EUROPEAN JOURNAL OF ENTOMOLOGY
ISSN (online): 1802-8829
http://www.eje.cz
*Eur. J. Entomol.* **117**: 474–480, 2020
doi: 10.14411/eje.2020.050
ORIGINAL ARTICLE

# Cytogenetic characterization of periodical cicadas (Hemiptera: Cicadidae: *Magicicada*)

**Gayane KARAGYAN[1], Natalia GOLUB[2] and Teiji SOTA[3]**

[1] Scientific Center of Zoology and Hydroecology, NAS RA, P. Sevak 7, Yerevan, 0014 Armenia;
e-mail: gaykaragyan@yahoo.com

[2] Zoological Institute, Russian Academy of Sciences, Universitetskaya nab. 1, 199034 St. Petersburg, Russia;
e-mail: nvgolub@mail.ru

[3] Department of Zoology, Graduate School of Science, Kyoto University, Sakyo, Kyoto, 606-8502 Japan;
e-mail: sota@terra.zool.kyoto-u.ac.jp

**Key words.** Hemiptera, Cicadidae, *Magicicada*, periodical cicadas, chromosome numbers, karyotype, C-heterochromatin, nucleolus organizer regions, AT-rich regions, 18S rDNA, telomeres

**Abstract.** The periodical cicadas of the genus *Magicicada* Davis, 1925 inhabiting eastern United States are characterized by a long juvenile period of 17 or 13 years and periodical mass emergence of adults. In this genus, only seven species are distinguished and are distributed into three species groups, Decim, Cassini and Decula. We performed comparative cytogenetic study of all seven *Magicicada* species, and 6 of them were explored for the first time. The techniques used included standard chromosome staining, differential chromosome staining techniques (C-banding, AgNOR-banding and fluorochrome DAPI-staining) and FISH (fluorescence in situ hybridization) with 18S rDNA and $(TTAGG)_n$-telomeric probes. All these species have holokinetic chromosomes, like other Hemiptera. Karyotypes of all species appeared to be remarkably conserved with 2n = 19/20 (18 + X0/XX) (male/female); one very large pair of autosomes and all other chromosomes constituting a decreasing size series; X chromosomes were indistinguishable, at least, at mitotic metaphases. The nucleolus organizer regions (NORs) revealed on one of the medium-sized chromosome pairs in all species and were shown to coincide with the rDNA clusters revealed by FISH. In all species, very small blocks of C-heterochromatin were variously located on chromosomes clearly using DAPI but less distinctly by C-banding. The chromosomes of all the species studied were confirmed to contain the "insect type" motif of telomeres $(TTAGG)_n$.

## INTRODUCTION

The genus *Magicicada* Davis, 1925 includes periodical cicadas that occur in the eastern United States, have a long juvenile period of 17 or 13 years and periodical mass emergence of adults. There are seven species in *Magicicada* in three species groups (Decim, Cassini and Decula). Each species group contains one species with a 17-year life cycle and one or two species with 13-year life cycles; 17-year species occur in the north and 13-year species in the south. The Decim group contains the 17-year species *M. septendecim* (Linnaeus, 1758) and two 13-year species *M. neotredecim* (Marshall & Cooley, 2000) and *M. tredecim* (Walsh & Riley, 1868). The Cassini group contains the 17-year species *M. cassini* (Fisher, 1852) and 13-year species *M. tredecassini* (Alexander & Moore, 1962). The Decula group contains the 17-year species *M. septendecula* Alexander & Moore, 1962 and the 13-year species from the Midwestern and southern part of the eastern United States: *M. tredecula* Alexander & Moore, 1962 (Alexander & Moore, 1962; Marshall & Cooley, 2000; Cooley et al., 2001; for other references see Williams & Simon, 1995). The 17- and 13-year species within the same species groups differ only in the length of juvenile period, except for *M. tredecim* for which the pitch of male song differs. The parallel divergence of 13- and 17-year life cycles in the three periodical cicada groups was recently evidenced by molecular phylogenetic and population genetic analyses (Sota at al., 2013; Fujisawa et al., 2018; Du et al., 2019).

