anjos et al. 2016).

In the order Hymenoptera, the presence of B chromosomes have already been reported in ants, wasps and bees. In ants, these chromosomes were detected in species of several genera (Lorite et al. 2002, Mariano et al. 2001, reviewed by Loiselle et al. 1990 and Gokhman 2009). In the parasitoid wasps, until now, these chromosomes were only found in *Nasonia vitripennis* Walker, 1836 (Pteromalidae), *Trichogramma kaykai* Pinto et Stouthamer, 1997 (Trichogrammatidae), *Encarsia asterobemisiae* Viggiani et Mazzone, 1980 (Aphelinidae) and in *Pnigalio agraules* Walker, 1830, *P. gyamiensis* Myartseva & Kurashev, 1990 and *P. mediterraneus* Ferrière & Delucchi, 1957 (Eulophidae) (Nur et al. 1988, Baldanza et al. 1999, Stouthamer et al. 2001, Gebiola et al. 2012, Gokhman et al. 2014). B chromosomes have also been identified in *Trypoxylon albitarse* Fabricius, 1804 (Crabronidae) (Araújo et al. 2000). Finally, in bees, B chromosomes have been reported in the genera *Melipona* Illiger, 1806 (*M. rufiventris* Lepeletier, 1836 and *M. quinquefasciata* Lepeletier, 1836), *Partamona* Schwarz, 1939 (*P. cupira* Smith, 1863, *P. helleri* Friese, 1900 and *P. rustica* Pedro et Camargo, 2003) and *Tetragonisca* Moure, 1946 (*T. fiebrigi* Schwarz, 1938) (revision in Tavares et al. 2017). They are also probably present in the species *P. criptica* Pedro et Camargo, 2003, *P. seridoensis* Pedro et Camargo, 2003, *P. gregaria* Pedro et Camargo, 2003, *P. chapadicola* Pedro et Camargo, 2003 and *P.* aff. *helleri* since molecular analysis demonstrated the presence of a sequence-characterized amplified region (SCAR) marker specific to the B chromosome of *P. helleri* in these genomes (Correia et al. 2014, Tosta et al. 2014, Machado et al. 2016). However, for these species, the presence of B chromosomes needs to be confirmed through cytogenetic techniques, as does the variation found in the sawfly *Tenthedro brevicornis* (Konow, 1886) (Sanderson 1970) and in the Braconidae, *Aphidius ervi*, Halliday, 1834 (Gokhman and Westendorff 2003).

The number of species with B chromosomes, however, increases as new species are studied cytogenetically (Camacho et al. 2000). For example, for many years it was considered that *M. quinquefasciata* had n = 18 and, consequently, 2n = 36 (Kerr 1972), a diploid

number very different from that of most *Melipona* species surveyed so far (n = 9 and 2n = 18; revision in Tavares et al. 2017). However, Kerr (1972) probably examined a colony that was yielding diploid males (Tarelho 1973). Then, Pompolo (1992) reported that analysis of one colony of *M. quinquefasciata* showed 2n = 20 chromosomes. It was only when a cytogenetic analysis was carried out several years later that *M. quinquefasciata* was found to have the same chromosome number as the majority of other *Melipona* species, 2n = 18, and that the numeric variations found in the karyotype of this species (2n = 19–22 and n = 9–13) were attributed to the presence of different numbers of supernumerary chromosomes (Rocha 2002, Rocha et al. 2007). However, despite comparing the general characteristics of the karyotype of *M. quinquefasciata* with that of other *Melipona* species, Rocha et al. (2007) did not specifically described the karyotype of *M. quinquefasciata*, the banding patterns obtained, or the variation in the number of B chromosomes found.

Thus, in the present study, we combined the data obtained by Rocha (2002) for two colonies of *M. quinquefasciata* with the analysis of five other colonies in order to: 1) describe in detail the karyotype of *M. quinquefasciata*, including the chromosome number, morphology and the location of heterochromatic regions, regions rich in AT/CG and ribosomal genes, and (2) verify the existence of B chromosomes in colonies from different locations, as well as their variation within colonies.

Materials and methods

Biological material

Post-defecating *M. quinquefasciata* larvae obtained from a colony from Brasília, DF (15°46'47"S, 47°55'47"W) and one from Luziânia, GO (16°15'09"S, 47°57'01"W) were analysed in 2000–2002 (Rocha 2002). Later, in 2013, we analysed three more colonies from Bicas, MG (21°43'31"S, 43°03'34"W), and two from Januária, MG (15°29'17"S, 44°21'42"W; State Park of Veredas of Peruaçú, PEVP).

Chromosome preparation and treatments

Chromosome preparations (Imai et al. 1988) were obtained using cerebral ganglion cells of larvae in the final stage of defecation. The number of individuals and number of metaphases per individual analysed varied from colony to colony (Suppl. material 1: Table S1).

To determine the number and morphology of the chromosomes, conventional staining was performed using Giemsa diluted in Sorensen buffer at a ratio of 1:30, for 20 minutes. The C-banding technique was used for heterochromatin detection (Rocha and Pompolo 1998). Metaphases were analysed on an Olympus BX60 microscope and the karyotypes were assembled using Image-Pro Plus (Version 6.3, Media Cybernetics 2009). The chromosomes were classified according to Levan et al. (1964), and the karyotypes were arranged by pairing chromosomes in decreasing order of size.

Sequential staining with fluorochromes 4',6-diamidino-2-phenylindole (DAPI) and chromomycin A_3 (CMA_3) was performed according to Schweizer (1980), using DAPI first for 30 min, followed by CMA_3 for 1 h. The use of distamycin was omitted. The fluorescent *in situ* hybridisation (FISH) technique (Viegas-Pequignot 1992) was performed using the 45S rDNA probe pDm 238 (Roiha et al. 1981). The best images were captured by a CCD camera coupled to an Olympus BX-60 epifluorescence microscope, using excitation filters WB (λ = 330–385 nm) and WU (λ = 450–480 nm), under immersion and at 100× magnification.

Results and discussion

The chromosome number of *M. quinquefasciata* ranged from 2n = 18 to 22 in females and from n = 9 to 13 in males, as already described by Rocha et al. (2007). Its karyotypic formula was 2K = 10M + 6SM + 2A (Fig. 1). Thus, the typical chromosome number of *M. quinquefasciata* was the same found in most *Melipona* species (2n = 18; Tavares et al. 2017), and numeric variations are due to the presence of 0–4 B chromosomes in females and males (Fig. 2).

In the analysed colonies, the majority of individuals had B chromosomes (Suppl. material 1: Table S1). In samples from Brasília and Luziânia, for example, all females analysed showed at least one B chromosome and only four of the eight analysed males from Luziânia had cells without B chromosomes. Even in these four males, the number of cells with B chromosomes was much higher than the number of cells without them. Similarly, in the colonies from Bicas and Januária, the number of female cells without B chromosomes was very low.

Variations were also observed in the number of B chromosomes between cells of the same individual, between individuals of the same colony, and between colonies from different localities (Fig. 2; Suppl. material 1: Table S1). In samples from Januária, for example, all individuals with B chromosomes had two chromosomes of that kind, while in samples from Brasília, Luziânia and Bicas, individuals with 0, 1, 2, 3 or 4 B chromosomes were found. Intra- and intercolonial variations relating to the presence of B chromosomes have also been described in *P. helleri,* another stingless bee species. In this species, the number of B chromosomes can range from 0–7 between and within colonies and the size of the B chromosome can also vary among colonies from different geographic locations (Costa et al. 1992, Brito et al. 1997, 2005, Tosta et al. 2004, Martins et al. 2014). Likewise, in *M. rufiventris* a small B chromosome was found in a few individuals (males and females) from one of the six colonies analysed (Lopes et al. 2008). Marthe et al. (2010) also described the presence of one B chromosome in some individuals of two colonies of *P. cupira* and Barth et al. (2011) observed that colonies of *Tetragonisca fiebrigi* can harbour individuals with 0, 1 or 2 B chromosomes. Together, our data and these published reports demonstrated that intra- and intercolony variation in the number of B chromosomes is common in stingless bees.

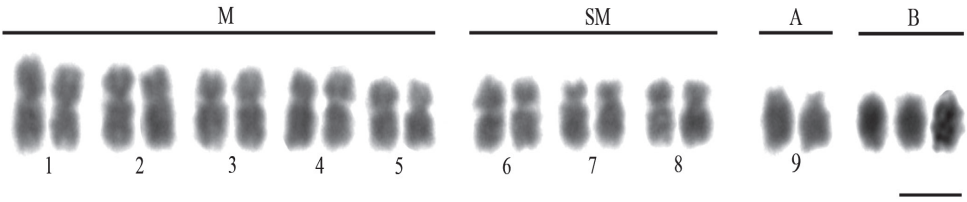

Figure 1. Representative karyotype of *Melipona quinquefasciata* female, with three B chromosomes, stained with Giemsa. M, SM, A and B: metacentric, submetacentric, acrocentric and B chromosomes, respectively. Scale bar: 5μm.

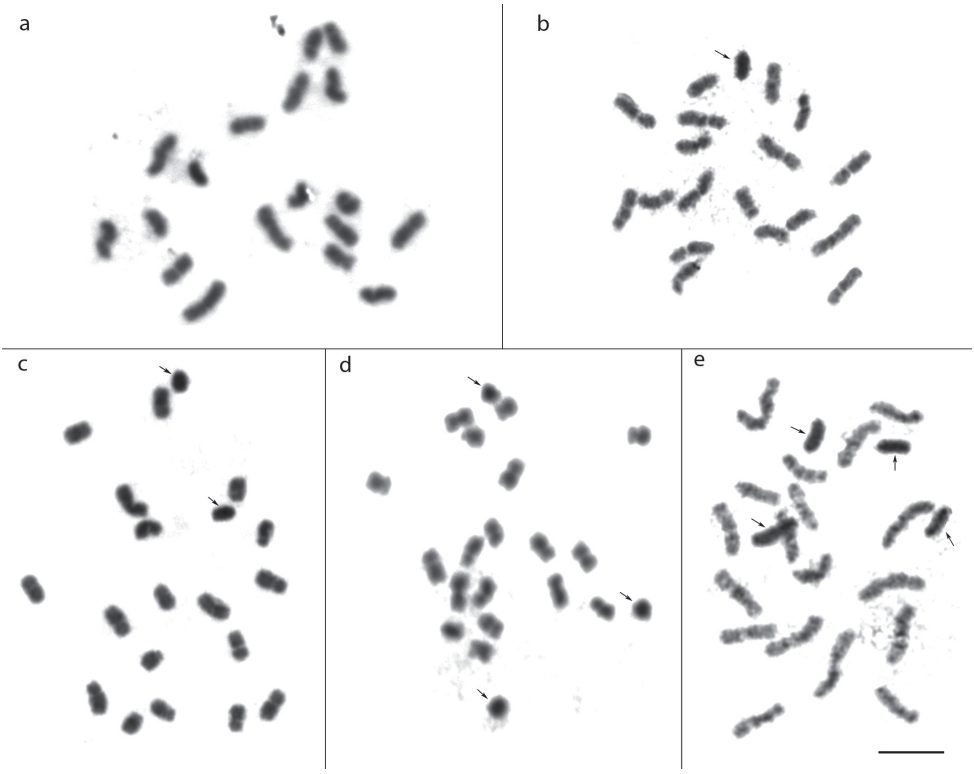

Figure 2. *Melipona quinquefasciata* metaphases, stained with Giemsa, showing the presence of 0, 1, 2, 3 and 4 B chromosomes (arrows). Scale bar: 5μm.

In different individuals and in the analysed colonies as a whole, the number of cells carrying two (411 cells) or three (268 cells) B chromosomes was considerably higher than those that had four B chromosomes (34 cells; Suppl. material 1: Table S1), as previously observed for *P. helleri* (Costa et al. 1992, Brito et al. 1997, Tosta et al. 2004). A more extensive cytogenetic analysis further demonstrated the presence of up to 7 B chromosomes in some *P. helleri* individuals (Martins et al. 2014) and, it is possible that analysis of colonies from other localities may change our perspective on B chromosome

numbers for *M. quinquefasciata*. Such analysis could provide insight as to whether there is a mechanism restricting the number of B chromosomes in stingless bees, as originally proposed by Martins et al. (2013). Interestingly, no study has reported a positive or negative effect on fitness related to the presence of different numbers of B chromosomes in this or other *Meliponini* species, as has been found for some other taxa (Camacho 2005).

Our data also revealed that, in *M. quinquefasciata*, the heterochromatin, identified by the C-banding technique, was located only in the pericentromeric region of pair 1 (Fig. 3a). Similar results have already been described for other *Melipona* species, such as *M. marginata* Lepeletier, 1836 (Maffei et al. 2001), *M. asilvai* Moure, 1971 (Rocha et al. 2002), *M. compressipes* (Fabricius, 1804) (Rocha et al. 2002), *M. rufiventris*, and *M. mondury* Smith, 1863 (Lopes et al. 2008). Therefore, it was possible to infer that the chromosomes of the A complement of *M. quinquefasciata* had low heterochromatin content. As the genus *Melipona* can be separated in two groups, one with low (Group I) and the other with high (Group II) heterochromatin amounts, *M. quinquefasciata* could be grouped into Group I together with *M. marginata*, *M. quadrifasciata* Lepeletier, 1836, *M. bicolor* Lepeletier, 1836, *M. asilvai*, *M. subnitida* Ducke, 1910, *M. mandacaia* Smith, 1863 and *M. puncticolis* Friese, 1902 (Rocha and Pompolo 1998, Rocha et al. 2002).

However, *M. quinquefasciata* belongs to the subgenus *Melikerria* Moure, 1992 and species clustered in Group I belong to the subgenera *Melipona* Illiger, 1806 or *Eomelipona* Moure, 1992; Group II clusters species of the subgenera *Melikerria* and *Michmelia* Moure, 1975 (Lopes et al. 2011). Additionally, *M. fasciculata* Smith, 1854 and *M. interrupta* Latreille, 1811, the only other species of the subgenus *Melikerria* that had their heterochromatin distribution pattern analysed, presented high heterochromatin quantities and were included in Group II (Lopes et al. 2011). This reinforces the need of additional cytogenetic studies concerning species of this subgenus.

By comparison, the B chromosomes of *M. quinquefasciata* were completely heterochromatic, as shown by the C-banding technique (Fig. 3a) and Giemsa staining (Fig. 1), regardless their number in the examined metaphases (Fig. 2). The staining with DAPI confirmed the heterochromatic nature of these chromosomes (Fig. 3c), indicating that, unlike the chromosomes of the A complement, B chromosomes of *M. quinquefasciata* were rich in AT base pairs. Unfortunately, due to their heterochromatic nature, it was not possible to study the morphology of B chromosomes of *M. quinquefasciata* in detail.

CMA_3 staining and FISH analysis using a 45S rDNA probe confirmed that ribosomal genes were located only in the pericentromeric region of pair 1 in the karyotype of *M. quinquefasciata* (Fig. 3b, d), as already reported for the two colonies analysed by Rocha et al. (2007). The presence of a unique autosome pair with a nucleolus organizer in *M. quinquefasciata* corroborated previous reports about the location of the rDNA clusters in other *Melipona* species, independent of the technique used (Ag-NOR impregnation, CMA_3 staining or FISH; Rocha et al. 2002, Brito et al. 2003, Lopes et al. 2011, Cunha et al. 2018, Piccoli et al. 2018). This seemed to be the most frequent pattern found in other Meliponini genera (Brito-Ribon et al. 1999, Rocha et al. 2003, Krinski et al. 2010), although the presence of multiple rDNA clusters has also been described (Rocha et al. 2003, Brito et al. 2005, Duarte et al. 2009, Martins et al. 2009, Godoy et al. 2013).

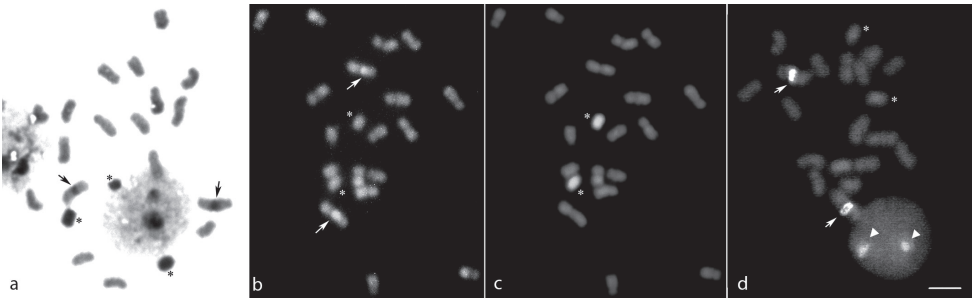

Figure 3. *Melipona quinquefasciata* metaphase with 2n = 18 + 2Bs submitted to C-banding (**a**), CMA_3 (**b**) and DAPI (**c**) staining, and to the FISH technique (**d**). The arrows indicate the rDNA location, while asterisks indicate the B chromosomes and arrowheads indicate an interphase nucleus with two signals. Scale bar: 5 μm.

Conclusion

The results of this study demonstrated that *M. quinquefasciata* has an A complement with a chromosome number characteristic of the *Melipona* genus (2n = 18; n = 9) and a karyotypic formula of 2K = 10M + 6SM + 2A. The numerical variation frequently described for this species might be explained by the presence of a variable number of B chromosomes in individual karyotypes. These chromosomes were found in individuals from different localities and were completely heterochromatic. By comparison, in the chromosomes of the A complement heterochromatin was located only in the pericentromeric region of pair 1, which corresponded to the nucleolus organising region, as demonstrated by CMA_3 staining and *in situ* hybridisation using a 45S rDNA probe.

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Supplementary material 1

Table S1

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Data type: species data

Explanation note: Sampled localities, number and sex of the individuals of *Melipona quinquefasciata* analyzed (N) and their cytogenetic characteristics..

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RESEARCH ARTICLE

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Karyotype and chromosomal characteristics of rDNA of *Cobitis strumicae* Karaman, 1955 (Teleostei, Cobitidae) from Lake Volvi, Greece

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Abstract

The karyotype of Greek cobitid fish *Cobitis strumicae* Karaman, 1955, from Lake Volvi, Greece, a representative of one of its two major intraspecific phylogenetic lineages, was analysed by means of sequential Giemsa-staining, C-banding, silver-staining, CMA_3 fluorescence banding and also by *in situ* hybridization (FISH) with rDNA probe. The diploid chromosome number was 2n = 50, karyotype composed of 10 pairs of metacentric to submetacentric and 15 pairs of subtelocentric to acrocentric chromosomes. The nucleolus organizer regions (NORs) as revealed by Ag- and CMA_3 staining and FISH were situated in the telomeric region of the fourth submetacentric chromosome pair. The chromosomes contained very low content of C-positive heterochromatin. No heteromorphic sex chromosomes were detected. This first karyotype report for any species of lineage *Bicanestrinia* Băcescu, 1962 shows a simple karyotype dominated by acrocentric chromosomes and possessing single NOR-bearing chromosome pair. Cytotaxonomic implications of this finding for the taxonomy of the genus *Cobitis* Linnaeus, 1758 are further discussed.

Keywords

chromosome banding, NOR phenotype, FISH, rDNA, cytotaxonomy of *Cobitis* loaches

Introduction

The genus *Cobitis* Linnaeus, 1758 attracted the interest of evolutionary biologists by producing several gynogenetic female-only lineages after hybridisation of species (Bohlen and Ráb 2001). As reasons for the asexual reproduction in these hybrids differences in the karyotype and chromosome structure between the parental species have been proposed. Indeed, within *Cobitis* a large variability of karyotypes and chromosomal markers have been observed (Janko et al. 2007). On the other hand, species of *Cobitis* are morphologically highly similar and difficult to identify on the basis of morphologic characters. They have a pronounced sexual dimorphism with males being smaller than females and developing an ossified plate-like structure on the dorsal side of the pectoral fins, called '*lamina circularis*'. The widespread presence of hybrid lineages further complicates the systematics and taxonomy of *Cobitis* loaches, therefore genetic methods are applied in identification of species. Chromosome studies have shown that most species have a diploid chromosome number of 2n = 50, but highly diversified karyotypes (reviewed in Ráb and Slavík 1996, Arai 2011). This genetic marker therefore appears to be one of the key parameter in the genetic and taxonomic studies of *Cobitis* loaches, e.g. Ráb and Slavík (1996), Boroň and Danilkiewicz (1998), Vasil'eva and Vasil'ev (1998), Ráb et al. (2007), and serves as one of the determination tools to identify genome composition in hybridogenous clonal asexual biotypes (Janko et al. 2007, Majtánová et al. 2016).

