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Evidence for TTAGG telomere repeats and rRNA gene clusters in leafhoppers of the genus *Alebra* (Hemiptera: Auchenorrhyncha: Cicadellidae)

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Abstract. The leafhopper genus *Alebra* Fieber, 1872 comprises a complex of morphologically similar species. The chromosome complements (karyotypes) of five *Alebra* species, i.e. *A. albostriella*, *A. coryli*, *A. viridis*, *A. wahlbergi* and a new, yet undescribed species, provisionally named Taxon 1, were here investigated, three of these species (*A. coryli*, *A. viridis*, and Taxon 1) for the first time. The techniques applied included standard chromosome staining, fluorescence in situ hybridization (FISH) for mapping of 18S rDNA and telomeric repeats (in every species), C-banding, AgNOR-banding and CMA_3/DAPI- staining (in *A. viridis*). The species have a holokinetic type of chromosomes, as in other hemipterans. Karyotypes of all species are remarkably conserved with 2n = 22 + X(0)/XX (male/female), one large and 10 medium pairs of autosomes and the X chromosome similar in size to larger chromosomes within this group. In every species, FISH identified the "classical" insect telomere repeat of TTAGG and rRNA gene clusters located on the homologues of a medium-sized pair of autosomes, presumably number 5. Thus, speciation in *Alebra* has apparently not involved significant karyotypic changes. In *A. viridis*, rDNA sites were both Ag- and CMA_3-positive and were located at an interstitial position. C-banding revealed heterochromatic bands in the X chromosome and also in all but four pairs of autosomes, the bands were located at one telomere of a chromosome. C-bands were positive for CMA_3 and negative for DAPI, suggesting that C-heterochromatin is mainly enriched in GC-pairs.

INTRODUCTION

Most previous cytogenetic studies on the suborder Auchenorrhyncha (Insecta: Hemiptera) were performed using conventional (routine) chromosome staining techniques. This approach allows the detection of gross karyotypic alterations, such as changes in chromosome number and size, and provides an overview of chromosome behaviour during mitosis and meiosis. So far, descriptions regarding specific chromosome regions, like C-heterochromatin and NORs, are scarce (reviewed in Kuznetsova & Aguin-Pombo, in press). Over the last few years, the use of fluorescence in situ hybridization (FISH) has opened up a new area of research in different groups of insects with holokinetic chromosomes, making possible cytological identification as well as the molecular mapping of individual chromosomes within karyotypes (e.g. Manicardi et al., 2002; Yoshido et al., 2005; Monti et al., 2011; Grozeva et al., 2011, 2014). In the Auchenorrhyncha, this technique has so far only been applied to nine cicadomorphan species, including *Mapuchea chilensis* (Nielson, 1996) (Myerslopiidae) and eight species of the spittlebug genus *Philaenus* Stal, 1864 (Aphrophoridae) in which the distribution of 18S rDNA sites and presence of the $(TTAGG)_n$ telomeric motif were detected (Maryańska-Nadachowska et al., 2013; Golub et al., 2014; Kuznetsova et al., 2014).

The genus *Alebra* Fieber, 1872 is classified within the subfamily Typhlocybinae Kirschbaum 1868, one of the largest and most diversified of the auchenorrhynchan family Cicadellidae (leafhoppers). The latter includes nearly 22,000 (McKamey, 2002) or even 25,000 (Dietrich & Rakitov, 2002) described species. To date, karyotypes in terms of chromosome numbers and sex chromosome systems are known for 289 species in 165 genera of Cicadellidae, among which 34 species (of the 5000 described) and 15 genera (of the 450 accepted) belong to the subfamily Typhlocybinae (de Lello et al., 1982; Aguin-Pombo et al., 2006, 2007; de Bigliardo et al., 2011; Juan, 2011; Kuznetsova et al., 2013; for other references and discussion, see reviews of Kirillova, 1988; Emeljanov & Kirillova, 1990; Kuznetsova & Aguin-Pombo, in press). Until now, neither the karyotypes of Typhlocybinae nor indeed Cicadellidae as a whole have ever been subjected to differential chromosome staining of any description.