All species of *Magicicada* are well studied with respect to their morphology, life-cycle, ecology, distribution and behaviour, information which has been successfully used for understanding the evolution and speciation in *Magicicada* (Cooley et al., 2001). However, there are very few cytogenetic studies of periodical cicadas. The only karyotyped species is *M. septendecim*, with 2n = 18 + X0/XX (Shaffer, 1920, as *Cicada* (*Tibicen*) *septendecim*). Here we studied the karyotypes of all seven species of *Magicicada* using differential staining techniques (C-banding, AgNOR-banding, DAPI-staining) and fluorescence in situ hybridization (FISH) with 18S rDNA and $(TTAGG)_n$-telo-

meric probes. The aim of this study was to conduct a comprehensive cytogenetic analysis of the genus in order to reveal the similarities and differences between the species.

## MATERIAL AND METHODS

Adult specimens of *Magicicada* were collected in 2011 and 2013 (Table 1). Male and female abdomens were dissected, immersed in 0.9% sodium citrate solution for 30 min and fixed in 3 : 1 fixative (96% ethanol : glacial acetic acid). The samples were stored at –20°C until chromosomal preparation.

The gonads were placed on slides in a drop of 70% acetic acid and then dissected. Squashed chromosomal preparations were obtained using a dry ice quick-freezing technique (Conger & Fairchild, 1953). Ag-banding was done using the method proposed by Howell & Black (1980) with minor modifications. The slides were hydrolysed in 2 N formic acid for 10 min, rinsed in running water and dried. Then 4–5 drops of 50% aqueous silver nitrate ($AgNO_3$) solution and 2 drops of colloidal developer solution (0.2 g gelatine, 10 ml distilled water and 0.1 ml concentrated formic acid – HCOOH) were placed on the slides. The slides were then each covered with a coverslip and incubated on a hotplate for 3–4 min at 60°C in a moist chamber (warmed beforehand). The slides were dried after rinsing in distilled water.

C-banding was done according to the protocol of Rożek (2000). The slides were treated for 1–3 min in 0.2 N HCL at room temperature, then rinsed in distilled water. Thereafter, the slides were placed in 5% $Ba(OH)_2$ solution at 20°C for approximately 4 min, then rinsed in running and distilled water. Then the slides were incubated in 2×SSC solution (0.3 M sodium chloride containing 0.03 M tri-sodium citrate) at 60°C for 1 h. After rinsing in distilled water, the slides were dried and stained with 4% Giemsa solution in phosphate buffer (pH 6.8) for 8 min.

DAPI staining was done according to Karagyan et al. (2012). In brief, after the C-banding procedure described above, slides were immersed in McIlvaine buffer (pH 7.0) for 5 min. After this, slides were stained with DAPI at a final concentration of 0.4 µg/ml in 10 mM McIlvaine buffer (pH 7.0) for 5 min. Then slides were rinsed in distilled water and mounted in antifade medium consisting of 1% n-propylgallate in a 10 M McIlvaine buffer (pH 7.0) solution with 70% glycerol.

Fluorescence in situ hybridization (FISH) was done according to the published protocol (Grozeva et al., 2015) with minor modifications. In brief, the target 18S rDNA probe (about 1200 bp fragment) was PCR amplified and labelled with biotin-11-dUTP (Fermentas, EU) using primers: 18SrRNA_F 5'-GATCCTGCCAGTAGTCATATG-3', 18SrRNA_R 5'-GAGTCAAATTAAGCCGCAGG-3' (Anokhin et al., 2010). Genomic DNA was extracted from the true bug *Pyrrhocoris apterus* (Linnaeus, 1758). An initial denaturation period of 3 min at 94°C was followed by 35 cycles of 30 s at 94°C, annealing for 30 s at 55.5°C and extension for 1.5 min at 72°C, with a final extension step of 3 min at 72°C. The telomere probe $(TTAGG)_n$ was amplified using PCR and labelled with rhodamine-5-dUTP (GeneCraft, Köln, Germany) using primers: TTAGG_F 5'-TAACCTAACCTAACCTAACCTAA-3' and TTAGG_R 5'-GGTTAGGTTAGGTTAGGTTAGG-3' (Grozeva et al., 2010). An initial denaturation period of 3 min at 94°C was followed by 30 cycles of 45 s at 94°C, annealing for 30 s at 50°C and extension for 50 s at 72°C, with a final extension step of 3 min at 72°C. The chromosome preparations were treated with 100 µg/ml RNase A and 5 mg/ml pepsin solution to remove excess RNA and protein. Chromosomes were denatured in the hybridization mixture containing labelled 18S rDNA and $(TTAGG)_n$ probes with an addition of salmon sperm blocking reagent and then hybridized for 42 h at 37°C. 18S rDNA probes were detected with NeutrAvidin-Fluorescein conjugate (Invitrogen, Karlsbad, CA, USA). The chromosomes were mounted in an antifade medium (ProLong Gold antifade reagent with DAPI, Invitrogen) and covered with a glass coverslip. As a control for the efficacy of our $(TTAGG)_n$ FISH experiments, we used chromosome preparations of *Psococerastis gibbosa* (Sulzer, 1766) (Psocoptera), which is known to be $(TTAGG)_n$-positive (Golub et al., 2019).