Recent phylogenetic studies (Buj et al. 2014, Ludwig et al. 2001, Perdices and Doadrio 2001, Perdices et al. 2016) demonstrated that the European representatives of *Cobitis* include five major lineages, namely the 'Siberian lineage', represented by a single species *C. melanoleuca* Nichols, 1925, Băcescu's (1962) subgenera *Acanestrinia* (now often referred to as 'Adriatic lineage'), *Iberocobitis*, *Bicanestrinia*, and *Cobitis* s. str. The subgenus *Bicanestrinia* is morphologically well characterized by having two *laminae circulares* on the pectoral fins of males. Species of *Bicanestrinia* occur in the Middle East (Turkey, Iran, Syria) and southeast Europe (Bulgaria, Greece) (Bohlen et al. 2006). Up to now, only one species of *Bicanestrinia*, *C. linea* (Heckel, 1847), has ever been analysed in a cytogenetic study, therefore little is known about cytogenetic similarities and differences between *Bicanestrinia* and *Cobitis* s. str. One of the European species of *Bicanestrinia*, *C. strumicae* Karaman, 1955, has long been known from rivers draining into the Aegean Sea, such as Struma, Maritza and the lakes adjacent to the Struma basin such as Volvi and Koronia in Greece. However, it has recently been found in the Danube basin, where it is genetically involved in asexual hybrid forms (Choleva et al. 2008). Since further studies on this example of a sperm-dependent hybrid switch of the sexual hosts require a proper identification of the genetic material of *C. strumicae*, the cytogenetic analysis of Struma spiny loach will complete identification tool box of hybrid biotypes of the genus *Cobitis*.

This study reports on the karyotype and other chromosomal characteristics of Greek cobitid fish *C. strumicae* from population inhabiting Lake Volvi, Greece, analysed by means of sequential Giemsa-staining, C-banding, silver-staining, CMA_3 fluorescence banding and by *in situ* hybridization (FISH) with 28S rDNA.

Material and methods

Ten males and two females were collected at the outlet of a thermal spring into Lake Volvi, Greece, by dip net and transferred alive to the laboratory. The examined specimens are deposited as voucher samples in the collection of the Laboratory of Fish Genetics, IAPG, CAS, Liběchov, under Accession Code CoS/97. Valid Animal Use Protocol was in force during study in IAPG (No. CZ 02386). Standard procedures for chromosome preparation followed Ráb and Roth (1988). Silver (Ag-) staining and Chromomycin A_3 (CMA_3) fluorescence banding, for detection of NORs, followed Howell and Black (1980) and Sola et al. (1992), respectively. The sequence of stainings followed protocol of Rábová et al. (2016). Fluorescence *in situ* hybridization (FISH) with a mouse rDNA biotinylated probe (clone I-19, a 4.2-kb EcoRI-SalI fragment containing most of the 28S rDNA-coding region) to detect chromosomal sites of rDNA, i.e. sites of NORs, followed the procedure of Reed and Phillips (1995) and Ozouf-Costaz et al. (1996). Briefly, a mouse rDNA clone, was biotin-labelled by nick translation (Oncor, Inc). Chromosomes were pretreated by incubating the slides in 2X SSC (pH 7.0) at 37 °C for 30 min, dehydrated in a 4 °C ethanol series, and air-dried. Chromosomal DNA was denatured by incubating the slides in a filtered 70% formamide/2X SSC solution (pH 7.0) at 70 °C for 2 min, followed by dehydration in 4 °C ethanol series. Labelled probe was diluted to 16.6 ng/µl in hybridization solution (Hybrisol VII, Oncor; 50% formamide), denatured by incubation at 70 °C for 5 min and placed immediately on ice until applied to slides. Hybridization was performed using 20–25 µl (~ 250 ng) of probe mixture/slide and incubated overnight in a 37 °C humidity chamber. After hybridization, slides were washed in a 50% formamide/2X SSC solution (pH 7.0) at 37 °C for 15 min, followed by an 8 min wash in 2X SSC (37 °C, pH 7.0). Slides were washed at room temperature for 2 min each in the following series: 4X SSC; 4X SSC + triton X; and a 1:1 mix of 4X SSC and PN buffer (0.1 M NaHP0, 0.1 M NaH P0, 5% NP-40 detergent, pH 8.0). Fluorescein-isothiocyanate (FITC)-conjugated avidin was used to detect hybridization signal. Chromosomes were counterstained with propidium iodide (0.375 µg/ml) in antifade (10 mg/ml p-phenylenediamine dihydrochloride (DAPI) in PBS/90% glycerol, pH 8.0) and chromosomes viewed under epifluorescence.

At least 25 Giemsa-stained or banded metaphases plates per individual were examined, most of them sequentially. Chromosomes were classified according to Levan et al. (1964), metacentric to submetacentric and subtelocentric to acrocentric chromosomes, respectively were grouped together in Fig. 1.

Results

Karyotype and banding analysis

Chromosome counts from all 12 individuals revealed an invariable diploid chromosome number 2n = 50. The karyotype consisted of 10 pairs of metacentric (m) to

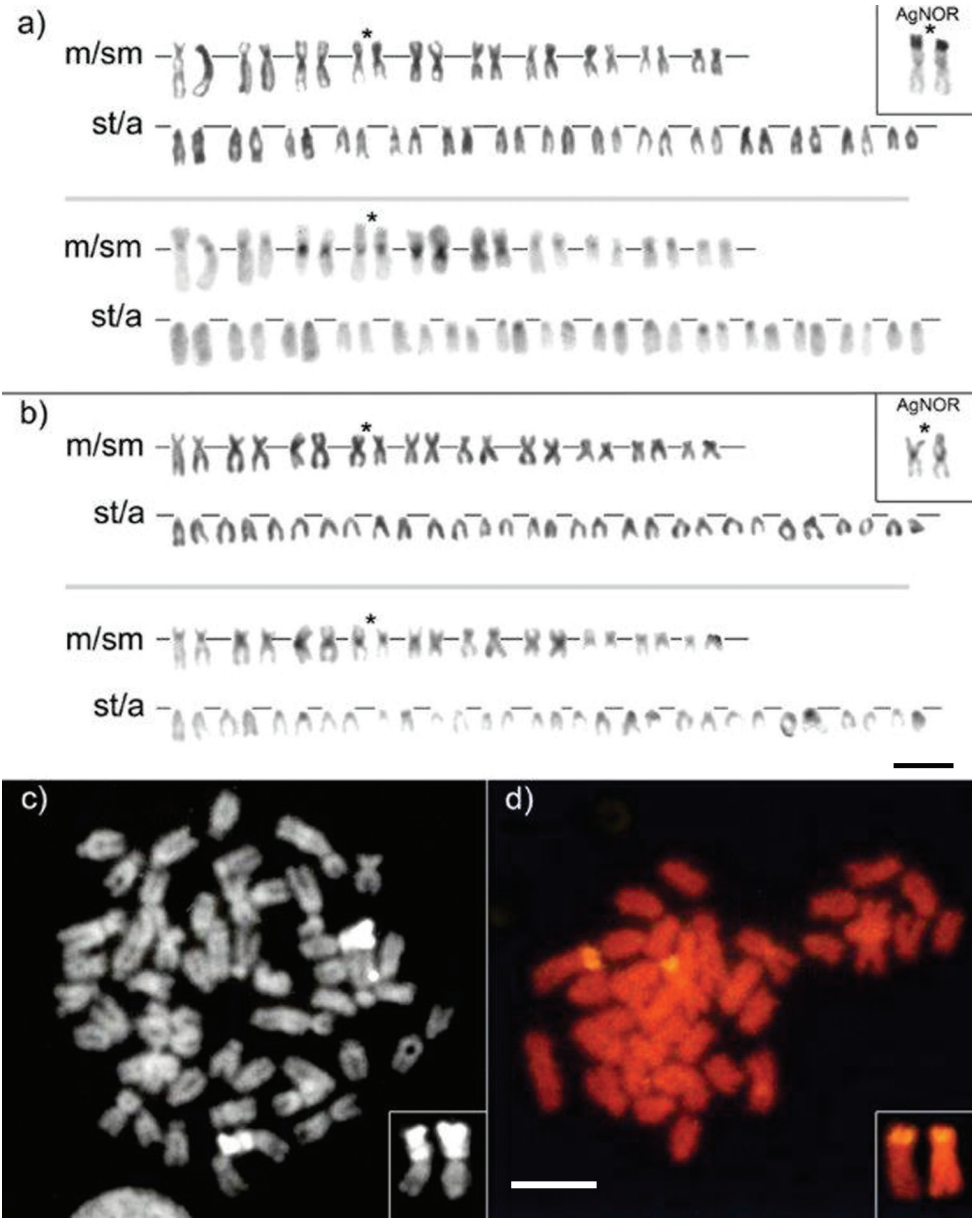

Figure 1. Karyotypes of a male (**a**) and female (**b**) of *C. strumicae* from Lake Volvi arranged from sequentially Giemsa-stained (upper row) and C-banded (lower row) chromosomes; sequentially Ag-stained chromosome pair with positive signal is framed in (**a**) and (**b**); metaphase cells of *C. strumicae* after CMA_3 staining (**c**) and FISH with rDNA probe (**d**) chromosomes bearing CMA_3 and FISH signals are framed; m – metacentric, sm – submetacentric, st – subtelocentric and a – acrocentric chromosomes. Scale bar: 10 μm.

submetacentric (sm) and 15 pairs of subtelocentric (st) to acrocentric (a) chromosomes (Fig. 1a, b). No heteromorphic sex chromosomes were detected in males (Fig. 1a) and females (Fig. 1b). The nucleolus organizer regions (NORs) as revealed by Ag- and

CMA_3 staining were situated in the telomeric region of the fourth m/sm chromosome pair. This pair of chromosomes was also observed to be end-to-end associated in some metaphases. No variation in number of NORs was observed while size polymorphism was frequently detected. C-banding revealed small heterochromatic blocks in pericentromeric regions of all pairs of chromosomes except fifth and sixth m pairs where the blocks of heterochromatin were large (Fig. 1).

Chromosomal location of rDNA

FISH with 28S rDNA probe showed strong labelling of a single chromosomal pair (Fig. 1d). Identification of chromosomes by propidium iodide counterstaining revealed the labelled pair to be the same as that identified by Ag- and CMA_3 staining. No other positively labelled chromosomal sites were found.

Chromosomal organization of NOR sites

CMA_3 staining revealed the positive signal on the NOR-bearing pair only (Fig. 1c). The CMA_3 positive blocks covered entire p arm from the pericentromeric region to telomeres with distinct gap close to centromere. However, C-banding showed positive heterochromatin blocks in pericentromeric region which clearly corresponded to smaller CMA_3-positive blocks (Fig. 1a, b). Ag-staining (Fig. 1a, b) and FISH (Fig. 1d) showed positive signals in distal parts of shorter arm only.

Discussion

Arrangement of nucleolar ribosomal DNA in *C. strumicae* chromosomes

We examined chromosomes of *C. strumicae* by means of several banding methods detecting sites of major ribosomal DNA, i.e. sites of NORs (Ráb et al. 1996). The application of GC-specific fluorochromes such as CMA_3 or Mithramycin (MM), together with enhancing AT-specific counterstains that specifically interact with GC-rich DNA sequences and/or examination of rDNA loci by FISH indicate that the sites of NORs of teleostean fishes detected by means of silver staining contain large fractions of GC-rich DNA, e.g. Mayr et al. (1985), Amemiya and Gold (1986), Schmid and Guttenbach (1988) and reviewed by Gornung (2013). The association of GC-rich DNA type of heterochromatin with rDNA sites is present in lower and higher teleostean groups, suggesting that it is evolutionarily conserved among teleosts (Gornung 2013). However, this character exits also in bichirs (Polypteriformes), partly in paddlefishes (Symonová et al. 2017a), gars (Symonová et al. 2017b) and bowfin (Majtánová et al. 2017), but not in sturgeons (Fontana et al. 2007). Among *Cobitis* loaches, this characteristic pattern was found in *C. vardarensis* Karaman, 1928 (Rábová et al. 2001),

C. elongatoides Băcescu et Mayer, 1969 (Ráb et al. 2000) and *C. taenia* Linnaeus, 1758 (Boroň et al. 2006), i.e. species from *Cobitis* s. s. clade. Our analysis of chromosomal characteristics of major rDNA in *C. strumicae* confirms such characteristic association of GC-rich DNA and sites of NORs for the so far uninvestigated subgenus *Bicanestrinia*.

Recent cytogenetic studies in fish (Gornung 2013), also suggested that not all CMA_3-positive signals represent sites of NORs but exclusively GC-rich heterochromatin blocks which are not associated with ribosomal DNA (Ráb et al. 1996). Our investigation of *C. strumicae* chromosomes using several methods to detect NORs revealed such type GC-rich DNA heterochromatin which is present exclusively on NOR-bearing chromosome arm including pericentromeric region. Interestingly, the sequential Ag-staining and C-banding together with CMA_3 fluorescence showed that NOR sites stained negative after C-banding procedure. Such an identical association of positive Ag-, CMA_3 and C- band signals at the NOR sites appears to be ubiquitous pattern for fish genomes. However, our present results for *C. strumicae* showing negative C-bands at NOR sites together with the same findings in *C. vardarensis* (Rábová et al. 2001), *C. elongatoides* (Ráb et al. 2000) and *C. taenia* (Boroň et al. 2006) may indicate the different structural organization of chromosomes at the NOR sites in the genomes of the genus *Cobitis*.

Cytotaxonomy of *Cobitis strumicae*

Diploid chromosome number (2n), karyotype structure, i.e. number of chromosomes in the particular categories and especially number and location of NORs, i.e. NOR phenotypes, have proven useful for fish cytotaxonomy. Ráb and Slavík (1996) and Arai (2011) overviewed all available data regarding chromosome studies of *Cobitis* loaches. However, many of listed studies did not provide exact localities, morphological descriptions, data about deposition of voucher specimens and/or depiction of analysed material and what`s more – many reports analysed species under the collective name *C. taenia*. This is the reason why data concerning the name of species given in that list must be used with caution for cytotaxonomic comparisons. As a result, many data should be verified and completed by the new data. Anyhow, the lists of Ráb and Slavík (1996) and Arai (2011) show that only one of the currently recognized species of the subgenus *Bicanestrinia* was subjected to karyotype analysis: *C. linea* from the Kor River basin, Iran, where authors reported 2n = 50 and a karyotype composed of 4 m, 40 sm and 6 st, NF value 94 (Esmaeili et al. 2015). This karyotype composition differs remarkably from that of *C. strumicae*, but one should bear in mind that both *C. strumicae* and *C. linea* belong to different mitochondrial lineages *sensu* Bohlen et al. (2006) and such variation might indicate the existence of a karyotype differentiation within Bicanestrinia, similarly as within *Cobitis* s. s. (Janko et al. 2007, Ráb et al. 2007).

The species under study, *C. strumicae*, shares the diploid chromosome number 2n = 50 with most of the species karyotyped so far. Its karyotype dominated by uniarmed (acrocentric) chromosomes and lack of morphologically differentiated sex chromosomes is rather common among *Cobitis* loaches.

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RESEARCH ARTICLE

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Pilot satellitome analysis of the model plant, *Physcomitrella patens*, revealed a transcribed and high-copy IGS related tandem repeat

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Abstract

Satellite DNA (satDNA) constitutes a substantial part of eukaryotic genomes. In the last decade, it has been shown that satDNA is not an inert part of the genome and its function extends beyond the nuclear membrane. However, the number of model plant species suitable for studying the novel horizons of satDNA functionality is low. Here, we explored the satellitome of the model "basal" plant, *Physcomitrella patens* (Hedwig, 1801) Bruch & Schimper, 1849 (moss), which has a number of advantages for deep functional and evolutionary research. Using a newly developed pyTanFinder pipeline (https://github.com/Kirovez/pyTanFinder) coupled with fluorescence *in situ* hybridization (FISH), we identified five high copy number tandem repeats (TRs) occupying a long DNA array in the moss genome. The nuclear organization study revealed that two TRs had distinct locations in the moss genome, concentrating in the heterochromatin and knob-rDNA like chromatin bodies. Further genomic, epigenetic and transcriptomic analysis showed that one TR, named PpNATR76, was located in the intergenic spacer (IGS) region and transcribed into long non-coding RNAs (lncRNAs). Several specific features of PpNATR76 lncRNAs make them very similar with the recently discovered human lncRNAs, raising a number of questions for future studies. This work provides new resources for functional studies of satellitome in plants using the model organism *P. patens*, and describes a list of tandem repeats for further analysis.

Keywords

Physcomitrella patens, Bryophyta, satellite DNA, chromosomes, fluorescence *in situ* hybridization, long non-coding RNAs, rDNA

Introduction

A substantial part of eukaryotic genomes is composed of different families of repetitive elements (REs). Some REs are ancient viruses (e.g., mobile elements), whereas others are *de novo* generated sequences without a specific structure. The latter include satellites, or tandem repeats (TRs), dispersed repeats and other repeat groups. TRs are the main components of heterochromatin, centromeres and telomeres (Henikoff et al. 2001, Plohl et al. 2008). TRs are important for genome stability and integrity and play a critical role in centromere function, meiotic chromosome segregation, gene regulation, X chromosome recognition and speciation (Dernburg et al. 1996, Ferree and Barbash 2009, Jagannathan et al. 2017, Menon et al. 2014, Talbert and Henikoff 2018). The genomic organization, chromosome distribution and sequence of TRs could differ significantly between closely related species and even between chromosomes of one organism (Almeida et al. 2012; Jagannathan et al. 2017, Jo et al. 2009, Kirov et al. 2017, Lim et al. 2004, Lower et al. 2018, May et al. 2005, Plohl et al. 2008, Robledillo et al. 2018, Ruiz-Ruano et al. 2016). Because TRs can mislead the recombination machine, they can also play a negative role and be the reason for genome rearrangements (Ma and Bennetzen 2006). Surprisingly, a recent study has demonstrated that TRs are not an inert part of a genome, some TRs, including those that have intergenic spacer (IGS), telomere and centromere origins, are expressed in a cell (Chen et al. 2008, May et al. 2005, Perea-Resa and Blower 2017, Yap et al. 2018, Zhao et al. 2018). Although the functions of the so-called satRNAs are enigmatic, there is a growing body of evidence that some of them can interact with different proteins and play nuclear architectural roles (Chujo et al. 2017, Staněk and Fox 2017, Sun et al. 2017, Yap et al. 2018).

The rapid evolution and high intra-monomer identity of TRs significantly hamper their study at the genome level. TRs are often collapsed or placed into an unassembled portion of the genome (e.g. Chr0, (Saint-Oyant et al. 2018)), which significantly reduces the amount of information available to study the organization of TRs. Long-read sequencing, optical mapping and other modern techniques can help to overcome these obstacles (Jain et al. 2018, Khost et al. 2017, Lower et al. 2018, Weissensteiner et al. 2017). High-throughput methods, including methods used to identify TRs from raw NGS data, have allowed researchers to gain a deeper insight into TR evolution and abundance (Lower et al. 2018, Novák et al. 2017). In addition, information about the TRs physical location is useful for understanding the TR evolution and function as well as for the improvement of the genome assembly (Saint-Oyant et al. 2018). Molecular cytogenetic techniques such as fluorescence *in situ* hybridization (FISH) or PRINS have been applied to study the genomic organization of TRs at a chromosome level (Cuadrado and Jouve 2010, Gosden et al. 1991, Jiang and Gill 2006, Kirov et al. 2017, Pavia et al. 2014, Rosato et al. 2016, Sone et al. 1999, Xiao et al. 2017). The unique nature of TRs allows their rapid localization on the chromosomes through non-denaturating FISH (ND-FISH, (Cuadrado and Jouve 2010, Jiang and Gill 2006, Kirov et al. 2017, Pavia et al. 2014, Xiao et al. 2017)). Although it is an important tool for studying the genome organization of TRs, the application of molecular cytoge-

netic methods is challenging and further improvement of chromosome preparation and FISH protocols are needed for some species (Kirov et al. 2016, Rosato et al. 2016).

The latest discoveries, including the specific transcription of some TRs as satRNAs and lncRNAs, which play important roles in regulatory processes, have moved satellite DNA biology from structural genomics to functional genomics. Satellite DNA annotation has been performed for a long list of plant species, but there are only a few model plants that are suitable for deep functional studies of TRs. In addition, no model basal plants are present on this list, although they could facilitate the study of the TR evolution mechanisms on a long timescale. Here, we performed a pilot satellitome analysis of the model basal plant, the moss *Physcomitrella patens* (Hedwig, 1801) Bruch & Schimper, 1849. It is a widely used model plant for molecular and developmental biology, evolution and biochemistry studies (van Gessel et al. 2017). The "basal" position of mosses in the land plant phylogeny makes this plant unique, bridging the gap between green algae and flowering plants (van Gessel et al. 2017). The chromosome level assembly of moss has been recently performed and different transcriptomic, epigenetic and proteomic datasets as well as tools are available (Amagai et al. 2018, Fesenko et al. 2017, Fesenko et al. 2016, Fesenko et al. 2015, Lang et al. 2005, Lang et al. 2018, Ortiz-Ramírez et al. 2016, Quatrano et al. 2007, Rensing et al. 2008, van Gessel et al. 2017). Using the newly developed pyTnaFinder pipeline (https://github.com/Kirovez/pyTanFinder), we identified five TRs that show prominent FISH signals on the nucleus and chromosomes (for two TRs). Nuclear organization revealed two TRs with distinct locations, in the heterochromatin and perinucleolar bodies. One TR, called PpNATR76, was located in the IGS of 45S rRNA genes. Using transcriptomic and genomic data, we found that PpNATR76 is transcribed into lncRNAs with unknown functions. Comparison of the distinct features of PpNATR76 organization and transcription and similarities with the recently discovered IGS-related lncRNAs in humans suggest that the transcription of a functionally important satellite containing lncRNAs from the IGS region is a conserved principle between plants and humans.

Material and methods

pyTanFinder development

pyTanFinder was written in python v3.6 using biopython (Cock et al. 2009) and networkx (Hagberg et al. 2008) libraries. Tandem Repeat Finder tool (Benson 1999) was run in the initial step of the pipeline followed by BLASTN (Altschul et al. 1997) similarity search between different monomers. Using similarity search data, the graphs were constructed by the networkx library (Hagberg et al. 2008) and a sequence with the maximum number of edges (hits) was selected for each graph. The most representative monomer sequence is then described according to its different features including accumulating abundance (the sum of the copy number of each monomer from graphs multiplied by the monomer length), monomer length and number of connections in the cluster using

matplotlib (Hunter and engineering 2007) library. The histograms are generated and represented as html document. pyTanFinder is licensed under the MIT License and is available from GitHub repository (https://github.com/Kirovez/pyTanFinder).

Slide preparation

For chromosome and nucleus preparation, the Gransden strain of *P. patens* was grown in Knop medium with 500 mg/L ammonium tartrate with 1.5% agar (Helicon, Moscow, Russian Federation) in a Sanyo Plant Growth Incubator MLR-352H (Panasonic, Osaka, Japan) with a 16-hour photoperiod at 24 °C and 61 μmol/m^{2}s. Gametophores at different stages (green – light green sporophyte colors) were used for analyses. Chromosome preparation was performed according to the "SteamDrop" protocol (Kirov et al. 2014) with modifications described earlier (Kirov et al. 2015). Briefly, young sporophytes were collected and fixed in Carnoy's solution (3:1, ethanol/acetic acid) for 3 h at room temperature and stored at –200 °C in 70% ethanol. The fixed material was washed twice in distilled water for 30 min and in 100 mM Citric buffer (pH 4.8). Then, the sporophytes were transferred into the enzyme mixture and incubated for 3 h at 37 °C. The 0.6% enzyme mixture containing Pectolyase Y-23 (Kikkoman, Tokyo, Japan), Cellulase Onozuka R-10 (Yakult Co. Ltd., Tokyo, Japan) and Cytohelicase (Sigma-Aldish Co.LLC, France), was prepared in 0.1 M citric buffer (pH4.8). Slides were prepared using a 1:1 (ethanol/acetic acid) mixture as the first drop and 100% acetic acid as the second drop. Then, slides were additionally incubated for 15–30 s in a drop of 60% acetic acid at 42 °C. One slide per cell suspension was checked by DAPI (100 μg/ml, 4' 6-diamidino-2-phenylindole) staining and mounted in Vectashield (Vector Laboratories, Burlingame, CA).

NGS sequencing of the moss genome

Isolated DNA was used in NGS sequencing. A sequencing library was prepared by the NEBNext ultra DNA Library Prep Kit for Illumina (New England Biolabs, UK). After preparation of the samples, the libraries were analyzed using Qubit (Invitrogen) and 2100 Bioanalyzer (Agilent Technologies). Amplification of the samples was performed according to the protocol (Illumina) using MiSeq. Raw Illumina fastq files were demultiplexed, quality filtered and analyzed using FastQC (Schmieder and Edwards 2011). RepeatExplorer tool (Novak et al. 2013) was run with default settings taking 500000 randomly selected single end reads (>100 bp) as input.

Fluorescence in situ hybridization (FISH) and microscopy

FISH was performed as previously described (Kirov et al. 2017) using TAMRA-labeled oligo probes synthesized by Evrogen (Table 1).

Table 1. Oligo probes on TRs used in FISH experiments.

ID	Sequence
17_50	(TAMRA)-AACCTTCTAGAAGAGAAGTTT
21_215	(TAMRA)-ACTTCCAGAGAGCATCGGCAA
602_86	(TAMRA)-AAGTGATGAACAAAATTTCTC
04_78	(TAMRA)-AACTTGCATTCTTCATTTTCA
592_108	(TAMRA)-ATTTCTTAGAAAATACGTTCT
20_76	(TAMRA)-AGTCCCGTCGCGAGTCCCGGA
19_95	(TAMRA)-ATAATTCTATCGGTTATGTTT
05_92	(TAMRA)-AATAATAGTAAAAGTTATAGC
21_43	(TAMRA)-ACCTTCAAGTGGACCTTAGTA
01_31	(TAMRA)-AATCAGCTCGAGTCGAGCTGA
08_44	(TAMRA)-AGCTGATGGCAGGTAAGGGAG
02_27	(TAMRA)-CTTCCGTCTTGGATCCGGAAT
08_217	(TAMRA)-AAAGTAGATCTAAAAATAAAA
05_178	(TAMRA)-ACACGAAACTCACAACTTACT
21_43	(TAMRA)-ACCTTAGTGGAGAAGTTCTGC
18_62	(TAMRA)-AGGGGAGTTTTCAAGTTTTTG
10_116	(TAMRA)-ATTGGAGAAGTATCATTGTAA
16_64	(TAMRA)-ATCGAAGAGCTAGCTTCAAGC
1004_43	(TAMRA)-AGAGAAGTTCTGTCCTTGCCT

Table 2. Primers used for qRT-PCR amplification of PpNATR76 transcripts.

Gene id	Forward	Reverse
Pp3c20_303V3.1	ATGGAGCGGGACAAGAGG	GAGTCCCGACCTCTGGCG
Pp3c20_283V3.1	CCCCCGCCAAAAATGGTTAC	CGGGACAAGGAAGAGGAGGA
Pp3c19_9271V3.1	ACTGGGCTCAAAGAAGGCAG	AGGAGGAAGAGGAGGAAGGC
Pp3c14_12290V3.2	CCCTAGCCTTTGGTTGCGTT	ACTCTCCCTTGCAATGGTCG
Pp3c4_8299V3.1	GTGTCGGGGTTAGGAAGTGG	TAGCTCTTGGAACTCGCTGC

qRT-PCR

Total RNA from protonemata tissue was isolated according to Cove et al. 2000. The RNA quality and quantity were evaluated by electrophoresis in an agarose gel with ethidium bromide staining. The exact concentration was measured using the Quant-iT RNA Assay Kit, 5–100 ng on a Qubit 3.0 (Invitrogen, US). The cDNA for RT-PCR was synthesized using the MMLV RT Kit (Evrogen, Russia). Primers (Table 2) were designed by the Primer 3.0. qRT-PCR with actin gene primer pairs was used as a positive control, whereas qRT-PCR with MQ and DNAse-treated RNA was used as a negative control. RT-PCR was performed using the qPCRmix-HS SYBR system and SYBR Green I (Evrogen) dye on a LightCycler® 96 (Roche, Mannheim, Germany). qPCR was performed in three biological and three technical replicates.

Results

Search for tandem repeats in *P. patens* genome by read clustering and pyTanFinder

To find the TRs in the *P. patens* genome, we used the Tandem Repeat Finder tool (TRF, (Benson 1999)). However, TRF provides all the TRs found in the genome; information about the copy number of individual TR monomers is unavailable. Moreover, the TRF output is redundant and it is difficult to manually handle it to find high-copy TRs that possess a certain monomer length and copy number. To overcome these obstacles we designed a python pipeline that we called pyTanFinder (https://github.com/Kirovez/pyTanFinder). It is a user-friendly command line tool to run TRF and parse the results followed by clustering of similar tandem repeats. The output of this program is a FASTA file of all tandem repeats and a table containing unique TR sequences with the estimated abundance in the genome. In addition, pyTanFinder also generates a html report containing histograms of the distribution of the TR monomer size and number of connections of each monomer into an individual cluster. We applied the pyTanFinder pipeline to the *P. patens* (v3.3) genome sequence. We identified 1518 TRs with a minimum length of genome occupy 1000 bp. Because TRs can be collapsed during genome sequence assembly, we performed low-coverage Illumina DNA sequencing followed by *de novo* annotation of TRs in next generation sequencing data using the RepeatExplorer tool (Novak et al. 2013). The clustering of the genomic reads did not reveal any clusters with a ring or globular shape that both corresponded to high-copy TRs. We then compared DNA sequences produced by the pyTanFinder pipeline and RepeatExplorer to find TRs with high copy number in both datasets. 19 TRs that were found in both datasets were used for further analysis (Table 3).

The monomer length of the TRs ranged from 27 to 217 bp (Fig.1A) and the GC content varied from 20 to 70% (Fig. 1B).

According to the pyTanFinder results, 7 (37%) TRs have high (>18000 bp, hcTRs) and 12 (63%) TRs have low (<15000 bp, lcTRs) total abundance. We were able to design primers for 5 hcTRs and obtained ladder-like or smear PCR products (Fig. 1C) that are known characteristic features of TRs (Kirov et al. 2017). Only 8 of 19 identified TRs (trTRs) were similar to the RepeatExplorer contigs from the top 200 clusters, whereas the other TRs were similar to low abundant repeat clusters. Interestingly, the pyTanFinder total abundance data did not correlate with the RepeatExplorer genome proportion data, as only 2 of the trTRs were in set of hcTRs (Table 1). Therefore, based on two approaches (pyTanFinder and RepeatExplorer) we were able to identify two sets of TRs in the moss genome that have a high and low copy number.

FISH localization of tandem repeats in *P. patens*

We used FISH to determine whether the identified TRs occupy large clusters in the moss genome. A molecular cytogenetic approach to visualize DNA sequence loci on

Table 3. General information about identified tandem repeats used for FISH analysis.

Id	Monomer length, bp	Repeat Explorer cluster	Abundancy, bp	Sequence
Pp17_50	50	10	285023	GAACCTTCTAGAAGAGAAGTTTCTAGAACCTTC TAGAAAAGAAGCCTCTG
Pp21_215	215	309	156974	CACTTCCAGAGAGCATCGGCAATTTGAACTCTC TTGTGGAGTTGAATTTGTATAGATGTCGATCCT TGAAGGCACTTCCAGAGAGCATCGGCAATTTGA ACTCTCTTGTGGAGTTGAATTTGTATGGATGTC GATCCTTGAAGGCACTTCCAGAGAGCATCGGC AATTTGAACTCTCTTGTGAAGTTGAATTTGGTA GATGTCGATCCTTGAAGG
Pp602_86	86	2626	60915	AAGTGATGAACAAAATTTCTCATTTTGCCAAGT GATGAACAAAATTTCTCATTTGCCAAGTGATGAA CAAAATTTCTCATTTTGCC
Pp04_78	78	340	38748	CAACTTGCATTCTTCATTTTCATGCTCAACTTA CATTCTCTATTTCCATGCTCAACTTGCATTCTCT ATTTCCATGCT
Pp592_108	108	1758	34258	ATTTCTTAGAAAATACGTTCTAAATGCAAAGATA CAATTTCTTAGAAAATACGTTCTAAATGCAAAG ATACAATTTCTTAGAAAATACGTTCTAAATGCAA AGATACA
Pp20_76	76	226	22386	TCCCAGTCCCGTCGCGAGTCCCGGACTTCCTC CTCCTCTTCCTTGTCCCGCCGCGACTCCCTAG TCCCGGCGCGAG
Pp19_95	95	363	18717	ATAATTCTATCGGTTATGTTTAAGGTATTCAAGA TATTATCATATACCAATGAATGAATAATGTGCCAT TGCCCACCCAAATATTGGAGTTTACC
Pp05_92	92	209	13907	CCTCTAATAATAGTAAAAGTTATAGCAATAAATAA TAATTATCAGACTTCCAATAATAGTAAAATTTATA GCAATAAATAATAATTATCGGA
Pp21_43	43	1161	10324	CCTTGCCTTCACCTTCAAGTGGACCTTAGTAGA GAAGTTTTGT
Pp01_31	31	178	5381	AATCAGCTCGAGTCGAGCTGATTTGCTTCTC
Pp08_44	44	193	3978	AGCTGATGGCAGGTAAGGGAGATTGCATGAATC AGCTCGAGTCG
Pp02_27	27	118	3648	CTTCCGTCTTGGATCCGGAATTGGCTC
Pp08_217	217	227	3472	TTTCTTAAAGTAGATCTAAAAATAAAAGTTTTGT CAAAAAAGTAGGCTTTGCTAAGTGATGACTAGA AGTGATTTCTATGTTTGAAGATGCAAAGCTCCT CTTGTTTGTTGTTAAGAAGTATAATTTACTAAAA TAAGTTATTAAATAAACAGGAAAATCAAGACGTA AGATTCCTCACAAGATTTGGGATTTACTTCAGA AAACCAACAATTCAAG
Pp05_178	718	2110	2848	CACACGAAACTCACAACTTACTCCGCACAC AACTGATCGTCGACAACGTCGTAAAGCAAG GCAACATCAGTGACAACAACGGGGAATCCT ACAGTTTTGTGTCCACAACCTTCTCCTCAC AAGTGAGATGAGGAACCCATCCGATATCTT-TGTGAGGGAGTGATGATACCGGAGGAAT
Pp21_43	43	1161	2648	GTGGACCTTAGTGGAGAAGTTCTGCCCTTGCC TTCACCTTCAA
Pp18_62	62	13	2608	AGGGGAGTTTTCAAGTTTTTGCAAGGTTACTA GTTCGGTTTCATTGGAGGTTTTTGAAGATC
Pp10_116	116	115	1619	ATTGGAGAAGTATCATTGTAAAGCAAGACTATGG AGGTATAAAAAGGGAGGTACATTTACAAGATATA GATGCCTTTGATTTAAGTTTTATTAAAAAAAAAA AAAAAAAAAAAAA
Pp16_64	64	116	1572	GGGGTTTTTTGGATCGAAGAGCTAGCTTCAAG CTCTTTTCAAGGTCACTAGGTTGGTTTCATTA

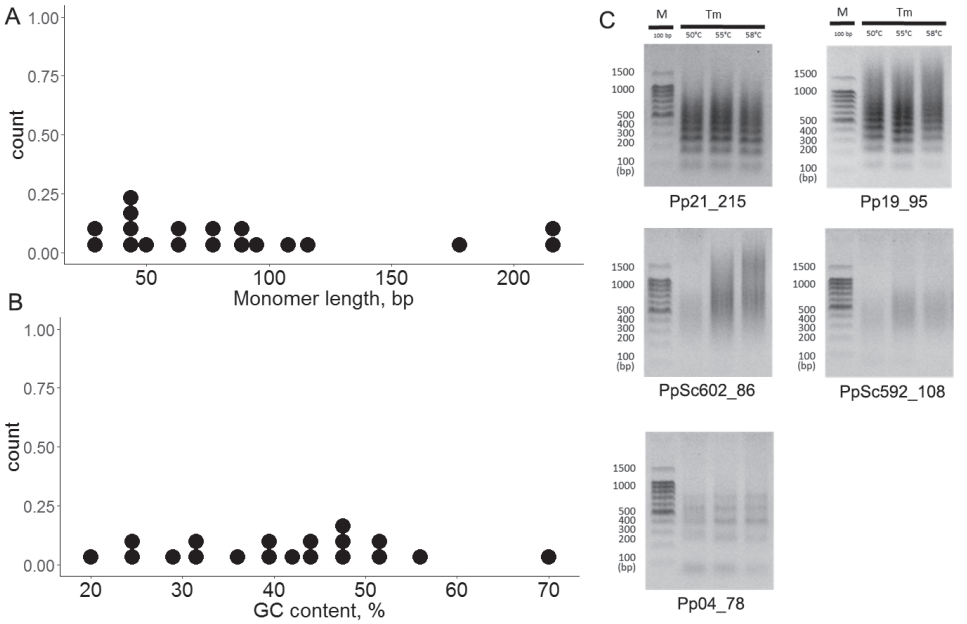

Figure 1. Features of 19 TRs. **A** Monomer length distribution **B** GC content distribution **C** Electrophoresis of PCR products from 5 TRs.

chromosomes and nuclei is challenging for bryophites (Rosato et al. 2016). To perform a pilot FISH experiment, we optimized the "SteamDrop" protocol (Kirov et al. 2014) for the preparation of the moss chromosome. Different types of material were used including protoplast, protonemata and unmatured sporophyte. No metaphase chromosomes were observed when protoplasts were used. The chromosome preparation from protonemata and unmatured sporophyte tissues resulted in a very low number of cells in the metaphase stage. Even the pretreatment of protonema tissue with different cytostatic chemicals (colchicine (3–4 h incubation in 0.05 – 0.3%), 1-bromnaphtalene (overnight incubation in saturated solution), and amiprofos-methyl (3–4 h incubation in 5 μM solution)) did not improve the results. The examples of anaphase, 1n (protonema, n=27) and 2n (sporophyte, 2n=54) metaphases as well as pachytene chromosomes after 4',6-diamidino-2-phenylindole (DAPI) staining are shown in Fig. 2.

We designed 19 TAMRA oligonucleotide probes to perform a nuclei-FISH assay. To validate that the obtained slides were suitable for FISH experiments, we used known tandemly organized sequences, Arabidopsis-type telomeric repeat ((TTTAGGG)n) and 45S rDNA, as positive controls. FISH experiments revealed many dot-like (Fig. 3A) and few distinct (Fig. 3B) signals for telomere and 45S rDNA probes, respectively, which suggested that the slides were suitable for FISH analysis in moss. We then performed nuclei-FISH experiments for 19 moss TRs. These experiments revealed 5 TRs for which FISH signals were detectable on the nuclei (Fig. 3).