The slides stained using the different chromosome banding techniques were analysed under a Boeco (BM-180/I/Pl) microscope (Germany) with a 100× objective. Images were taken using a ToupCam 9.0 MP digital camera. Chromosome slides stained with FISH were analysed under a Leica DM 6000 B microscope (Germany) with a 100× objective. Images were taken using a Leica DFC 345 FX camera using Leica Application Suite 3.7 software with an Image Overlay module.

**Table 1.** Specimens used in the chromosome analysis.

| Species | Locality | Date of collection | No. of specimens | Staining |
|---|---|---|---|---|
| *Magicicada cassini* | Goochland Co./Louisa Co., VA | 25.05.2013 | 4♂ 6♀ | Ag-banding<br>C-banding<br>DAPI-staining<br>FISH |
| *M. tredecassini* | South of Norris, IL | 26.05.2011 | 2♀ | Ag-banding<br>C-banding<br>DAPI-staining<br>FISH |
| | Dixon Springs, IL | 19.05.2011 | 1♀ | |
| | Greenback, TN | 21.05.2011 | 1♂ 2♀ | |
| *M. neotredecim* | Stephen A. Forbes State Park, IL; South of Norris City, IL | 24.05.2011 | 2♂ 4♀ | Ag-banding<br>C-banding<br>FISH |
| | | 26.05.2011 | 4♀ | |
| *M. septendecim* | Martinsville, VA | 11.05.2013 | 4♂ 6♀ | Ag-banding<br>C-banding |
| *M. tredecim* | Greenback, TN | 21.05.2011 | 1♂ 2♀ | Ag-banding<br>C-banding<br>FISH |
| *M. tredecula* | Murfreesboro, TN | 20.05.2011 | 2♀ | Ag-banding<br>C-banding<br>DAPI-staining<br>FISH |
| | Buffalo Valley, TN | 20.05.2011 | 2♂ | |
| | Greenback, TN | 21.05.2011 | 2♀ | |
| | South of Norris, IL | 26.05.2011 | 1♂ 2♀ | |
| *M. septendecula* | Mayodan, Rockingham Co., NC | 10.05.2013 | 3♂ 6♀ | Ag-banding<br>DAPI-staining |