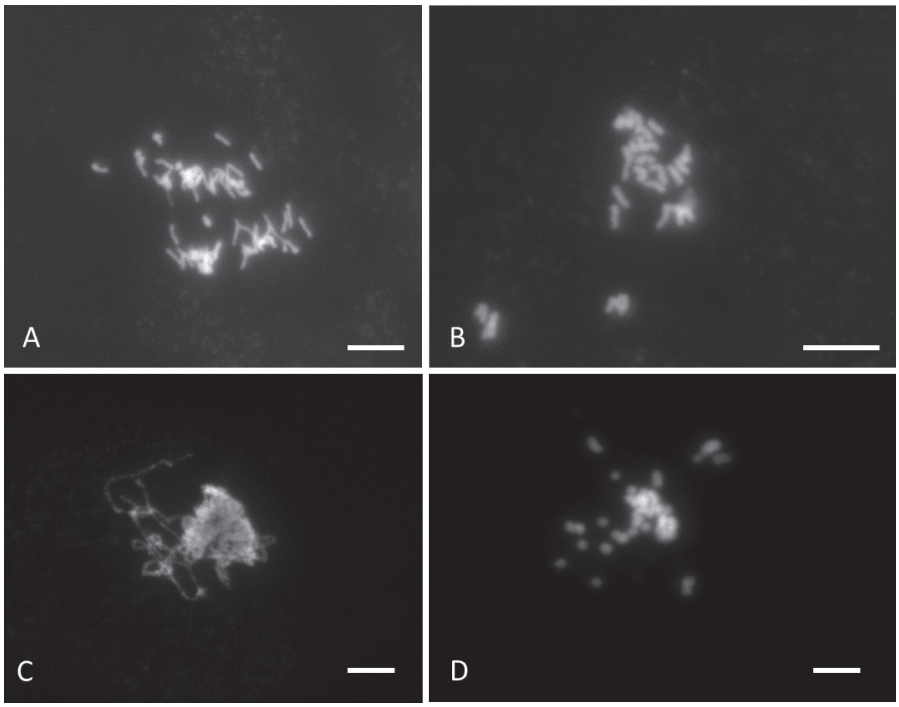

Figure 2. Mitotic and meiotic chromosomes of *P. patens* after DAPI staining. Anaphase (**A**), 1n ((**B**) protonema, n=27) and 2n ((**D**) sporophyte, 2n=54) metaphases and pachytene (**C**) stages. Scale bar: 5 μm.

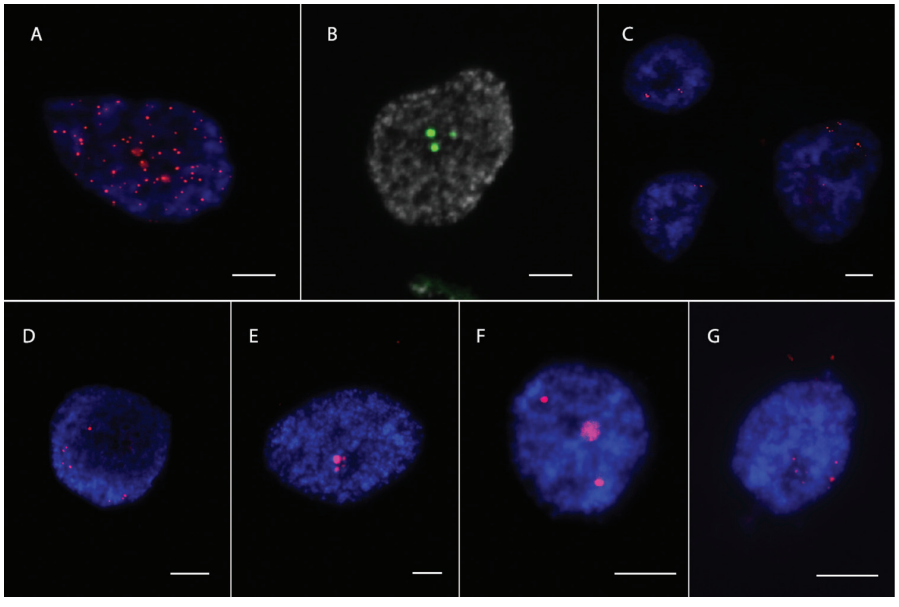

Figure 3. Results of FISH with labeled probes designed on Arabidopsis-type telomere repeat (**A**), 45S rDNA (**B**) and 5 identified TRs: Pp602_86 (**C**), Pp21_215 (**D**), Pp20_76 (**E**), Pp19_95 (**F**) and Pp592_108 (**G**).

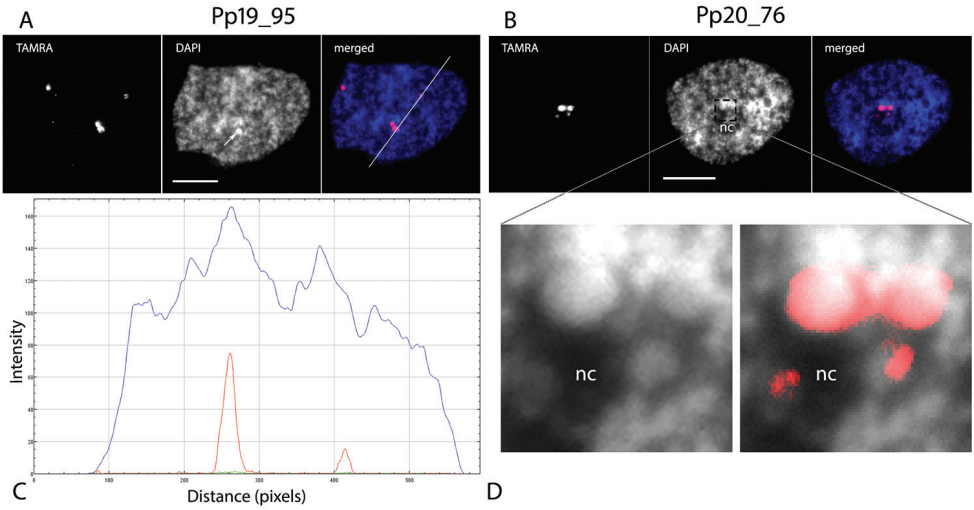

Figure 4. Nuclear organization of Pp19_95 (**A, C**) and Pp20_76 (**B, D**) TRs. **A** and **B** picture series shows fluorescence on DAPI and TAMRA channels and merged pictures **C** RGB profile of the nucleus; blue and red lines show the pixel intensity for two Pp19_95 FISH signals and DAPI staining, respectively **D** Digitally zoomed in part of the nucleus with red Pp20_76 FISH signals. nc marks the nucleolus. Scale bar: 5 μm.

Three repeats (Pp602_86, Pp21_215, Pp592_108) gave several signals that occupied two distinct territories in the nucleus. FISH signals from one TRs, Pp19_95 (95bp monomer size), were associated with heterochromatin regions of the nucleus (Fig. 4A, C) detected by DAPI. FISH signals from another TR, Pp20_76, were located at one nuclear region that was in close proximity to the nucleolus (perinucleolar region), which can be well-distinguished by DAPI staining (Fig. 4B). In contrast to Pp19_95 TR, the DAPI profile from Pp20_76 hybridization loci does not show any clear differences from neighboring nuclear regions. A closer look at the FISH signals shows that Pp20_76 loci are organized as a droplet-like structure (Fig. 4D).

Thus, nuclei FISH analysis of 19 TRs identified by pyTanFinder pipeline showed 5 TRs with pronounced signals. Moreover, one (Pp19_95) of the repeats was associated with heterochromatin structures while another one (Pp20_76) was associated with perinucleolar bodies. The 5 TRs were used for further analysis.

Location of the TRs in moss genome

To integrate our data with the *P. patens* genome sequence, we mapped 5 TRs back to the assembled *P. patens* genome sequence and estimated the genomic distribution of the TRs. Up to 45% (for Pp19_95) of BLAST hits belonged to the sequences that were not included in any assembled chromosomes (scaffolds), suggesting a challenge in the assembly of the genomic regions carrying the TRs (Fig. 5A). All BLAST hits were

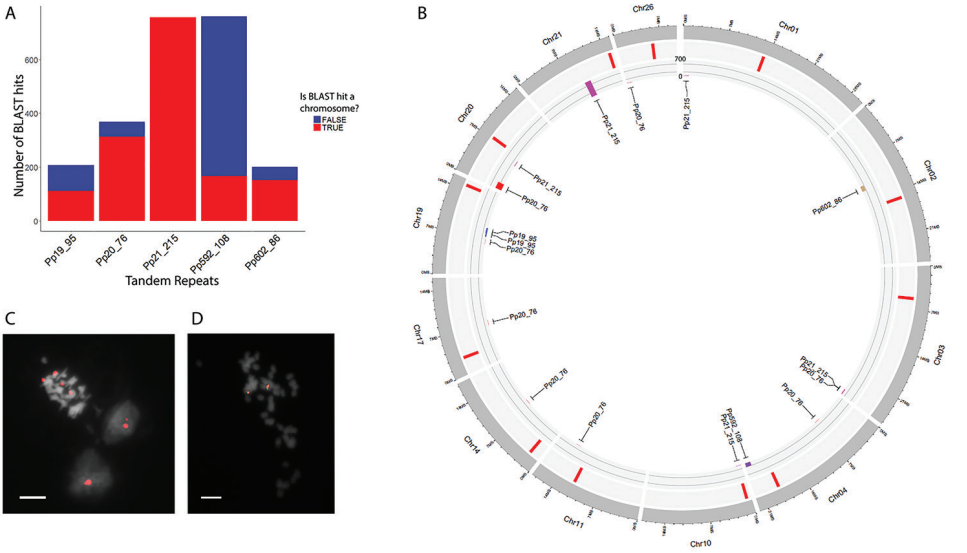

Figure 5. Chromosome location of 5 TRs. **A** Bar plot showing the number of BLAST hits derived from scaffolds and chromosome sequences **B** Circos plot: the inner layer corresponds to the bar plot showing the number of BLAST hits of the TRs on the chromosomes; FISH localization of Pp20_76 (**C**) and Pp602_86 (**D**). Scale bar: 5 μm.

distributed along 12 *P. patens* chromosomes. The Pp602_86, Pp21_215, Pp20_76, Pp19_95 and Pp592_108 TRs had 1, 5, 8, 2 and 1 loci in the assembled genome, respectively. Most of the identified loci contained only a few monomers; each of the repeats possessed a single locus with a high (up to 700) number of tandemly organized repeats including Pp21_215 (Chr21), Pp602_86 (Chr02), Pp592_108 (Chr01), Pp19_95 (Chr19) and Pp20_76 (Chr20). Two TRs, Pp21_215 and Pp20_76, had a bias toward distal parts of the chromosomes, with 60% (3) and 34% (3) loci located at the ends of the assembled chromosomes, respectively (Fig. 5B). A comparison of the putative centromere (RLC5 retrotransposon, Lang et al. 2018) and the TR locations revealed co-localization of 2 Pp21_215 (25%) loci on Chr10 and Chr20 with the RLC5-enriched regions, suggesting possible pericentromeric localization of this TR.

To further verify the results of nuclei-FISH and bioinformatics mapping, we performed FISH on moss chromosomes using two probes, Pp602_86 (single locus) and Pp20_76 (multiple loci). Although the chromosome preparation protocol needs to be further improved for *P. patens*, we were able to identify FISH signals from Pp20_76, located at the ends of two chromosome pairs, and from Pp602_86, located in the proximal positions of one chromosome pair (Fig. 5). FISH results for Pp60_86 correlated well with bioinformatics analysis which also showed a single locus on chromosome 2. In contrast, Pp20_76 has multiple loci in the moss genome assembly; two loci were revealed by FISH. One of the explanations of this discrepancy in bioinformatics and *in situ* experiments may be the limitation of FISH method sensitivity. The sensitivity of

FISH does not allow to physically map the DNA sequences if they occupy on the chromosomes less than 3–10 Kb (Valárik et al. 2004, Khrustaleva and Kik 2001). Therefore only the longest Pp20_76 array, located on Chr20, could potentially be visualized by this method. In addition, the FISH signals we observed were located at the end of the chromosomes, which is also in concordance with bioinformatics search. At the same time, a second FISH signal may be derived from Pp20_76 locus that was probably not well assembled. Therefore, the genomic mapping results together with FISH results provided evidence that the TRs that were detected occupied long clusters in the moss genome and allowed further integration of the TR location with the genomic context data available for *P. patens* (Lang et al. 2018).

Pp20_76 is located in actively transcribed chromatin

Because of the special location of Pp20_76 in the nucleus (near nucleolus) and the detected nucleus bodies enriched by this TR, we named this TR as PpNATR76(76 bp ***P. p****atens* peri**N**ucleolar **A**ssociated **T**andem **R**epeat) and analyzed it further. The alignment of 200 PpNATR76 sequences found in the moss genome showed a high conservation level between monomers. In addition, sequence analysis of the consensus PpNATR76 monomer revealed a long polypyrimidine track ($(CCT)_n$ motif). To determine why PpNATR76 DNA was located proximal to the nucleolus, we mapped the 45S rDNA to the moss genome. Using *A. thaliana* 45S rDNA gene (GenBank: X52320.1), we found two minor rDNA loci in the moss genome located on chromosomes 18 and 26 and one major rDNA locus on chromosome 20. The chromosomal location of 45S rDNA and PpNATR76 were identical on chromosomes 20 and 26, where they occupied c. 250Kb and 16Kb regions, respectively. Moreover, a detailed analysis of the loci revealed that PpNATR76 was located between 45S rDNA genes, in the IGS regions (Fig. 6A). Using the data available for moss, as a model organism, we checked the DNA and histone epigenetic landscape in the largest cluster on Chr20. We found a clear reduction in CG, CHG and CHH DNA methylation in the 45S rDNA/ PpNATR76 region (Fig. 6). In addition, the level of 'active' (H3K4me3, H3K9Ac, H3K27Ac) histone marks was significantly higher in this region compared with the flanking ones (Fig. 6). We also checked RNAseq data and found high level of RNAseq read coverage for this region, as expected for rDNA loci (Fig. 6).

PpNATR76 is transcribed into lncRNAs

Because of the transcription activity of the PpNATR7-occupying region, our next aim was to find *P. patens* transcripts possessing the PpNATR76 TR. This analysis revealed 16 transcripts whose genes were located on 5 chromosomes (Chr20, Chr19, Chr4, Chr17, Chr14). Only 4 of the transcripts possessed annotated canonical ORFs (Pp3c19_9270V3.1, p3c19_9271V3.1, Pp3c4_8299V3.1 and Pp3c14_12290V3.1). Pp3c14_12290V3.1 was the only transcript that had ORF with homology to known

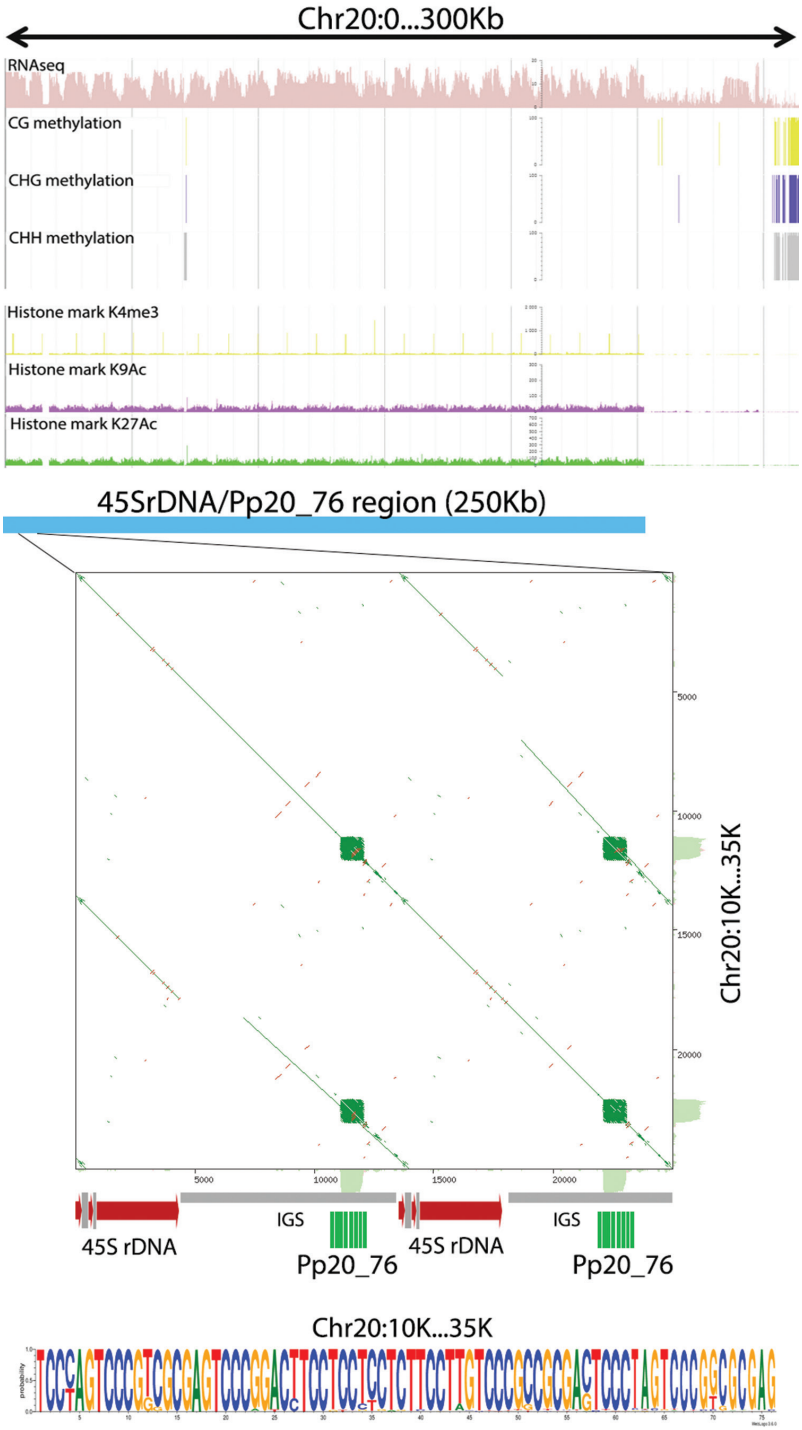

Figure 6. Genomic organization and epigenetic landscape of 45SrDNA/PpNATR76 locus. Top panel is a snapshot of CoGe GBrowser for *P. patens* (https://genomevolution.org/). Logo picture from multiple alignment of 200 PpNATR76 monomers is shown at the bottom.

proteins and was annotated as NADH:ubiquinone reductase, whereas predicted proteins from other PpNATR76 possessing transcripts did not show any homology to known proteins. These data suggested that the PpNATR76 transcripts mostly belonged to lncRNAs. To assess the robustness of the results, we performed a quantitative RT-PCR (qRT-PCR) validation of 5 PpNATR76 transcript genes (Pp3c20_303V3.1, Pp3c19_9271V3.1, Pp3c20_283V3.1, Pp3c14_12290V3.2, Pp3c4_8299V3.1) using protonemata RNA samples. For this experiment, DNA was taken as a positive control, whereas extracted RNA and MQ were negative controls. We then calculated the difference between the Cq values of pure RNA (DNA contamination control) and cDNA specific amplification. The results of qRT-PCR showed that all transcripts were expressed on detectable levels of > 5 delta. In addition, for 3 out of 5 genes, sense as well as antisense transcriptions were observed, whereas for two genes (Pp3c20_283, Pp3c14_12290) only one-way directed transcription was detected. Collectively this data proved the existence of the pPNATR76 transcripts in somatic cells and strongly suggested that PpNATR76 was transcribed as part of both protein coding and lncRNAs.

Discussion

TRs with different monomer sizes are integral parts of most eukaryotic organisms, in which they are involved in diverse biological processes. Although many efforts have been made to understand the genomic organization, structure and evolution of TRs, their functions in a cell are still poorly understood. Here, we performed a pioneering identification and FISH verification of satellite repeats, forming a long array in the genome of the model plant, *P. patens*. We developed a pipeline, pyTanFinder, and identified 19 TRs, of which 5 TRs produced FISH signals. We found both heterochromatin associated and transcribed TRs. Genomic and transcriptomic analyses identified IGS-associated moss TR, PpNATR76, which was sequestered in the perinucleolar space and transcribed as a part of lncRNAs.

pyTanFinder pipeline identified heterochromatin located satellite DNA sequences in moss

Advances in genome sequencing and bioinformatics approaches in the last decades has triggered the progress in satellite repeat isolation (reviewed by (Lower et al. 2018)). We explored the satellitome of the model plant, *P. patens*, using our pyTanFinder pipeline and repeat library generated by RepeatExplorer (Novák et al. 2013). Although a large number of TR identification tools have been developed (reviewed by (Lower et al. 2018), the pyTanFinder pipeline can be very useful if the available full genome sequence is highly fragmented. It is very common for satellite repeats to collapse during genome assembly (Saint-Oyant et al. 2018). Therefore, the identification of a TR in a single locus produced by some tools may lead to some spurious results. This limitation

is overcome in the pyTanFinder pipeline by clustering of similar TRs identified across all chromosome and scaffold sequences followed by calculation of the TR abundance based on all sequences in a cluster. This approach also makes it possible for pyTanFinder to be applied for the identification of satellite repeats in long-read single molecule real time genome sequencing data generated by modern PacBio and Oxford NanoPore platforms. Our preliminary results obtained on PacBio data of *Aegilops taushii* Coss., 1850 (SRA archive at NCBI: SRX3098055) supports this suggestion (data not shown). The pioneering satellite DNA identification and its FISH mapping in the moss nucleus performed in this study resulted in a set of cytogenetic markers that can be useful for future genomic and cytogenetic data integration. As shown in many other plants, the integration of chromosomal and sequence data may help to shed more light on genome evolution and to correct genome assembly ((Fransz et al. 2016, Kirov et al. 2015, Saint-Oyant et al. 2018, Shearer et al. 2014)). Molecular cytogenetic techniques, such as FISH, have never been applied to mosses; therefore, the chromosome preparation and FISH mapping procedures described in this study are important for further improvement of the *P. patens* genome assembly and annotation. Interestingly, recent (Lang et al. 2018) as well as earlier works (Melters et al. 2013) have shown low TR abundancy in the genomes of basal plants. In concordance with this observation, Lang et al. (2018) also observed a lack of clear heterochromatin regions on nuclei that typically contain TRs. Although we also did not observe large heterochromatin blocks, our slide preparation procedure allowed us to identify some small heterochromatin blocks in the moss nucleus (Figs 3, 4). In addition, the pyTanFinder pipeline allowed us to isolate at least one TR Pp19_95, which was enriched in the identified heterochromatin regions. Moreover, this repeat exhibits strong DNA methylation compared with that of the neighboring regions, which also suggested that it was located in the heterochromatin. It would be interesting to check in the future whether the heterochromatin organization is similar between basal plants and angiosperms.

Intergenic 45S rDNA spacer is a source of satellite non-coding transcripts: a principle that is conserved from first land plants to human

We found one IGS-related satellite repeat, named PpNATR76, that had several distinguishable features at the genome and transcriptome levels: 1) its DNA occupied distinct perinucleolar-associated chromatin bodies and most of its copies were located in IGS 45S rDNA spacer; 2) its DNA was hypomethylated and associated histones were enriched in 'active' chromatin marks and 3) it was transcribed into lncRNAs. The number (four signals for diploid nucleus used in this study) of PpNATR76 FISH signals was in agreement with previously observed 1–2 rDNA loci in moss and other bryophytes (Berrie 1958a, b, Rosato et al. 2016, Sone et al. 1999). As this TR was a part of the IGS region and its FISH signals on the nucleus (Fig 4B, D) were identical to 45S rDNA (Fig. 3B), we supposed that the observed PpNATR76 perinucleolar bodies were knob-like rDNA chromatin. From a first glance, this was not congruent with 'active' histone

marks and the almost absence of DNA methylation in the 45S rDNA/IGS/PpNATR76 region because the knob structure consisted of heterochromatin. However, condensed knobs and decondensed transcriptionally active rRNA genes are interspersed in one NOR region (Pontes et al. 2003). Indeed, we also found high concentration of 'inactive' chromatin marks in this region of the *P. patens* genome (H3K9me2, H3K27me3, data not shown). Because of the identity of 'active' and 'inactive' 45S rDNA sequences, the bioinformatics mapping of Chip-seq reads to the genome is not able to distinguish them and leads to erroneous results when 'active' and 'inactive' chromatin marks co-occurred. Therefore, PpNATR76 TR is a part of both knob-like ('inactive', visualized by FISH) and transcriptionally active (invisible by FISH because of the low local nuclear density of labeled loci and limited FISH sensitivity) chromatin.

Satellite DNA repeats frequently originate in plant IGS DNA and have similar organization between closely related species (Almeida et al. 2012, Falquet et al. 1997, Jo et al. 2009, Lim et al. 2004). However, the PpNATR76 length (76bp) was much shorter than the previously described IGS-associated TRs (>170 bp). IGS-associated short TRs (STR) with a monomer length range from 2 to 12 have also been described in humans (Goodwin and Swanson 2014, Yap et al. 2018). Interestingly, we showed the existence of PpNATR76 containing lncRNAs in moss cell. Recently, Yap et al. (2018) also found multiple STR-enriched lncRNAs (PNCTR) in human cell. In addition, PpNATR76 lncRNAs possess poly-pyrimidine (purine) track, which was also identified in PNCTR RNAs, where it is recognized by pyrimidine tract-binding protein (PTBP1)-specific motifs, allowing it to sequester a significant fraction of PTBP1 in the perinucleolar compartment. Poly-purine stretches were also found in another rDNA IGS-related lncRNA, PAPAS (Bierhoff H et a., 2017, Zhao et al. 2018), in which this motif is involved in forming a DNA-RNA triplex that tethers this lncRNAs to the enhancer region of rRNA genes. The described features make genomic and transcriptomic organization of moss PpNATR76 lncRNAs and human IGS related lncRNAs quite similar. Although future studies of PpNATR76 lncRNAs are required, it can be speculated that the transcription of functionally important satellite-possessing lncRNAs from the IGS region is a conserved principle between plants and humans. Because of the activity of rDNA loci, IGS-related TRs have exceptional location in the genome that promotes their transcription, resulting in the origin of novel classes of lncRNAs. This remarkable feature distinguishes this type of TR from heterochromatin-associated TRs. Our results pose a number of questions about the possible function of PpNATR76 lncRNAs as well as the existence of similar IGS-related lncRNAs in other basal species and angiosperms.

Conclusions

In this study we extended the list of model plant species for TR studies with a well-known model "basal" plant, *P. patens,* and provided a set of new FISH-verified TRs for further functional and evolutionary analysis in moss. We described a new pipeline pyTanFinder for the identification of TR in fragmented genome sequences and demon-

strated the conservation principle of IGS-related TR lncRNA expression between human and early diverged land plants. The results of our work will accelerate further studies of TR biology and function in a plant cell using the model "basal" plant *P. patens*.

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DATA PAPER

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New data on karyotypes of lace bugs (Tingidae, Cimicomorpha, Hemiptera) with analysis of the 18S rDNA clusters distribution

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Abstract

The karyotypes of 10 species from 9 genera of the family Tingidae (Hemiptera, Heteroptera, Cimicomorpha) are described and illustrated for the first time. These species are: *Agramma atricapillum* (Spinola, 1837), *Catoplatus carthusianus* (Goeze, 1778), *Dictyla platyoma* (Fieber, 1861), *Lasiacantha hermani* Vásárhelyi, 1977, *Oncochila simplex* (Herrich-Schaeffer, 1830), *Tingis* (*Neolasiotropis*) *pilosa* Hummel, 1825, and *T.* (*Tropidocheila*) *reticulata* Herrich-Schaeffer, 1835, all with 2n = 12A + XY, as well as *Acalypta marginata* (Wolff, 1804), *Derephysia* (*Paraderephysia*) *longispina* Golub, 1974, and *Dictyonota strichnocera* Fieber, 1844, all with 2n = 12A + X(0). Moreover, genera *Catoplatus* Spinola, 1837, *Derephysia* Spinola, 1837, and *Oncochila* (Herrich-Schaeffer, 1830) were explored cytogenetically for the first time. Much as all other hitherto studied lace bugs, the species studied here have 12 autosomes but differ in their sex chromosome systems. The ribosomal clusters were localized on male meiotic cells of all ten species already mentioned and, additionally, in *Acalypta carinata* Panzer, 1806 known to have 2n = 12A + X (Grozeva and Nokkala 2001) by fluorescence *in situ* hybridization (FISH) using a PCR amplified 18S rDNA fragment as a probe. In all cases, rDNA loci were located interstitially on a pair of autosomes. Furthermore, two species possessed some additional rDNA clusters. Thus, *Acalypta marginata* showed clearly defined interstitial clusters on one more pair of autosomes, whereas *Derephysia longispina* had a terminal cluster on the X-chromosome. FISH performed with the telomeric $(TTAGG)_n$ probe

did not reveal labelling in chromosomes of any species studied. Hence, the results obtained provide additional evidence for the karyotype conservatism, at least regarding the number of autosomes, for variation in chromosomal distribution of rDNA loci between species and for the lack of the ancestral insect telomeric sequence TTAGG in lace bugs. Preliminary taxonomic comments are made basing on some cytogenetic evidence.

Keywords
Karyotype, chromosome number, sex chromosomes, FISH, rDNA, $(TTAGG)_n$, lace bugs, Tingidae, Heteroptera

Introduction

Tingidae (lace bugs) are a relatively large family belonging to one of the evolutionarily advanced true bug infraorders Cimicomorpha. The family comprises approximately 2600 species and more than 270 genera in the two currently recognized subfamilies, the Tinginae and the Cantacaderinae (Golub and Popov 2016). The currently available cytogenetic evidence is confined to the largest and most diverse subfamily Tinginae (Grozeva and Nokkala 2001, Golub et al. 2015, 2016, 2017, for other references see Ueshima 1979). Based on the present knowledge, the subfamily exhibits karyotype conservatism, at least in terms of the number of autosomes which is 12 in all hitherto studied species. On the other hand, the species can differ in sex chromosome systems which are of either an XY or an X(0) type, the former being clearly more characteristic of lace bugs. By now, 38 species from 18 genera have been karyotyped and the great majority of these species, 34 in 16 genera, were shown to have 2n = 14 (12A + XY) in males.

In recent years, cytogenetic studies with the use of fluorescence *in situ* hybridization (FISH) have advanced our understanding the karyotype structure of lace bugs (Golub et al. 2015, 2016, 2017). It became evident that, despite very similar karyotypes, these insects show significant interspecific differences in the major rDNA loci distribution. The 18S rDNA sites can appear either on sex chromosomes or on autosomes being in turn located either interstitially or terminally on a chromosome. Likewise, our studies suggest that lace bugs lack the insect-type telomeric sequence TTAGG (Golub et al. 2015, 2017).

To further explore the karyotype structure and evolution in lace bugs, we examined distribution of the rRNA gene loci in eleven additional species including *Acalypta carinata* (Panzer, 1806), *A. marginata* (Wolff, 1804), *Agramma atricapillum* (Spinola, 1837), *Catoplatus carthusianus* (Goeze, 1778), *Derephysia* (*Paraderephysia*) *longispina* Golub, 1974, *Dictyonota strichnocera* Fieber, 1844, *Dictyla platyoma* (Fieber, 1861), *Lasiacantha hermani* Vásárhelyi, 1977, *Oncochila simplex* (Herrich-Schaeffer, 1830), *Tingis* (*Neolasiotropis*) *pilosa* Hummel, 1825, and *T.* (*Tropidocheila*) *reticulata* Herrich-Schaeffer, 1835. In each species, we mapped the insect-type telomere motif $(TTAGG)_n$. All species (besides *A. carinata*) as well as the genera *Catoplatus* Spinola, 1837, *Derephysia* Spinola, 1837, and *Oncochila* Stål, 1873 were studied here for the first time in terms of standard chromosome complement.

Table 1. Material used for chromosome analysis.

Species	Data and place of collection	Number of males examined	Number of nuclei studied by	
			routine staining	FISH
1. *Acalypta carinata*	30.04.2017, Voronezh Province, Russia	1	23	12
2. *Acalypta marginata*	30.4 – 05.05.2017, Voronezh Province, Russia	12	28	24
3. *Agramma atricapillum*	01.06.2017, Bogdinsko-Baskunchakski Nature Reserve, Astrakhan Province, Russia	2	–	17
4. *Catoplatus carthusianus*	31.07.2017, Voronezh Province, Russia	20	65	47
5. *Derephysia* (*Paraderephysia*) *longispina*	7.06.2017, Voronezh Province, Russia	22	31	45
6. *Dictyla platyoma*	29 – 31.05.2017, Bogdinsko-Baskunchakski Nature Reserve, Astrakhan Province, Russia	2	–	14
7. *Dictyonota strichnocera*	20.06 – 01.07.2017, Voronezh Province, Russia	3	38	24
8. *Lasiacantha hermani*	2.06 – 16.06.2017, Voronezh Province, Russia	2	22	11
9. *Oncochila simplex*	22.06 – 03.07.2017, Voronezh Province, Russia 27.07.2017, Lipetsk Province, Russia	7	32	23
10. *Tingis* (*Tropidocheila*) *reticulata*	20.06 – 4.07.2017, Voronezh Province, Russia	20	–	31
11. *Tingis* (*Neolasiotropis*) *pilosa*	8.06 – 25.06.2017 Voronezh Province, Russia	10	–	22

Material and methods

Specimens of 11 lace bug species from 9 genera were sampled from the Voronezh and Astrakhan provinces of Russia (Table 1). Species identification was made by V. Golub. Only male specimens were used. Males were fixed in 3:1 fixative (96% ethanol: glacial acetic acid) and stored at 4 °C. Chromosomal preparations were obtained from the testes and made permanent using a dry ice quick-freezing technique. For standard karyotype analysis, a Feulgen-Giemsa method developed by Grozeva and Nokkala (1996) was used. FISH with 18S rDNA- and $(TTAGG)_n$-telomeric probes was carried out according to Grozeva et al. (2010). In brief, the probes were simultaneously used in double FISH experiments. Telomeric sequences and 18S rDNA probes were labelled by PCR with Rhodamine-5-dUTP (GeneCraft, Köln, Germany) and Biotin-16-dUTP, respectively. The probe for 18S rDNA was detected by NeutrAvidin fluorescein conjugate (Invitrogen, Karlsbad, CA, USA). Chromosomes were counterstained with DAPI (Sigma-Aldrich). As a positive control for the efficacy of our $(TTAGG)_n$ FISH experiments, we used chromosome preparations from the jumping plant bug species (Hemiptera, Psylloidea) known to be $(TTAGG)_n$ – positive (Maryańska-Nadachowska et al. 2018).

Chromosome slides were analyzed under a Leica DM 6000 B microscope (Leica Microsystems Wetzlar GmbH, Germany) with a 100× objective. Images were taken with a Leica DFC 345 FX camera using Leica Application Suite 3.7 software with an Image Overlay module.

All cytogenetic preparations and remains of the specimens from which the preparations were made are stored at the Zoological Institute of RAS, St. Petersburg.

Results

Acalypta carinata

2n = 12A + X (Fig. 1a – FISH)

This species was previously karyotyped by Grozeva and Nokkala (2001), and our observations corroborate with their data. At spermatocyte metaphase I (MI), six bivalents of autosomes and a univalent X-chromosome are present (Fig. 1a: n = 6AA + X). Bivalents are more or less close in size, and the X is about half the size of the bivalents.

Numerous 18S rDNA FISH signals are located interstitially on both homologues of one of the autosome pairs. FISH with the pentamer $(TTAGG)_n$ as the probe did not label the telomeres in chromosomes of *A. carinata*.

Acalypta marginata

2n = 12A + X (Fig. 1b – standard staining; Fig. 1c – FISH)

At spermatocyte MI, six bivalents of autosomes and a univalent X-chromosome are present (Fig. 1b: n = 6AA + X). Bivalents are of similar size, and the X is about half the size of the bivalents.

During late prophase, 18S rDNA FISH signals are visible on several bivalents being numerous and most pronounced on two of them (Fig. 1c). FISH with $(TTAGG)_n$ as the probe did not label the telomeres in chromosomes of *A. marginata*.

Agramma atricapillum

2n = 12A + XY (Fig. 1d – FISH).

At early MI, six bivalents of autosomes and X and Y chromosomes as univalents are present (Fig. 1d: n = 6AA + X + Y). Bivalents are of similar size. Sex chromosomes are approximately similar in size and placed separately from each other at this stage – that is characteristic of the true bugs (Ueshima 1979).

18S rDNA FISH signals are located interstitially on one of the bivalents being clearer defined on one of its homologues. FISH with $(TTAGG)_n$ as the probe did not label the telomeres in chromosomes of *A. atricapillum.*

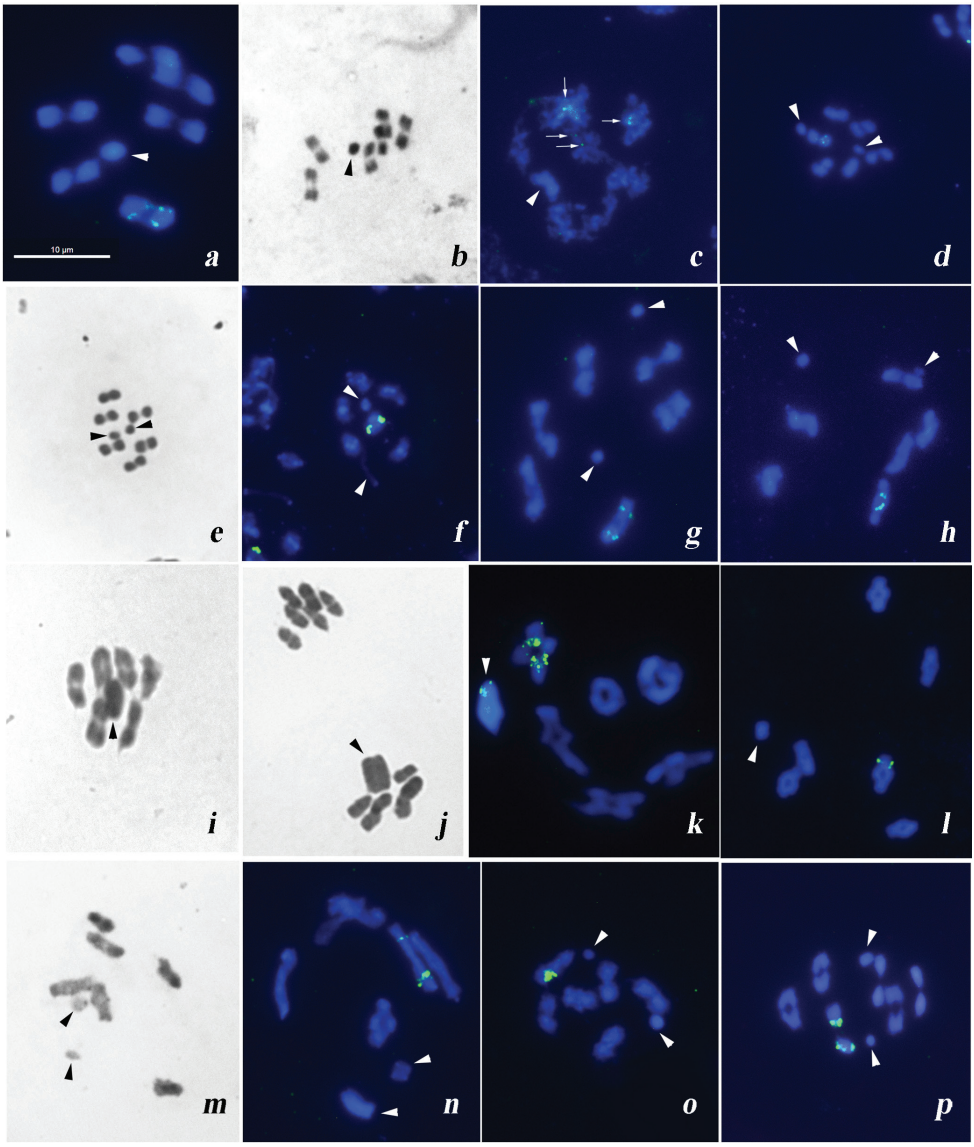

Figure 1. Male meiotic chromosomes of lace bug species after standard Schiff-Giemsa staining (**b, e, i, j, m**) and FISH with 18S rDNA and $(TTAGG)_n$ telomeric probes (**a, c, d, f-h, k, l, n–p**). **a** *Acalypta carinata* metaphase I (MI) **b, c** *Acalypta marginata* MI (**b**) late prophase (**c**); 18S rDNA FISH signals on several bivalents are indicated by arrows **d** *Agramma atricapillum* MI **e, f** *Catoplatus carthusianus* MI (**e**) late prophase (**f**) **g** *Lasiacantha hermani* prophase I/MI transition **h** *Dictyla platyoma* prophase I/MI transition **i, g, k** *Derephysia* (*Paraderephysia*) *longispina* MI (**i**) anaphase I (**j**) diakinesis (**k**) **l** *Dictyonota strichnocera* prometaphase I **m, n** *Oncochila simplex* prophase I to MI transition **o** *Tingis* (*Tropidocheila*) *reticulata* prometaphase I **p** *Tingis* (*Neolasiotropis*) *pilosa*. Sex chromosomes are indicated by arrowheads. Scale bar: 10 µm.