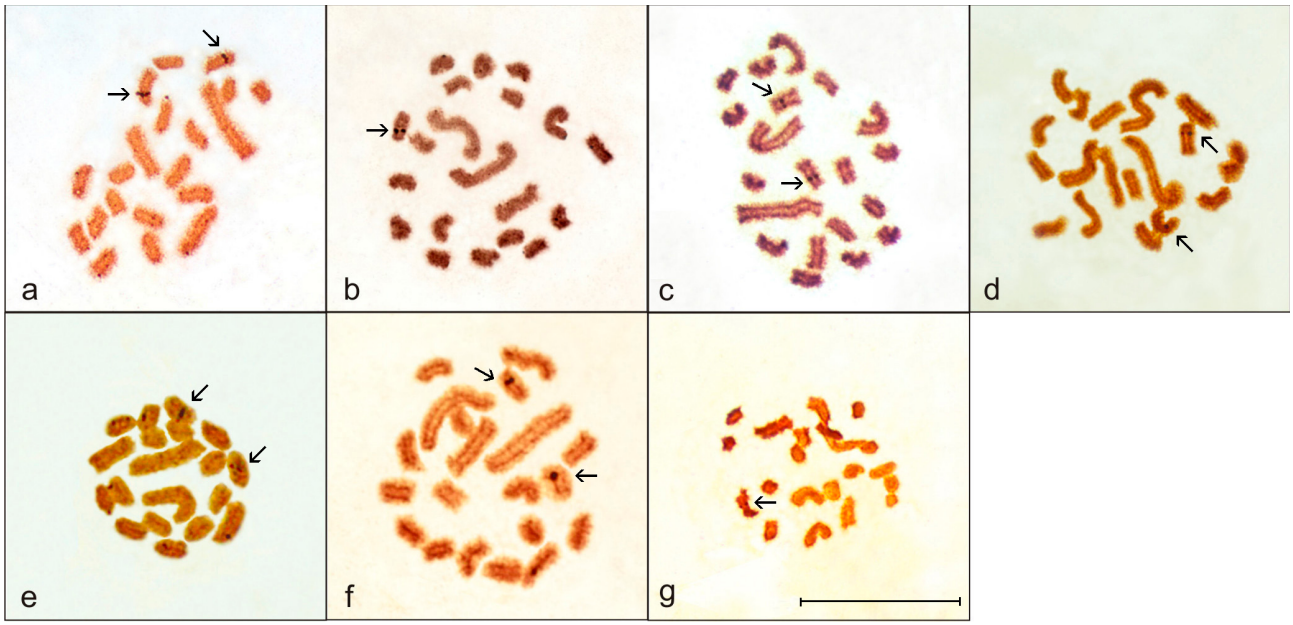


**Fig. 1.** Nucleolus organizer regions in *Magicicada*. a – *Magicicada cassini* (female), b – *M. tredecassini* (female), c – *M. neotredecim* (female), d – *M. septendecim* (male), e – *M. tredecim* (male), f – *M. tredecula* (female), g – *M. septendecula* (female). Arrows indicate argentophilic signals (NORs). Bar equals 10 μm.

## RESULTS

### Chromosome numbers

Nuclei at the mitotic metaphase stage in spermatogonia and oogonia were studied. At the mitotic metaphase stage, chromosomal sets of all species include 19 chromosomes in male and 20 chromosomes in female (Figs 1–4, Table 2). Thus, the diploid karyotype of each species was inferred to be 2n = 19, 20 (18 + X0/XX). In the karyotypes of all the species, one chromosomal pair (AA1) is significantly larger than the others. All other chromosomal pairs constitute a decreasing size series. Sex chromosomes are not distinguishable in either the male or female karyotype.

### NOR-bearing chromosomes

All the species were Ag-banded and argentophilic signals (NORs) were revealed in the interstitial regions of a pair of medium-sized chromosomes in each species (Fig. 1a–g).

**Table 2.** Karyotypes of Cicadidae.

| No. | Species | 2n | References |
|---|---|---|---|
| 1 | *Cyclochila australasiae* (Donovan, 1805) | ♂ 19 (18 + X0) | Whitten, 1965 |
| 2 | *Lyristes bihamatus* (Motschulsky, 1861) | ♂ 19 (18 + X0) | Perepelov et al., 2002; as *Tibicen bihamatus* |
| 3 | *Macrosemia saturata* (Walker, 1858) | ♀ 20 (18 + XX) | Chatterjee & Ghosh, 1978; as *Platylomia satura* |
| 4 | *Magicicada cassini* (Fisher, 1851) | ♂ 19 (18 + X0)<br>♀ 20 (18 + XX) | Present study |
| 5 | *M. tredecassini* (Alexander & Moore, 1962) | ♂ 19 (18 + X0)<br>♀ 20 (18 + XX) | Present study |
| 6 | *M. neotredecim* (Marshall & Cooley, 2000) | ♂ 19 (18 + X0)<br>♀ 20 (18 + XX) | Present study |
| 7 | *M. septendecim* (Linnaeus, 1758) | ♂ 19 (18 + X0)<br>♀ 20 (18 + XX) | Present study<br>Shaffer, 1920; as *Cicada* (*Tibicen*) *septendecim* |
| 8 | *M. tredecim* (Walsh & Riley, 1868) | ♂ 19 (18 + X0)<br>♀ 20 (18 + XX) | Present study |
| 9 | *M. tredecula* Alexander & Moore, 1962 | ♂ 19 (18 + X0)<br>♀ 20 (18 + XX) | Present study |
| 10 | *M. septendecula* Alexander & Moore, 1962 | ♀ 20 (18 + XX) | Present study |
| 11 | *Meimuna opalifera* (Walker, 1850) | ♂ 19 (18 + X0) | Kurokawa, 1953 |
| 12 | *Neotibicen tibicen tibicen* (Linnaeus, 1758) | ♂ 24 * | Wilcox, 1895; as *Cicada tibicen* |
| 13 | *Platypleura kaempferi* (Fabricius, 1794) | ♂ 19 (18 + X0) | Kurokawa, 1953 |
| 14 | *P. kuroiwae* Matsumura, 1917 | ♂ 19 (18 + X0) | Perepelov et al., 2002 |
| 15 | *Polyneura ducalis* Westwood, 1840 | ♀ 20 (18 + XX) | Chatterjee & Ghosh, 1978 |
| 16 | *Pycna repanda* Linnaeus, 1758 | ♂ 19 (18 + X0) | Chatterjee & Ghosh, 1978 |
| 17 | *Terpnosia maculipes* Walker, 1850 | ♂ 19 (18 + X0)<br>♀ 20 (18 + XX) | Chatterjee & Ghosh, 1978 |