Catoplatus carthusianus

2n = 12A + XY (Fig. 1e – standard staining; Fig. 1f – FISH).

At MI subjected to a routine staining as well as in a late prophase cell after FISH six bivalents of autosomes and univalent X and Y chromosomes are present (Fig. 1e, f: n = 6AA + X + Y). Bivalents are of similar size. Sex chromosomes are approximately similar in size and form a pseudo-bivalent at MI.

18S rDNA FISH revealed massive signals on one of the autosome pairs (Fig. f). FISH with $(TTAGG)_n$ as the probe did not label the telomeres in chromosomes of *C. carthusianus*.

Lasiacantha hermani

2n = 12A + XY (Fig. 1g – FISH)

During the prophase I to MI transition, six bivalents of autosomes and univalent X and Y chromosomes are revealed (n = 6AA + X + Y). Bivalents are of similar size. Sex chromosomes are similar in size and placed separately from each other at this stage.

Bright 18S rDNA FISH signals are located interstitially on both homologues of one of the bivalents. FISH with $(TTAGG)_n$ as the probe did not label the telomeres in chromosomes of *L. hermani*.

Dictyla platyoma

2n = 12A + XY (Fig. 1h – FISH)

During the prophase I to MI transition, six bivalents of autosomes and univalent X and Y chromosomes are revealed (n = 6AA + X + Y). Bivalents are of similar size. Sex chromosomes are similar in size and placed separately from each other at this stage.

Bright 18S rDNA FISH signals are located interstitially on both homologues of one of the bivalents. FISH with $(TTAGG)_n$ as the probe did not label the telomeres in chromosomes of *D. platyoma*.

Derephysia (*Paraderephysia*) *longispina*

2n = 12A + X (Fig. 1i, j – standard staining; Fig. 1k – FISH)

At MI, six bivalents of autosomes and a univalent X-chromosome are present (Fig. 1i: 6AA + X). Bivalents are very large and of similar size. The X is the largest element of the set and appears positively heteropycnotic at this stage. It goes to one of the daughter nuclei (pre-reduction) at anaphase I (AI), resulting in different MII cells, respectively, that with 6 autosomes only and that with 6 autosomes plus X-chromosome, the latter being split into the chromatids (Fig. 1j).

Figure 1k shows a diakinesis after FISH with 18S rDNA probe demonstrating the presence of multiple signals on one of the bivalents as well as on the X. These FISH signals are interstitial on the bivalent while telomeric on the X. FISH with $(TTAGG)_n$ as the probe did not label the telomeres in chromosomes of *D. longispina.*

Dictyonota strichnocera

2n = 12A + X (Fig. 1l – FISH)

The prometaphase I shows six bivalents of autosomes and a univalent X-chromosome (n = 6AA + X). Bivalents are of similar size, while the X is about half the size of the bivalents.

Bright 18S rDNA FISH signals are located interstitially on one of the bivalents, being however visible on one homologue only. FISH with $(TTAGG)_n$ as the probe did not label the telomeres in chromosomes of *D. strichnocera.*

Oncochila simplex

2n = 12A + XY (Fig. 1m – standard staining; Fig. 1n – FISH)

During the prophase I to MI transition, six bivalents of autosomes and univalent X and Y chromosomes placed separately from each other are revealed (Fig. 1m, n: n = 6AA + X + Y). Bivalents are approximately similar in size, and the X is twice as large as the Y.

Signals of the 18S rDNA probe are located interstitially on both homologues of one of the bivalents being more massive and bright on one of them (Fig. 1n). FISH with $(TTAGG)_n$ as the probe did not label the telomeres in chromosomes of *O. simplex*.

Tingis* (*Tropidocheila*) *reticulata

2n = 12A + XY (Fig. 1o – FISH)

Prometaphase I shows six bivalents of autosomes and X and Y chromosomes which are placed separately from each other at this stage. Bivalents are of similar size, and the X is twice as large as the Y (Fig. 1o).

Massive 18S rDNA FISH signals are located interstitially on one of the bivalents. FISH with $(TTAGG)_n$ as the probe did not label the telomeres in chromosomes of *T. reticulata*

Tingis* (*Neolasiotropis*) *pilosa

2n = 12A + XY (Fig. 1p – FISH)

During the MI to AI transition, six bivalents of autosomes and a pseudo-bivalent formed by X and Y chromosomes are revealed (n = 6AA + XY). At this stage, bivalents appear as similar in size, while X -chromosome is twice as large as the Y (Fig. 1p).

One of the bivalents shows bright 18S rDNA signals, the signals locating most likely interstitially as seen on one homologue of this bivalent at least. FISH with $(TTAGG)_n$ as the probe did not label the telomeres in chromosomes of *T. pilosa*.

Discussion

Chromosome numbers and sex chromosome systems

For the first time, we studied the standard karyotypes of 10 lace bug species belonging to 9 genera of the subfamily Tinginae. Our data on chromosome numbers and sex chromosome systems of these species reinforce the statement (Ueshima 1979, Grozeva and Nokkala 2001, Golub et al. 2015, 2016, 2017) that lace bugs exhibit extraordinary stability of karyotypes in terms of the number of autosomes. Much as all previously studied species (38 species, 18 genera), all the species explored in the present study showed 12 autosomes in their diploid karyotypes suggesting thus that this number is under stabilizing natural selection. On the other hand, these species, despite the same autosome number, differ by sex chromosome systems which are of an X(0) type in 3 species (in genera *Derephysia, Acalypta* Westwood, 1840, and *Dictyonota* Curtis, 1827) and of an XY type in 8 species (in genera *Agramma* Stephens, 1829, *Catoplatus*, *Dictyla* Stål, 1874, *Lasiacantha* Stål, 1873, *Oncochila*, *Tingis* Fabricius, 1803) respectively. The predominance of the XY-system is typical for the family Tingidae as a whole, being found in 41 of the 48 hitherto studied species. Since more than 70% of the cytogenetically studied species of Heteroptera have the XY system and only about 14% possess the X(0) system, the former system is considered typical for this suborder as a whole (Papeschi and Bressa 2006).

In summary, based on the currently available evidence, the karyotype of 2n = 12A + XY/XX (male/female) can be taken as the modal one for the family Tingidae, at least for the subfamily Tinginae. Moreover, we like to suggest that the XY system is the ancestral one in lace bugs and the X(0) is secondary resulting from the loss of the Y chromosome (see also Nokkala and Nokkala 1984).

The distribution of the sex chromosome systems in Tingidae seems to allow some preliminary taxonomic speculations. All the seven X(0)- lace bug species belong to the phylogenetically close genera *Acalypta* (*A. parvula* Fallén, 1897, *A. carinata*, *A. nigrina* Fallén, 1897, *A. marginata*; Grozeva and Nokkala 2001, present paper), *Derephysia* (*D. longispina*; present paper), *Kalama* Puton, 1876 (*K. tricornis* Schrank, 1801; Grozeva and Nokkala 2001), and *Dictyonota* (*D. strichnocera*; present paper). On the other hand, according to Southwood and Leston (1959), *Acalypta parvula* and another species of *Dictyonota* (*D. fuliginosa* Costa, 1853) both originating from British Islands have an XY system. However, neither illustrations nor descriptions of the karyotypes

were provided in the above-mentioned publication, so the credibility of these data is questionable. It is of interest that all the above genera share, besides common X(0) system, some morphological similarities, including the absence of the cuticular frame (peritrema) of the metatoracic scent glands in adults and bucculae not closed anteriorly (Horváth 1906, Kerzhner and Jaczewski 1964, Péricart 1983). Thus, these cytogenetic and morphological characters can be considered as synapomorphies for the genera *Acalypta*, *Derephysia*, *Kalama*, and *Dictyonota*. Furthermore, these genera have almost exclusively Holarctic distribution (Drake and Ruhoff 1965, Golub 1975, Péricart 1983, Péricart and Golub 1996, Froeschner 2001).

Karyotype structure

In the tingid karyotypes, autosomes are more or less close in size or, most probably, form gradually decreasing series in size (Grozeva and Nokkala 2001, Golub et al. 2015, 2016, 2017) and this is also true for the species used in the present study. Because of the uniform chromosome size and, additionally, of the holokinetic nature of chromosomes, it is almost impossible to identify separate chromosome pairs in a given karyotype when standard chromosome staining techniques are applied. Moreover, C-banding appeared to be not very helpful for the identification due to scarce and uniform C-patterns of the chromosomes although various species show some differences in the C-banding picture (Grozeva and Nokkala 2001). Sex chromosomes, both X and Y, are always small, smaller than any of autosomes of the set. The only so far known exception is *Derephysia longispina* from the present study. The karyotype of this species is unique in having rather large chromosomes, the X-chromosome being at least twice as large as any autosome. The observed differences may be of taxonomic significance. It would be of interest to compare the genome size in lace bug species with different chromosomal length. Furthermore, in *D. longispina* we were able to observe that the X-chromosome separated reductionally during first meiotic division (pre-reduction). The orthodox sex chromosomes pre-reduction seems to be characteristic of the Tingidae as a whole (Ueshima 1979, Grozeva and Nokkala 2001, present study). Interestingly, pre-reduction distinguishes lace bugs from all other Cimicomorpha families, for which sex chromosome post-reduction, i.e. the inverted sequence of sex chromosome divisions in male meiosis, is typical (Ueshima 1979).

rDNA-FISH

All 11 species studied here by FISH with 18S rDNA probes showed major rRNA gene clusters on an autosome pair. Unfortunately, based on the present data we cannot conclude whether these species share a syntenic location of their rDNA arrays since the chromosome pairs are of similar size and morphology within karyotypes. In one species, *Derephysia longispina*, an additional rDNA site was revealed on the X-

chromosome. Furthermore, *Acalypta marginata* displayed several rDNA loci housed on two pairs of autosomes, at least. These two species represent two novel patterns of rDNA distribution in lace bugs. Thus, the following patterns are currently known in Tingidae: on the X-chromosome, on both X and Y chromosomes, on one or two pairs of autosomes, and both on the X and one pair of autosomes. A wide variety of rDNA location between species sharing the same chromosome number has also been reported in some other Cimicomorpha families (Severi-Aguiar and de Azeredo-Oliveira 2005, Morielle-Souza and Azeredo-Oliveira 2007, Bardella et al. 2010, Grozeva et al. 2011, 2014, Poggio et al. 2011, Pita et al. 2013, Panzera et al. 2012, 2014, 2015).

Noteworthy is an interstitial location of the rDNA sites discovered in all lace bug species from the present study, at least in terms of autosomal location. Such is the case in the majority of lace bugs studied so far (Golub et al. 2016, 2017) suggesting this localization to be most characteristic of Tingidae. On the other hand, a terminal rDNA location has frequently been reported in other families of Cimicomorpha, e.g. Reduviidae and Cimicidae (Poggio et al. 2011, 2014, Panzera et al. 2012, Bardella et al. 2014, Grozeva et al. 2010, 2014). It is worth noting however that evidence was usually based on MI plates which characteristically show highly condensed chromosomes and may thus result in a misinterpretation.

$(TTAGG)_n$-FISH

Like all previously studied lace bug species in the genera *Agramma*, *Catoplatus*, *Dictyla*, *Elasmotropis* Stål, 1874, *Galeatus* Curtis, 1833, and *Tingis* (Golub et al. 2015, 2017), all species used in the present study representing 4 further genera, namely, *Acalypta*, *Dictyonota*, *Lasiacantha*, and *Oncochila*, showed no labelling with the pentameric repeat $(TTAGG)_n$. At the moment, all accumulated information on different insect groups supports the hypothesis suggested by Frydrychová et al. (2004) that the TTAGG telomeric repeat is ancestral one in the class Insecta. However, this repeat was either changed to another sequence (e.g. TCAGG in some beetles; Mravinac et al. 2011) or lost many times along various branches of the insect phylogenetic tree (Frydrychová et al. 2004), including some branches of Heteroptera. Within Heteroptera, the $(TTAGG)_n$ telomeric sequence is present in all hitherto studied basal families (Kuznetsova et al. 2012, Angus et al. 2017, Chirino et al. 2017) while was not found in all but one remaining families belonging to the evolutionarily advanced infraorders Cimicomorpha and Pentatomomorpha (Frydrychová et al. 2004, Grozeva et al. 2011, Golub et al. 2015, 2017, present paper). Specifically, the family Reduviidae (Cimicomorpha) is the only exception in this respect (Pita et al. 2016). The finding of the ancestral telomere motif $(TTAGG)_n$ in the youngest reduviid subfamily Triatominae (Pita et al. 2016) is of obvious interest and invites further investigation.

In sum, the data presented here add to the considerable body of previously published evidence that the lace bugs (1) are characterized by very conservative karyotypes

with 12 autosomes and the XY as the most typical sex chromosome system, (2) lack the insect telomeric sequence TTAGG and (3) differ from each other in the location of the rRNA genes in their genomes. The results have identified *D. longispina* as the species with the largest X- chromosome in the family Tingidae. The comparative survey has also shown that the evolutionarily secondary sex chromosome system X(0) is restricted to the genera sharing some specific morphological characteristics and can be useful thus to clarify the phylogenetic relations between the lace bug higher taxa.

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New insights into the karyotype evolution of the genus *Gampsocleis* (Orthoptera, Tettigoniinae, Gampsocleidini)

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Abstract

Five species belonging to the genus *Gampsocleis* Fieber, 1852 were analyzed using fluorescence *in situ* hybridization (FISH) with 18S rDNA and telomeric probes, as well as C-banding, DAPI/CMA_3 staining and silver impregnation. The studied species showed two distinct karyotypes, with 2n = 31 (male) and 2n = 23 (male) chromosomes. The drastic reduction in chromosome number observed in the latter case suggests multiple translocations and fusions as the main responsible that occurred during chromosome evolution. Two groups of rDNA distribution were found in *Gampsocleis* representatives analyzed. Group 1, with a single large rDNA cluster on the medium-sized autosome found in four species, carried in the haploid karyotype. Group 2, represented only by *G. abbreviata*, was characterized by the presence of two rDNA signals. TTAGG telomeric repeats were found at the ends of chromosome arms as expected. The rDNA clusters coincided with active NORs and GC-rich segments.

Keywords

Orthoptera, *Gampsocleis*, chromosome evolution, FISH, 18S rDNA, telomeric repeats, Ag-NOR, fluorochrome staining

Introduction

The *Gampsocleis* Fieber, 1852 belongs to Gampsocleidini Brunner von Wattenwyl, 1893, a relatively small tribe of Tettigoniinae Krauss, 1902, which includes 17 currently recognized species and 7 subspecies mainly distributed in the Palearctic region (Cigliano et al. 2018). The taxonomic status of some taxa is still confusing and difficult to interpret. Molecular phylogenetic studies on *Gampsocleis* have also shown the taxonomic problem (Zhang et al. 2011, Zhou et al. 2011). In this paper, we focus on molecular and classical cytogenetics, providing data on the karyotype structure and evolution of the group.

Changes in chromosome number (karyotype variability) or structure can contribute to speciation (e.g. Dion-Côté et al. 2017; Gould et al. 2017). Information on cytogenetic markers is therefore useful for understanding the chromosomal organization and assessing the karyotype diversity of organisms. In this sense, chromosome rearrangements, such as Robertsonian fusions and inversions, can be important in tettigoniid karyotype evolution and also could have a role as drivers in the speciation process (Warchałowska-Śliwa 1998).

The chromosome number (2n) and fundamental number (FN = numbers of chromosome arms) have been described for more than 110 species from 37 genera of Tettigoniinae. Most Palaearctic species have a diploid number of 31 (male) and 32 (female) acrocentric chromosomes with an X0/XX sex chromosome determination system. This karyotype has been suggested to be ancestral/modal for most tettigoniids (White 1973, Warchałowska-Śliwa 1998). The genus *Gampsocleis* is an interesting group with diverse chromosome numbers. So far, eight species are cytogenetically known (reviewed in Warchałowska-Śliwa 1998). Two different karyotypes have been characterized in *Gampsocleis* 31 (FN = 31) and 23 chromosome (FN = 36) karyotype in the male. However, the knowledge of cytogenetic patterns is still fragmentary (Warchałowska-Śliwa et al. 1992).

The present study reports the chromosomal characters of five *Gampsocleis* species using both molecular fluorescent *in situ* hybridization (FISH), and conventional methods. These data are an initial step towards better understanding of the evolutionary relationships within this genus.

Material and methods

A total of 18 specimens (adults and nymphs) belonging to five *Gampsocleis* species collected over several years (1990–2016) were selected for the study (Table 1). Gonads were excised and incubated in a hypotonic solution (0.9% sodium citrate), fixed in Carnoy's solution (ethanol: acetic acid – 3:1) and then stored at +2 °C until use. The fixed material was squashed in 45% acetic acid. Cover slips were removed using the dry ice procedure, and the preparations were then air-dried.

Fluorescence *in situ* hybridization (FISH) was performed as described by Grzywacz et al. (2018). The 18S rDNA probe was amplified with the 18S forward (5'-ACA AGG

GGC ACG GAC GTA ATC AAC -3') and 18S reverse (5'- CGA TAC GCG AAT GGC TCA AT -3') primers (Grozeva et al. 2011). The primers TTAGG_F (5'- TAA CCT AAC CTA ACC TAA CCT AA-3'), and TTAGG_R (5'-GGT TAG GTT AGG TTA GGT TAG G-3') (Grozeva et al. 2011) were used for visualizing the telomeric DNA. The rDNA and telomeric probes were labeled using biotin-16-dUTP (Roche Diagnostics GmbH, Germany) and digoxigenin-11-dUTP (Roche, Diagnostics GmbH, Germany), respectively. The rDNA probe was detected with avidin-FITC (Invitrogen, USA) and the telomeric probe with anti-digoxigenin rhodamine (Roche Diagnostics GmbH, Germany). The chromosomes were analyzed under a Nikon Eclipse 400 microscope fitted with a CCD DS-U1 camera and NIS-Elements BR2.

The distribution of heterochromatin was revealed by C-banding techniques, as described by Sumner (1972) with a slight modification. In order to reveal the molecular composition of constitutive heterochromatin, some slides were stained with CMA_3 to reveal GC-rich regions and DAPI to reveal AT-rich regions (Schweizer 1976). The silver staining of nucleolus organizer regions (NORs) was performed as previously reported in Warchałowska-Śliwa and Maryańska-Nadachowska (1992). At least 10 meiotic divisions (from diplotene to metaphase I) and three spermatogonial metaphases per male, and one to three males per species/population were analyzed using all the techniques. In all the analyzed species, the rDNA-FISH pattern, the locations of active NORs and heterochromatin pattern were recorded for meiotic bivalents in prophase I in the same individuals.

Table I. Localities of taxa, comparison of chromosome number and chromosomal localization of rDNA clusters, all forming active NOR.

Species	Collection sites and data	Geographical coordinates	No.	2n male	rDNA-FISH/ NOR
Gampsocleis gratiosa Brunner von Wattenwyl, 1862	China: Beijing area; 1995	no data	2	31	6
Gampsocleis sedakovii sedakovii (Fischer von Waldheim, 1846)	Russia: Altai Mts, valley of Edigan River; 1998	51.1235N, 86.5149E	3	31	6
Gampsocleis ussurensis Adelung, 1910	Korea: near Hamgyong Province, near Chongjin; 1990	41.79556N, 129.77583E	2	31	6
Gampsocleis abbreviata ebneri Uvarov, 1921	(FYR) Macedonia: Sveti Nikola municipality, Bogoslovec ridge; 2008	41.78663N, 22.01893E	2	23	5, 8/9
Gampsocleis abbreviata renei Miksic, 1973	Albania: Galichitsa Mts., above Pikina Voda place, above 1600 m; 2013	40.91136N, 20.85197E	1		
Gampsocleis abbreviata ssp.	Greece: Central Greece, Phthiotis, Palaiochori; 2015	38.70813N, 22.45736E	2		
Gampsocleis glabra Herbst, 1786	Bulgaria: Dobrich, Dobrich; 2006	43.60573N, 27.83478E	2	23	5
	Kazakhstan: (1) Aktobe, Safonowka, (2) Shimkent, Gavrilovka	42.20608N, 70.21833E	3		
	(3) Almaty, Uzunbylack; 2016	43.20317N, 78.98846E	1		

Results

We observed two different karyotypes with the sex determination system X0 in males of five species of the genus *Gampsocleis* (Table 1). The standard karyotype of *G. gratiosa*, *G. sedakovii sedakovii* and *G. ussuriensis* was characterized by a chromosome number of 2n = 31. In this case all chromosomes were acrocentric, consisting of four long, three medium and eight short pairs; the X chromosome was the largest element (Fig. 1a–e). In the second karyotype of *G. abbreviata* and *G. glabra* the chromosome number was reduced to 2n = 23 (Fig. 2a–j) with 11 pairs of autosomes arranged into three groups, 2 large, 3 medium, and 6 short pairs; among them, six pairs and the X chromosome were biarmed (Fig. 2b, marked with an asterisks). In both karyotypes, minor differences in the length of the short pairs of chromosomes sometimes made their precise identification difficult.

The localization of 18S rDNA in *Gampsocleis* was revealed by FISH and its activity analyzed by silver impregnation is summarized in Table 1. In four species we detected a single large rDNA cluster (per haploid genome) on a medium-sized autosome. This was

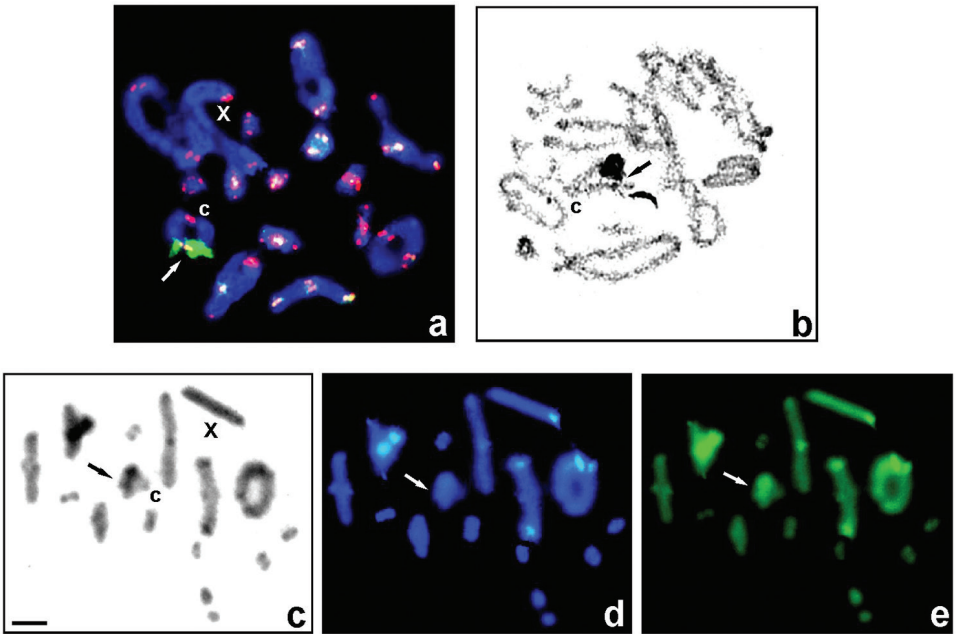

Figure 1. Examples of *Gampsocleis* species with 2n = 31 chromosomes (male): *G. s. sedakovii* (**a, c–e**) and *G. ussuriensis* (**b**) studied using different techniques: FISH with both 18S rDNA (green) and telomeric TTAGG (red) probes (**a**) in diakinesis revealed a single rDNA locus located distally on the 6th bivalent (white arrow) and one active NOR visualized by $AgNO_3$ staining (**b**) in diplotene (black arrow). C-banding (**c**) as well as fluorochrome staining of heterochromatin with DAPI (blue) and CMA_3 (green) bands in diakinesis (**d** and **e,** respectively); black arrows indicate a C-band, and white arrows indicate very weak DAPI+ and bright CMA_3+ signals located in a distal region on the 6th bivalent. C **(a–c**), centromere; X (**c–e**), sex chromosome. Scale bar: 10 μm.

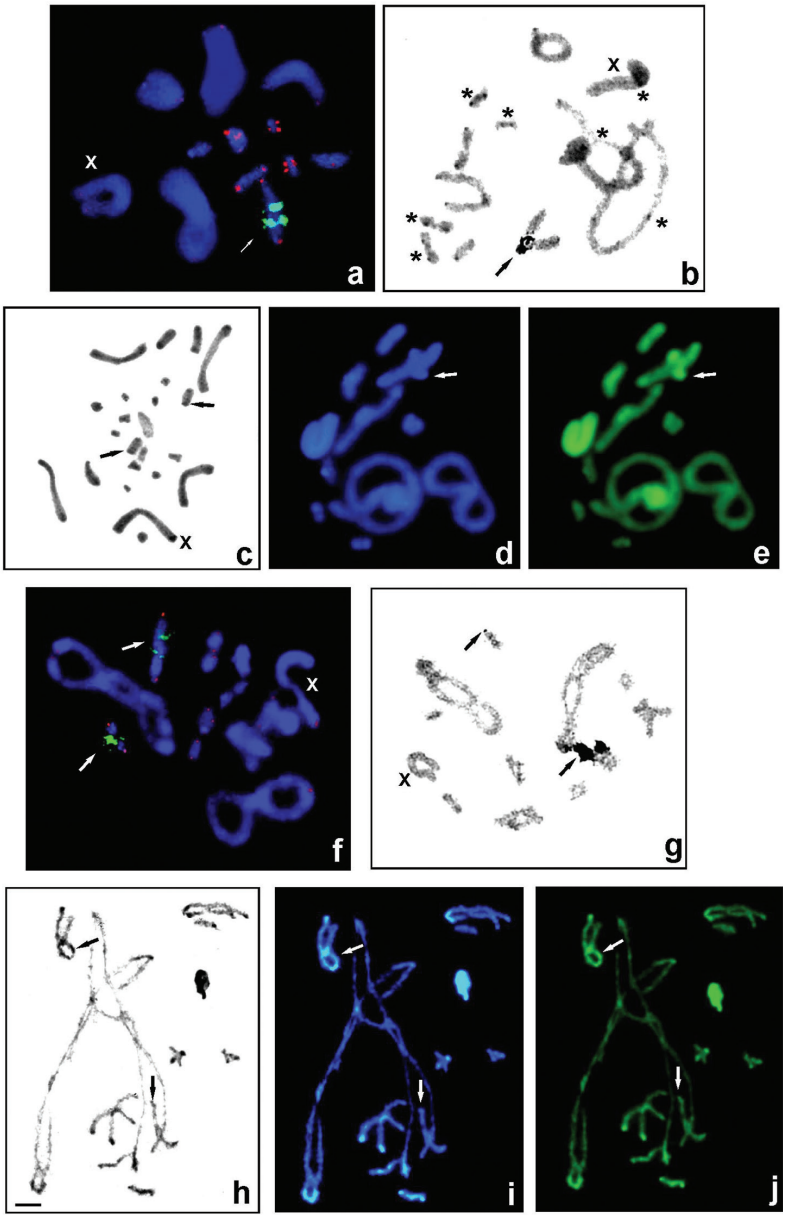

Figure 2. *Gampsocleis* species with 2n = 23 chromosomes (male): *G. glabra* (**a–e**) and *G. abbreviata* (**f–j**) studied using different techniques: FISH using 18S rDNA (green) and telomeric TTAGG (red) probes (**a, f**) and silver staining in diakinesis (**b,g**), C-banding of spermatogonial metaphase (**c**) and diplotene (**h**), and fluorochrome staining of heterochromatin with DAPI (blue) and CMA_3 (green) (**d, i, e, j**). Arrows indicate rDNA clusters located near the telomeric region on the 5th bivalent (**a, f**) and in a telomeric position on the short bivalent (**f**); active NORs co-localized with rDNA (**b, g**, black arrows)**;** thin C-bands (**c, h**, black arrows) and weak DAPI+ (**d, i**, white arrows) and bright CMA_3+ signals located near the telomeric region on the medium-sized bivalent (**e, j**, white arrows) as well DAPI-/CMA_3+ signals on the telomeric region of the short bivalent (**i,j**, white arrows). Bi-armed chromosomes are marked by asterisks (**b**). X, sex chromosome. Scale bar: 10 μm.

evident distally/terminally to the centromere on the 6th bivalent in males of *G. graciosa*, *G. s. sedakovii*, and *G. ussuriensis* (2n = 31) (Fig. 1a) or subterminally/subdistally on the 5th bivalent in male individuals from four localities of *G. glabra* (2n = 23) (Fig. 2a). In contrast, two FISH signals were detected subterminally and terminally on the 5th and 8/9th bivalents, respectively, in *G. abbreviata* males (2n = 23) (Fig. 2f). FISH with the $(TTAGG)_n$ probe (tDNA-FISH) localized the telomeric sequences to the ends of chromosomes of the analyzed species as expected; no hybridization signals of the probe were found in the centromere region of biarmed chromosomes in species with 23 chromosomes. Generally, FISH signals of the telomeric probe in species with 31 chromosomes were stronger than in those with 23 chromosomes (Figs 1a, 2a).

After both C-banding and DAPI/CMA_3 double staining, chromosome regions in the analyzed species showed discrete quantitative and qualitative variation in their constitutive heterochromatin. In *G. s. sedakovii*, *G. s. obscura*, *G. glabra*, and *G. ussuriensis* paracentromeric C-bands was uniformly present in long and medium-sized chromosomes, distal and interstitial bands are found to vary in size between these species, as described previously (Warchałowska-Śliwa et al. 1992, Table 1) and as example Fig. 1c (present study). In karyotypes with 23 chromosomes in both species, interstitial small C-bands near the distal region were present in the 5th pair (Fig. 2c). Generally, paracentromeric thin C-bands on most of the autosomes were very weakly DAPI-positive (DAPI+) and CMA_3-positive (CMA_3+), whereas the thick paracentromeric C-bands showed bright homogenous DAPI+ (AT-rich) and bright CMA_3+ (GC-rich) signals in some of the large and medium-sized autosomes and the X chromosome (Figs 1c–e; 2c–e, h–j). In addition, all species revealed weak C/DAPI+ and bright CMA_3+ signals in the distal/subdistal region of a medium-sized bivalent (6th or 5th) (Figs 1d,e; 2d,e,i,j). Additionally, in one short bivalent of *G. abbreviata*, a thin C-band in the telomeric region was visualized with the DAPI-/CMA_3+ signal (Fig. 2i,j). Thus, the heterochromatin composition in these chromosomes exhibits distinct GC-rich bands coincident with active NORs and rDNA-FISH signals (Figs 1a,b,e; 2 a,b,e,g,j).

Discussion

Our results are in line with previous studies (for a review see Warchałowska-Śliwa 1998), which revealed the advanced karyotype evolution in the genus *Gampsocleis*. The ancestral chromosome number 2n = 31 (FN=31) in Asian species was reported for males of two subspecies of *G. sedakovii* (*G. s. sedakovii*, *G. s. obscura*), *G. ussuriensis* and *G. gratiosa* (Hareyama 1932, Ueshima 1986, Kim et al. 1987, Warchałowska-Śliwa et al. 1992, Zhang et al. 2011), and for *G. buergeri* (Hareyama 1932). Only in *G. ryukyuensis* a metacentric X chromosome was observed (Ueshima 1986); in this case (FN=32), a pericentric inversion modified the centromere position, changing the morphology of the modal acrocentric sex chromosome to a biarmed X chromosome. Two Eurasian species, *G. glabra* and *G. abbreviata* (Warchałowska-Śliwa 1984, Warchałowska-Śliwa et al. 1992, present study), have reduced the chromosome number to 2n = 23

(FN = 36). This karyotype is probably the result of multiple translocations and fusions that occurred during the chromosome evolution in these species, as was suggested by Warchałowska-Śliwa (1984) and Warchałowska-Śliwa et al. (1992). In the last work, authors challenge the taxonomic status of *G. glabra* based on cytogenetic evidence (i.e. chromosome number). Currently, the Orthoptera Species File (Cigliano et al. 2018) include this species within *Gampsocleis*, based on morphological evidence.

In cytogenetic studies, the application of a variety of staining methods (classical and molecular) generally enables a better characterization of tettigoniid karyotypes and identification of genus/species-specific patterns (Grzywacz et al. 2017, Warchałowska-Śliwa et al. 2017). In this study, information revealed by FISH (rDNA and tDNA) is the first antecedent in species of *Gampsocleis.* Present result and previous cytogenetic data helps to interpret the chromosome evolution in this group. According to differences in the number and location of 18S rDNA signals, two groups were specified within the genus. The taxa belonging to group I were characterized by rDNA signals on one rDNA cluster in four species – *G. gratiosa*, *G. s. sedakovii*, *G. glabra*, and *G. ussuriensis*, while, in group II two rDNA loci in *G. abbreviata.* The karyotypes of three species (2n = 31) described both in this paper and previous work (Warchałowska-Śliwa et al. 1992), have a single active NOR and rDNA cluster on a medium sized autosome, probably M_6, near the distal region. This localization suggests the occurrence of the same chromosome reorganization in the karyotype of the latter two species (2n = 23), whose evolution is difficult to explain. The presence of a distally located active NOR in only a single middle-sized bivalent has also been described in others European Tettigoniinae (Warchałowska-Śliwa et al. 2005). In most cases, a single 18S rDNA cluster/NOR is located near the paracentromeric/interstitial region within the subfamily (Grzywacz et al. 2017, Warchałowska-Śliwa et al. 2017), as in other tettigoniids (e.g. Warchałowska-Śliwa et al. 2013). Two rDNA/NOR loci restricted to subdistal/distal regions on different chromosome pairs (M_5 and $S_{8/9}$) were found in *G. abbreviata*. However, this difference between species with 2n=23 must be confirmed by analyzing a larger number of individuals to clarify whether it is a specific marker for *G. abbreviata*. The occurrence of TTAGG telomeric repeats was detected at chromosome ends in all the *Gampsocleis* species. This telomeric motif plays an important role in karyotype stability and is a common trait in insects (Vítková et al. 2005). Some interspecific differences in signal intensity may have been due to the presence of different numbers of telomeric repeats, whereas the lack of these sequences in the centromere region of the bi-armed chromosomes of *G. glabra* and *G. abbreviata*, which originated by chromosome fusion, is probably due to the loss of telomeric repeats during karyotype evolution (e.g. Warchałowska-Śliwa et al. 2013, 2017).

Discrete quantitative and qualitative differences in constitutive heterochromatin were discovered in the chromosomes of the analyzed species after both C-banding and DAPI/CMA_3 double staining. The constitutive heterochromatin of all species analyzed was located in the paracentromeric and distal regions in some chromosomes and differed in size between species; similar observations were reported in previous studies of Gampsocleidini (Warchałowska-Śliwa et al. 1992) and other Tettigoniinae (e.g.

Grzywacz et al. 2017, Warchałowska-Śliwa et al. 2017). DAPI and CMA_3 staining showed very weak DAPI-positive (DAPI+) and CMA_3-positive (CMA_3+) segments. The thick C-bands coincided with bright homogenous DAPI+ (AT-rich) and bright CMA_3+ (GC-rich) signals in the distal regions of the large and medium-sized autosomes, as well as in the paracentromeric region of the X chromosome. The presence of weak C/DAPI+ and bright CMA_3+ signals near the distal region of a medium-sized bivalent is common for all *Gampsocleis* species, even in those with different chromosome numbers in their karyotype. The DAPI-/CMA_3+ signal was only found in one short bivalent of *G. abbreviata* in a thin distal C-band. Generally, the position of the major rDNA sites in the currently analyzed species corresponds to the active Ag-NOR sites and some GC-rich bands.

Previous data (Warchałowska-Śliwa et al. 1992) threw light on the problematic taxonomic status of *G. glabra,* which was found to differ from the other examined species in this genus on its chromosome number. This is in agreement with the present results, which confirmed the chromosome number of *G. glabra* and showed similar results for *G. abbreviata.* These findings suggest important genetic differences between species between Eastern/Central Asia and Europe. However, there are a number of taxa in Western Asia (25% of all described *Gampsocleis* species) that have not yet been studied.

Species of *Gampsocleis* can be assigned into two groups distinguished by both the chromosome number and geographic range, in accordance with previous studies (Hareyama 1932, Warchałowska-Śliwa 1984, Warchałowska-Śliwa et al. 1992, Ueshima 1986, Kim et al. 1987). Geography plays an important role in generating genetic diversity. Our and previously published (Warchałowska-Śliwa et al. 1992) data suggest that *G. glabra* and *G. abbreviata* should be considered as belonging to a separate group. This is justified on the basis of their significant karyotype differentiation and could be either confirmed or rejected in future detailed genetic, morphological and/or behavioral studies. Further analyses on inclusive taxonomic sample of *Gampsocleis* may refine generic and intrageneric classification.

In conclusion, the present study offers new insights into the karyotype characteristics of bushcrickets that may be useful for interpret or understand relationships within the genus *Gampsocleis* as well as the subfamily Tettigoniinae. Changes observed in karyotypes may probably also play an important role in speciation. Additional species and methods (morphological and genetic characters) should be examined in order to further elucidate the relationships within the genus *Gampsocleis* and the tribe Gampsocleidini.

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FISH-based karyotyping of *Pelmatohydra oligactis* (Pallas, 1766), *Hydra oxycnida* Schulze, 1914, and *H. magnipapillata* Itô, 1947 (Cnidaria, Hydrozoa)

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Abstract

An account is given of the karyotypes of *Hydra magnipapillata* Itô, 1947, *H. oxycnida* Schulze, 1914, and *Pelmatohydra oligactis* (Pallas, 1766) (Cnidaria, Hydrozoa, Hydridae). A number of different techniques were used: conventional karyotype characterization by standard staining, DAPI-banding and C-banding was complemented by the physical mapping of the ribosomal RNA (18S rDNA probe) and H3 histone genes, and the telomeric $(TTAGGG)_n$ sequence by fluorescence *in situ* hybridization (FISH). We found that the species studied had 2n = 30; constitutive heterochromatin was present in the centromeric regions of the chromosomes; the "vertebrate" telomeric $(TTAGGG)_n$ motif was located on both ends of each chromosome and no interstitial sites were detected; 18S rDNA was mapped on the largest chromosome pair in *H. magnipapillata* and on one of the largest chromosome pairs in *H. oxycnida* and *P. oligactis*; in *H. magnipapillata*, the major rRNA and H3 histone multigene families were located on the largest pair of chromosomes, on their long arms and in the centromeric areas respectively. This is the first chromosomal mapping of H3 in hydras.