* This value needs confirming.

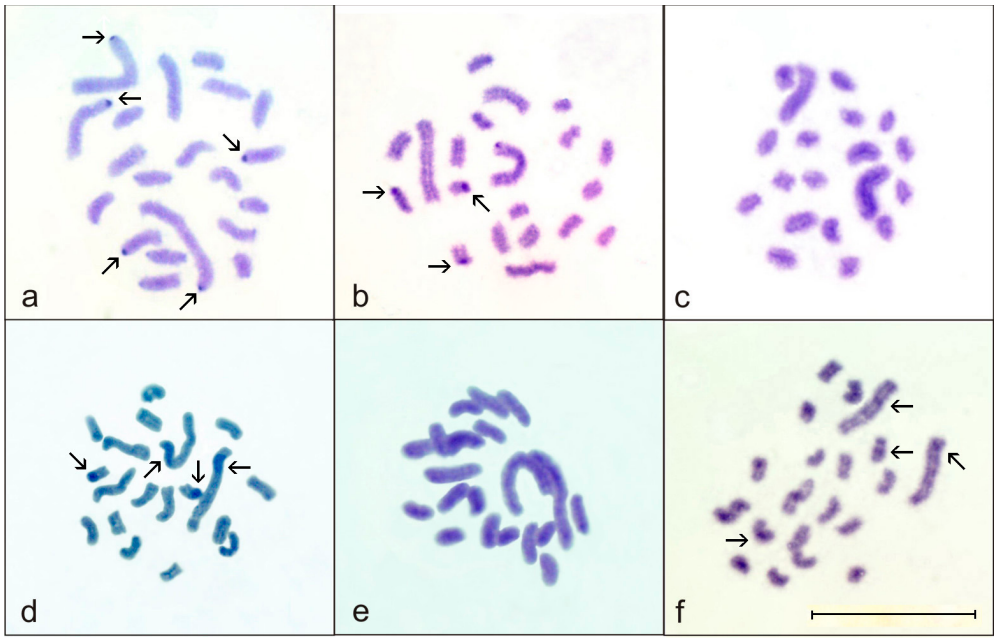


**Fig. 2.** Distribution of C-heterochromatin in *Magicicada*. a – *Magicicada cassini* (female), b – *M. tredecassini* (female), c – *M. neotredecim* (male), d – *M. septendecim* (male), e – *M. tredecim* (female), f – *M. tredecula* (female). Arrows indicate C-blocks. Bar equals 10 μm.

These signals were quite definite on one of the homologues (on both chromatids), while on the other the signals were not always well pronounced.

### Distribution of C-heterochromatin

All species of *Magicicada*, except *M. septendecula*, were C- banded. This revealed a small amount of heterochroma-

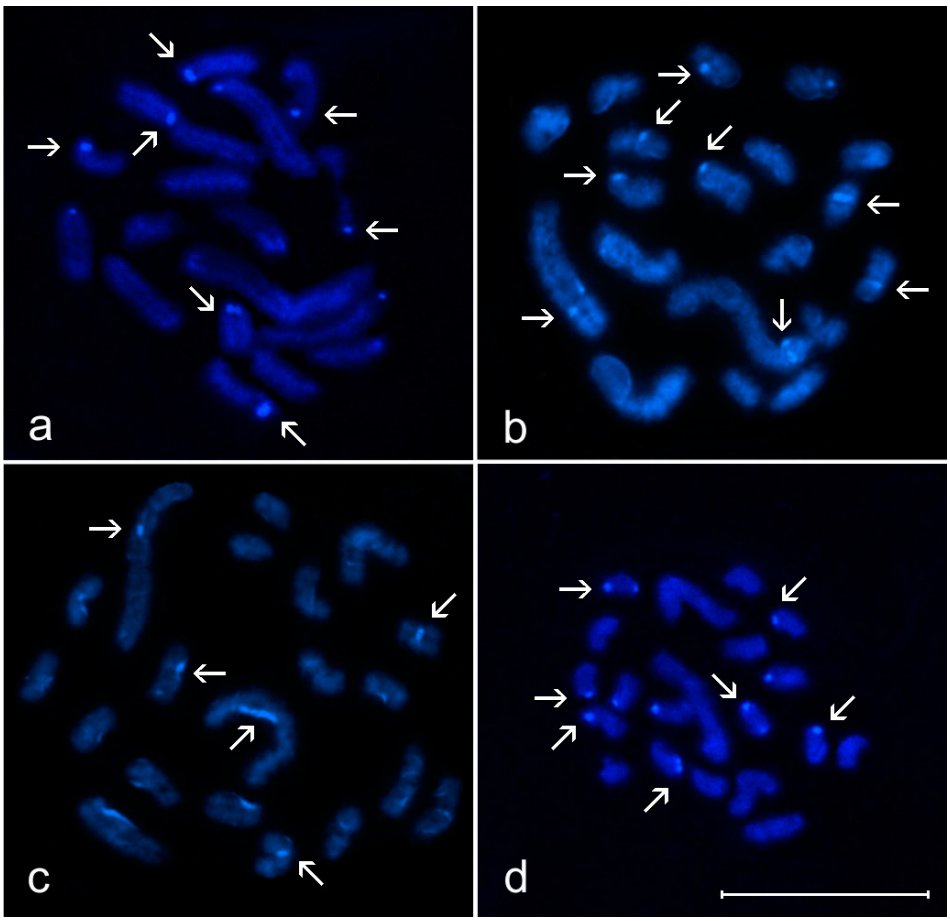


**Fig. 3.** AT-rich regions on DAPI-stained chromosomes of *Magicicada*. a – *Magicicada cassini* (male), b – *M. tredecassini* (male), c – *M. tredecula* (female), d – *M. septendecula* (female). Arrows indicate AT-positive regions. Bar equals 10 μm.