Keywords

Hydra, *Pelmatohydra*, Hydridae, karyotype, chromosomes, FISH, $(TTAGGG)_n$, 18S rDNA, histone H3

Introduction

Hydras are simple freshwater invertebrates belonging to one of the most ancient members of the animal kingdom, the phylum Cnidaria (class Hydrozoa, order Hydrida, family Hydridae). Hydras are of general interest since they display fundamental principles that underlie development, differentiation, regeneration and symbiosis (e.g. Bosch 2007, 2008, Khalturin et al. 2009, Augustin et al. 2010, Bosch et al. 2010). Some species of hydras are relatively easy animals to culture and maintain in the laboratory, then, they have been used as model organisms in many different areas of biological research, primarily in developmental biology often referred to as "evo-devo", i.e. evolutionary developmental biology research (Slobodkin and Bossert 2001, Galliot 2012).

Without detailed knowledge of these basal metazoans, it is impossible to provide an effective comparative framework for animal evolution (Zacharias et al. 2004). Nevertheless, the species level diversity, taxonomy and phylogenetic relationships of the hydra species are far from well understood. Jankowski et al. (2008) suggested 12–15 really different hydra species, whereas Bouillon et al. (2006) reported approximately 30 valid species, and the World Register of Marine Species lists 40 species (Schuchert 2018). All hydras were originally included in the single genus *Hydra* Linnaeus, 1758. However Schulze (1914, 1917) divided hydras into three genera, *Hydra*, *Chlorohydra* Schulze, 1914, and *Pelmatohydra* Schulze, 1914, and their validity was substantiated elsewhere (e.g. Collins 2000, Stepanjants et al. 2000, Anokhin 2002).

During the past decade or so, several molecular phylogenetic studies using mitochondrial and nuclear genes shed light on the diversity within *Hydra* sensu Linnaeus, 1758 (Hemmrich et al. 2007, Kawaida et al. 2010, Martínez et al. 2010, Schwentner and Bosch 2015). The genome of one species, *Hydra magnipapillata* Itô, 1947, has been recently assembled (Chapman et al. 2010).

Chromosomes are known to be the carriers of genetic material, and chromosome changes provide the basis of speciation (White 1973). As many as 8 species from all three above-mentioned hydra genera have been karyotyped so far (Xinbai et al. 1987, Ovanesyan and Kuznetsova 1995, Anokhin et al. 1998, 2010, Anokhin and Kuznetsova 1999, Anokhin 2002, 2004, Anokhin and Nokkala 2004, Zacharias et al. 2004, Stepanjants et al. 2006, Traut et al. 2007). These species were mainly studied using conventional chromosome staining techniques, including C-banding. They were shown to have 2n = 30, almost exclusively meta/submetacentric (m/sm) chromosomes of similar size, and C-heterochromatin blocks localized in the centromeric regions of the chromosomes. Sex chromosomes were not distinguished in any species. Thus, hydras can now been considered as the group with the greatest stability in their karyotype, at least regarding the number of chromosomes. In two studies only (Traut et al. 2007, Anokhin et al. 2010), the fluorescence *in situ* hybridization (FISH) was used to characterize hydras in terms of telomeric sequences and the chromosomal distribution of the rRNA and some other genes.

Our study was aimed to add new data on hydra chromosomes studied using C-banding and FISH with probes for the "vertebrate" telomere motif $(TTAGGG)_n$, 18S rDNA, and histone H3. We adopt here the generic hydra classification of Schulze (1914, 1917).

Material and methods

Experiments were carried out with three species, *Hydra magnipapillata*, *H. oxycnida* Schulze, 1914, and *Pelmatohydra oligactis* (Pallas, 1766). *H. magnipapillata* (strain 105) was obtained from the Institute of Zoology, University of Kiel (Germany); *H. oxycnida* and *P. oligactis* were collected from nature (58°48'46.9"N, 29°59'02.7"E, the Oredezh river, Leningrad Province, Russia). Polyps were cultured at 18 ± 0.5 °C for a long period of time in the case of *H. magnipapillata* or for one-two weeks in the cases of *H. oxycnida* and *P. oligactis*. They were fed regularly with freshly hatched nauplii of *Artemia salina* (Linnaeus, 1758) (Crustacea, Branchiopoda).

Different methods were tried to characterize the chromosomes of the above-mentioned species: C-banding for *H. magnipapillata* and *P. oligactis*; FISH mapping of 18S rRNA and histone H3 genes for *H. magnipapillata* and of the "vertebrate" telomere motif $(TTAGGG)_n$ for *H. oxycnida* and *P. oligactis*.

Spread chromosome preparations were made from asexual polyps. Hydras were subjected to a hypoosmotic shock with 0.4% trisodium citrate for 30 min followed by fixation in ethanol and acetic acid (3:1) for 15 min. Specimens were transferred to a drop of 70% ethanol on the glass slides and dissected with needles. The cell suspension was spread by the warm air stream (37–70 °C).

In DNA isolation, 18S rDNA and $(TTAGGG)_n$ probes generation and FISH experiments we followed the protocol described in Anokhin et al. (2010). The probe for the histone H3 was PCR amplified and labeled by Rhodamine-5-dUTP (GeneCraft, Germany) using primers H3F: 5'-ATG GCT CGT ACC AAG CAG ACV GC-3' and H3R: 5'-ATA TCC TTR GGC ATR ATR GTG AC-3' (Huang et al. 2011).

Microscopic images were taken using a Leica DM 6000B microscope with a 100× objective, Leica DFC 345 FX camera and Leica Application Suite 3.7 software with an Image Overlay module (Leica Microsystems Wetzlar GmbH, Germany). The filter sets applied were A, L5, N21 (Leica Microsystems, Wetzlar, Germany).

Results

Cytogenetic analyses were carried out on 10 specimens of every species (asexual forms), *Hydra magnipapillata*, *H. oxycnida*, and *P. oligactis*. Representative mitotic images of the species subjected to routine chromosome staining, C-banding, and FISH with the 18S rDNA, histone H3 and telomere $(TTAGGG)_n$ probes are shown in Figures 1–3.

Hydra magnipapillata

The karyotype was found to consist of 30 m/sm chromosomes (2n = 30), it is symmetrical in structure, with chromosomes showing a regular gradation in size. No heteromorphic chromosome pair (putative sex chromosomes) is identified. The homologues

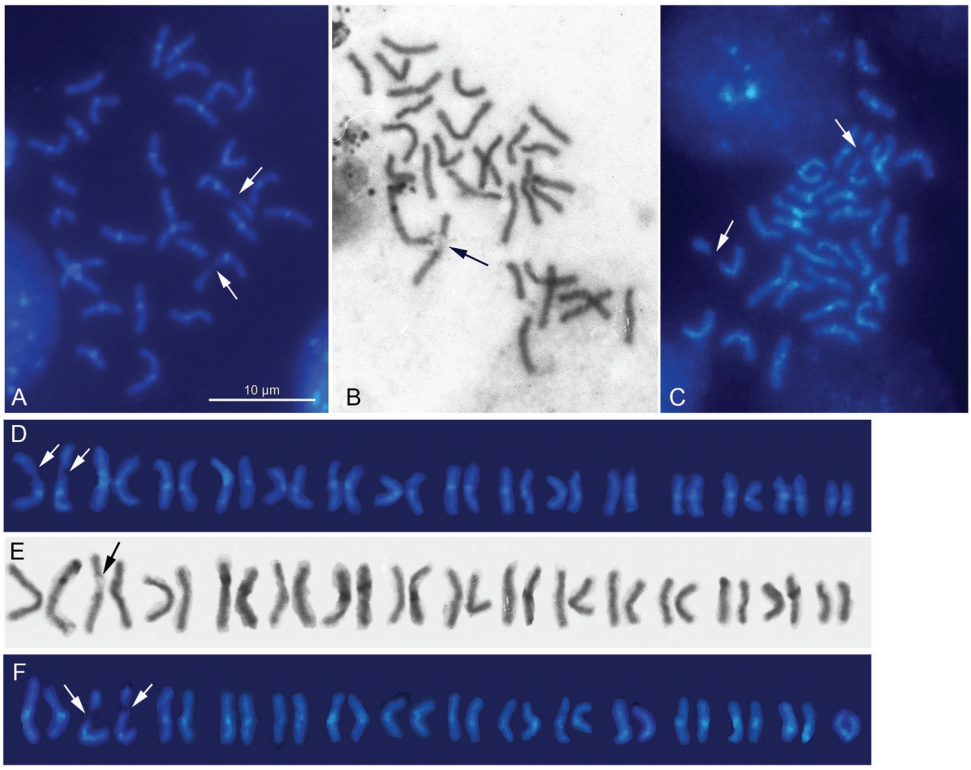

Figure 1. Mitotic chromosomes of *Hydra magnipapillata* after C- banding (**A**), *Hydra oxycnida* after routine staining (**B**), and *Pelmatohydra oligactis* after C- banding (**C**). C-bands are visible in the centromeric areas of the chromosomes. Karyograms of *H. magnipapillata* (**D**), *H. oxycnida* (**E**) and *P. oligactis* (**F**). Arrows indicate achromatic gaps.

of the largest pair carry achromatic gaps on their long arms. C-banding procedure revealed blocks of constitutive heterochromatin (C-blocks) localized in the centromere areas of the chromosomes (Fig. 1 A, D). FISH mapping of the 18S rDNA and histone H3 probes revealed hybridization signals on the largest pair of autosomes, on their long arms and around the centromeres respectively (Fig. 3A). The rDNA signals position corresponds to that of achromatic gaps, that's to be expected (Fig. 1 A, D).

Hydra oxycnida

As with *H. magnipapillata*, this species has 2n = 30; its karyotype is symmetrical in structure, with chromosomes showing a regular gradation in size, and no heteromorphic chromosome pair is observed. One of the largest chromosome pairs (the largest or the second largest) carries secondary constrictions on the long arm of every homologue (Fig. 1 B, E). Furthermore, the 18S rDNA signals were detected on the long arms of

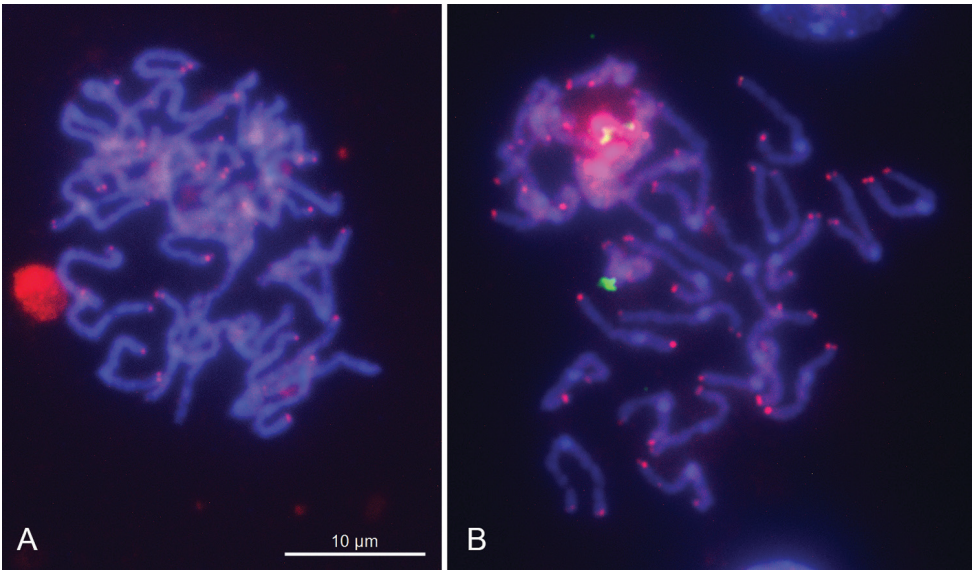

Figure 2. FISH with the "vertebrate" $(TTAGGG)_n$ telomeric probe (red signals) on mitotic chromosomes of *H. oxycnida* (**A**) and *P. oligactis* (**B**). The chromosomes are counterstained with DAPI.

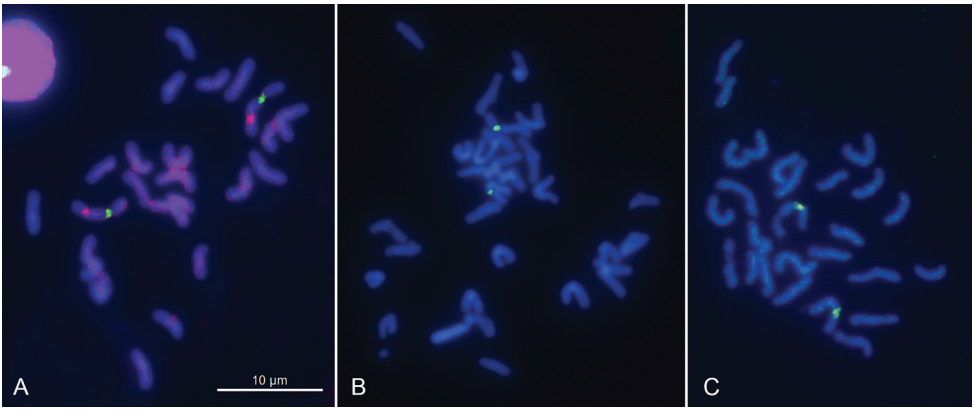

Figure 3. FISH with the 18S rDNA (green signals) and H3 histone (red signals) probes on mitotic chromosomes of *Hydra magnipapillata* (**A**), and with the 18S rDNA probe only on mitotic chromosomes of *Hydra oxycnida* (**B**) and *Pelmatohydra oligactis* (**C**). In *H. magnipapillata*, the FISH signals derived from the 18S and H3 probes are visible on the largest pair of chromosomes, on their long arms and in the centromeric areas respectively. Chromosomes are counterstained with DAPI.

one of largest chromosome pairs (Fig. 3 B). Again, as in the routinely stained preparations, more precise identification of this pair, whether it is the largest or the second largest one, appeared to be difficult. The $(TTAGGG)_n$ probe hybridized to the termini of every chromosome suggesting this sequence to be characteristic of the species (Fig. 2 A).

Pelmatohydra oligactis

As with both above-mentioned species, this species has 2n = 30; its karyotype is symmetrical in structure, with chromosomes showing a regular gradation in size, and no heteromorphic chromosome pair is observed. C-banding procedure followed by DAPI staining revealed C-blocks in the centromere regions of the chromosomes. All but one chromosome pairs were found to be m/sm. The exception was the smallest pair of chromosomes with very short arms which can be preliminarily identified as a subtelocentric/acrocentric pair (st/a). One of the largest chromosome pairs (the largest but maybe the second largest one) carries secondary constrictions on the long arm of every homologue (Fig. 1 C, F). Furthermore, the 18S rDNA signals were detected on the long arms of one of largest chromosome pairs (Fig. 3 C). Again, as in the routinely stained preparations, more precise identification of this pair, whether it is the largest or the second largest one, appeared to be difficult. The $(TTAGGG)_n$ probe hybridized to the termini of every chromosome suggesting this sequence to be characteristic of the species (Fig. 2 B).

Discussion

Characterization of karyotypes using standard staining and C-banding technique

Basic features of karyotypes revealed here in *Hydra magnipapillata*, *H. oxycnida*, and *Pelmatohydra oligactis* agree with those reported for these species previously (Anokhin and Kuznetsova 1999, Anokhin and Nokkala 2004, Anokhin et al. 2010). All hydra species studied so far have 2n = 30 with chromosomes showing a regular gradation in size, suggesting thus these features are under stabilizing natural selection. Among chromosomes, there is no pair to be taken as that of sex chromosomes. The centromere position is generally difficult to distinguish after conventional staining, and only C-banding is able to solve this question since C-heterochromatin in the hydra chromosomes is invariably located in the centromere regions (Anokhin and Nokkala 2004, Zacharias et al. 2004, present paper). The karyotypes of *H. magnipapillata* and *P. oligactis* as well as karyotypes of previously studied *H. circumcincta* Schulze, 2014 and *H. vulgaris* Pallas, 1766 (Anokhin and Nokkala 2004) are symmetrical and consist of mainly m/sm chromosomes. At the same time, a comparison between C-banded karyotypes of *P. oligactis* and *H. magnipapillata* showed that the former species had two subtelo/acrocentric (st/a) chromosomes, whereas the last-mentioned species had m/sm chromosomes only. This observation makes it apparent that some chromosome rearrangements have occurred during hydra species evolution, and thus, the species with the same chromosome number can differ one from another in chromosome morphology. The resolving of the issue needs to study in depth.

Characterization of karyotypes using FISH with the "vertebrate" $(TTAGGG)_n$ telomeric probe

Previous studies on *Hydra vulgaris* (Traut et al. 2007) and *H. magnipapillata* (Anokhin et al. 2010) have shown that these species possess the "vertebrate" $(TTAGGG)_n$ motif of telomeres. Our FISH analyses also showed the presence of this motif at the ends of chromosomes of *H. oxycnida* and *Pelmatohydra oligactis*. Furthermore, the "vertebrate" telomeric sequence is present in representatives of all basal metazoan groups (Traut et al. 2007) and, with some notable exceptions (nematodes and arthropods), is conserved in most Metazoa. Bearing in mind that the "vertebrate" TTAGGG telomeric repeat is widely distributed and is present in most major eukaryotic groups, it is assumed to be the ancestral motif of telomeres in eukaryotes as a whole (Traut et al. 2007, Gomes et al. 2010, Fulnečková et al. 2013).

Characterization of karyotypes using FISH with 18S rDNA and H3 probes

The chromosomal location of the 18S rRNA genes was studied here in all three species. *Hydra magnipapillata* was shown to have 18S rDNA sites on the large arms of the largest chromosome pair. In *H. oxycnida* and *Pelmatohydra oligactis*, these sites were revealed on one of the largest pairs, the largest or maybe on the second largest one. In every case, the location of these sites coincides with the achromatic gaps, which are generally referred to as secondary constrictions, the nucleolus organizer region (NOR) involved in the formation of nucleolus (McStay 2016). The chromosomal location of the histone H3 gene family was studied in *H. magnipapillata* only. Noteworthy that mapping of H3 has been achieved for the first time in hydras. *H. magnipapillata* showed the H3 sites in the centromeric areas of the largest pair of chromosomes. It is the species that has received the most study by FISH to investigate the chromosomal distribution of different genes and sequences including genes coding for 18S rRNA and 28S rRNA, a head-specific gene ks1, a gene family DMRT suggested to be involved in sex determination and *Tol2*-like transposable element (Anokhin et al. 2010). The rRNA genes were shown to be co-localized on the homologues of the largest pair of chromosomes, on their long arms. A sex-related gene DMRT was revealed on a pair of chromosomes suggesting thus that it is a dose-regulated sex-determining gene in hydras. Probes specific for the ks1 hybridized to three distinct chromosome pairs, and multiple copies of a Tol2 transposable element gene were found on every chromosome. We have shown here that the major rDNA and the H3 genes are positioned on the same pair of chromosomes of *H. magnipapillata*, on their long arms and in the centromeres respectively, and should be thus inherited together. Furthermore, our results suggest that, in *H. magnipapillata*, the canonical histone H3 appears in the form of its centromere-specific variant CENH3, which is known to be the key histone component of the centromere in eukaryotes (Malik et al. 2002, Black and Bassett 2008).

In conclusion, this study delivers insight into the organization of genomes of hydras by reporting first data on (1) *the chromosomal location* of the H3 histone genes by the example of *Hydra magnipapillata*; (2) the telomere motif and the distribution of the 18S rRNA genes on chromosomes of *Hydra oxycnida* and *Pelmatohydra oligactis*. Our results provide a foundation for further studying the mechanisms involved in the chromosome evolution of this phylogenetically important group having an ancient origin within Metazoa.

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