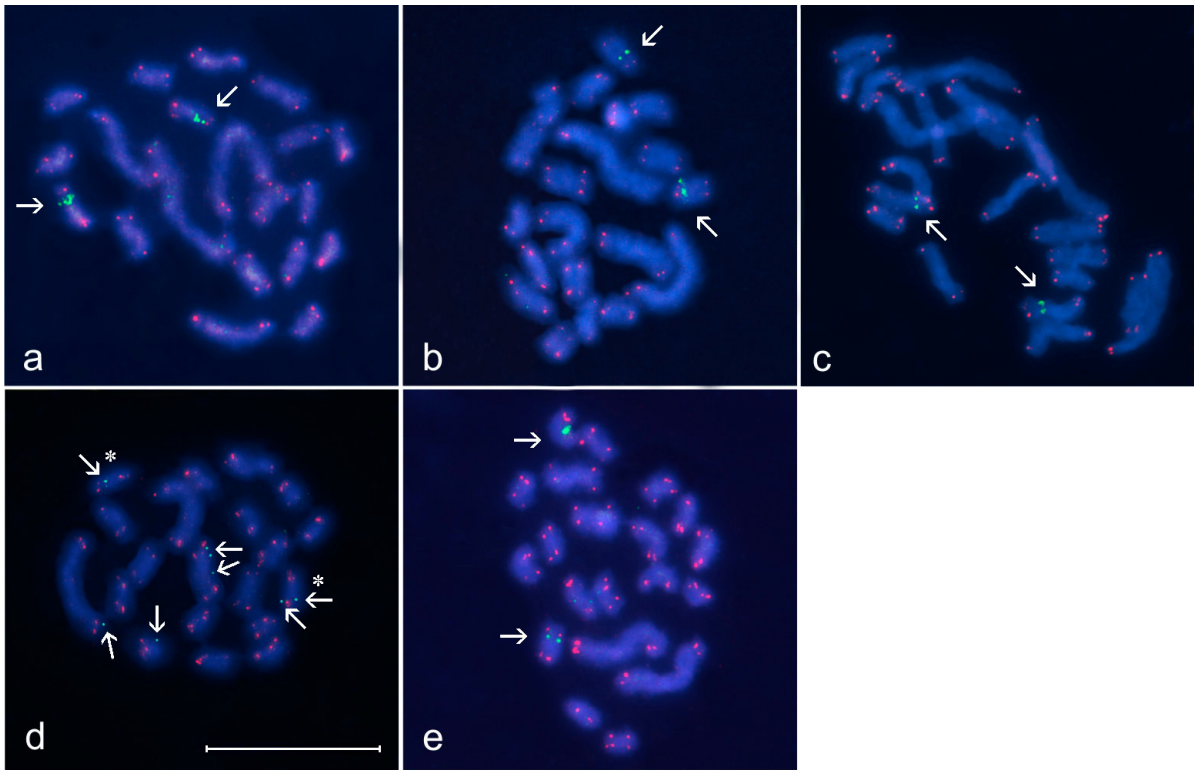


**Fig. 4.** FISH mapping of TTAGG telomeric sequences (red signals) and 18S rDNA (green signals) on chromosomes of *Magicicada*. a – *Magicicada cassini* (female), b – *M. tredecassini* (female), c – *M. neotredecim* (female), d – *M. tredecim* (female, presumable NOR-bearing chromosomes indicated by an asterisk), e – *M. tredecula* (female). Arrows indicate the18S rDNA clusters. Bar equals 10 μm.

tin in all the species (Fig. 2a–f). C-blocks were in the terminal position in *M. cassini* (Fig. 2a) and *M. tredecassini* (Fig. 2b), in the terminal and subterminal positions in *M. septendecim* (Fig. 2d) and in the subterminal and interstitial positions in *M. tredecula* (Fig. 2f). On the chromosomes of *M. neotredecim* and *M. tredecim* there were no distinct blocks of C-heterochromatin (Figs 2c, e).

### Localization of AT-rich regions

The chromosomes of *M. cassini*, *M. septendecula*, *M. tredecassini* and *M. tredecula* were stained with DAPI. Distinct AT-rich regions were revealed in terminal positions on chromosomes of *M. cassini* and *M. septendecula* (Figs 3a, d), in subterminal and interstitial positions in *M. tredecula* (Fig. 3c) and terminal, subterminal and interstitial positions on chromosomes of *M. tredecassini* (Fig. 3b).

### Distribution of 18S rDNA and the nucleotide telomeric sequence

FISH with 18S rDNA and $(TTAGG)_n$-telomeric probe was applied to *M. cassini*, *M. tredecassini*, *M. neotredecim*, *M. tredecim* and *M. tredecula*. In all these species, except *M. tredecim*, bright 18S rDNA signals were revealed interstitially on one of the medium-sized chromosomal pairs, being well pronounced on one of the homologues in most cases (Fig. 4a–c, e). In *M. tredecim* no massive clusters of rDNA were revealed. Small specific 18S rDNA signals were distributed on several chromosomes of the karyotype (Fig. 4d).

In all studied species FISH with the $(TTAGG)_n$ probe revealed bright fluorescent signals at the ends of every chromosome (Fig. 4a–e), indicating that the telomeres of the chromosomes in *Magicicada* are composed of the $(TTAGG)_n$ nucleotide sequence.

## DISCUSSION

### Chromosome numbers in Cicadidae

Wilcox (1895) observed spermatogenesis in *Cicada tibicen* (*Neotibicen tibicen tibicen* (Linnaeus, 1758)) and reports "12 chromatic rods in spermatogonia" and "24 spherical chromosomes in the spermatocytes" (i.e., 2n = 24; Chatterjee & Ghosh, 1978; Kirillova, 1986). This first report of chromosomes in Cicadidae was, however, based on an ambiguous drawing, and the chromosome number needs further investigation. Including our data, the karyotypes of 17 species of Cicadidae belonging to 9 genera are presented in Table 2. The modal diploid chromosome number in Cicadidae is 2n = 19, 20 (18 + X0/XX). The karyotypes of all these species are characterized by the presence of a pair of large chromosomes. Sex chromosomes are not distinguishable at mitotic metaphase; however, they are certainly not the largest (AA1), the second (AA2) and the NOR-bearing pairs of autosomes because these chromosomes appeared as pairs of homologues, both in male and female karyotypes.

Cicadidae like all other Hemiptera have holokinetic chromosomes (Kuznetsova & Aguin-Pombo, 2015), which have no localized centromeres and the large kinetochore plate extends along all or most of the length of the chromosome. It is generally accepted that holokinetic chromosomes are more likely to be subjected to fissions and

fusions, which lead to karyotype rearrangements, than are monocentric chromosomes (reviewed in Kuznetsova et al., 2011). Despite the fact that Hemiptera have holokinetic chromosomes, many hemipteran taxa of different taxonomic levels have stable karyotypes. The most impressive examples of chromosome stability within this group are reviewed by Kuznetsova and Aguin-Pombo (2015). The family Cicadidae is characterized by stable chromosome numbers suggesting that fusions and fissions have not played a major role in the speciation and evolution of this group.

### Differential staining in Cicadidae

Until now, cytogenetic studies on Cicadidae, with one exception, were done using conventional staining techniques. Perepelov et al. (2002) report the first data on C-banding staining of the chromosomes of *Lyristes bihamatus* (Motschulsky, 1861) (as *Tibicen bihamatus*) and *Platypleura kuroiwae* Matsumura, 1917. At metaphase I (MI), there are C-heterochromatic blocks with different distributions on all the chromosomes of both these species. In *L. bihamatus* C-blocks were distinct and at terminal positions on the chromosomes. The chromosomes of *P. kuroiwae* had a smaller amount of constitutive heterochromatin at a subterminal or interstitial position (Perepelov et al., 2002).

In our study, the chromosomes of the species of *Magicicada* characteristically had a small amount of constitutive heterochromatin variously located. Distinct C-blocks were located terminally in *M. cassini* and *M. tredecassini*, terminally and subterminally in *M. septendecim* and subterminally and interstitially in *M. tredecula.*

The results of DAPI staining partly confirmed those obtained using C-banding. Well-pronounced AT-positive signals were observed terminally on the chromosomes of *M. cassini*, which accords with results obtained using C-banding. On the chromosomes of *M. tredecassini* and *M. tredecula*, subterminal and interstitial DAPI-signals mainly coincided with C-blocks. It is noteworthy that in our study DAPI was more successful in revealing constitutive heterochromatin than C-banding.

Ag-banding in all the species studied revealed interstitially located NOR on the medium-sized chromosomal pair, which is likely to be the same chromosomal pair in all the karyotypes studied.

### Distribution of 18S rDNA and nucleotide telomeric sequence in Cicadidae

FISH with the 18S rDNA probe revealed two different patterns in the locations of the ribosomal genes: (1) large clusters on one of the chromosomal pairs, (2) small clusters on several chromosomal pairs (in *M. tredecim*). In species with pattern (1), 18S rDNA clusters were located interstitially on a pair of medium-sized chromosomes, which accords with the results obtained using Ag-banding. In *M. tredecim*, some of the signals detected using FISH in all likelihood coincide with those obtained using silver impregnation. It is not possible, however, to make a final conclusion due to the equal FISH signals intensity on the different chromosomes.

FISH with the $(TTAGG)_n$ probe revealed that this sequence occurs at the ends of all the chromosomes of the five species studied. Thus, the telomeres of species of *Magicicada* consist of TTAGG tandem repeats. This telomere motif is reported in the majority of insects and in many arthropod lineages and is considered to be the ancestral sequence of arthropod telomeres (Sahara et al., 1999; Frydrychová et al., 2004). In Auchenorrhyncha, this motif is reported in the genera *Philaenus*, Aphrophoridae (Maryańska-Nadachowska et al., 2013), *Mapuchea*, Myerslopiidae (Golub et al., 2014), *Alebra*, Cicadellidae (Kuznetsova et al., 2015) and *Mahanavra*, Cercopidae (Anjos et al., 2016). Our data on the genus *Magicicada*, Cicadidae confirm that the $(TTAGG)_n$ telomeric repeat motif has been conserved in the Auchenorrhyncha.

**ACKNOWLEDGEMENTS.** We thank J. Yoshimura and S. Kakishima for helping with sampling. The present study was supported by research project no. AAAA-A19-119020790106-0 and research grants from the Russian Foundation for Basic Research no 19-54-18002 (to N.G.) and JSPS KAKENHI (no. 22255004 to J.Y.; no. 19H05550 to T.S.).

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Received October 23, 2020; revised and accepted November 30, 2020
Published online December 15, 2020