The taxonomy of the genus *Alebra* is difficult because most species do not show any apparent differences in male genital structures. Based on slight but consistent morphological differences three different groups within the genus are recognized occurring in North America (Hamilton, 1995), Europe (Drosopoulos & Loukas, 1988; Gillham, 1991; Aguin-Pombo, 2002) and Asia (Wagner, 1940;

Dworakowska, 1968, 1971). The European group is represented by five morphologically similar, but biologically distinct species: *A. albostriella* (Fallen, 1826), *A. sorbi* Wagner, 1940, *A. wahlbergi* (Boheman, 1845), *A. coryli* Le Quesne, 1977, *A. viridis* Rey (Gillham, 1991) and several cryptic yet unnamed taxa (Aguin-Pombo, 2002). Nymphs and adults of these species are mesophyll-feeders being associated with a large number of broad-leaved trees and shrubs. Although these leafhoppers are usually found in small numbers, some species can occasionally become pests on chestnuts, hazel and other plants (Viggiani, 1971; Lauterer, 1986; Drosopoulos et al., 1987).

Cytologically, the genus *Alebra* is poorly explored. To date, only two species, *A. albostriella* and *A. wahlbergi*, have been studied, both of which possess the karyotype 2n = 22 + X(0)/XX (male/female). In a total of 13 analyzed populations of these species from Greece, a lot of meiotic abnormalities and polymorphism for B-chromosomes were detected (Kuznetsova et al., 2013).

In order to continue cytological investigation of the genus, we have in the present study investigated karyotypes of three further species, *A. coryli*, *A. viridis* and one presumably new yet undescribed species named here as Taxon 1. In addition, we reinvestigated karyotypes of *A. albostriella* (in two earlier unexplored populations) and *A. wahlbergi* (for the first time in females). Here we show the results of FISH experiments to investigate the distribution of 18S rRNA gene clusters and the presence/absence of the $(TTAGG)_n$ telomeric motif in all the aforementioned species. This is only the third physical mapping effort reported for the auchenorrhynchan genomes using FISH. In addition, in *A. viridis*, a more detailed cytogenetic analysis was performed using C-banding, silver staining and fluorochrome DAPI/CMA_3-staining.

MATERIAL AND METHODS

Source of material

The four species of the "*albostriella*" group presented in the western Palaearctic were included in this study, namely: *A. albostriella*, *A. wahlbergi*, *A. coryli*, and *A. viridis*. In addition, specimens assigned to a presumably new, yet hitherto undescribed species, provisionally named here as Taxon 1, were studied. This last species was distinguished according to its food plant associations, distribution, colouration, shape of male abdominal apodemes and genetic differences (Aguin-Pombo, 2002). Leafhoppers were sampled with a sweep net from different food plants foliage in several localities of Greece and Portugal. For each species, collection sites, food plants, number of specimens examined and the chromosome staining techniques applied are presented in Table 1.

Chromosome preparations

Adult males and females were fixed immediately after collection in the field in Carnoy fixative (96% ethanol and glacial acetic acid, 3 : 1) and kept at 5°C and in part at room temperature until slides were made. The storage temperature was found to have no effect on the quality of chromosome preparations. In males, preparations were made from testes, which were dissected in a drop of 45% acetic acid and squashed under a coverslip on a glass microscope slide. In females, preparations were made from ovaries and from mature eggs. The slides were frozen using dry ice, the coverslips were removed with a razor blade, and the preparations then dehydrated in fresh fixative (3 : 1) for 20 min and air dried. Slides were first examined under a phase-contrast microscope to check for the availability of meiotic divisions and the quality of chromosome spreads.

Standard chromosome staining

The preparations were stained with 2% aceto-orcein or/and using the Schiff-Giemsa method developed by Grozeva & Nokkala (1996) for the true bugs and previously applied successfully to many auchenorrhynchan species (e.g. Kuznetsova et al., 2009). In brief, the preparations were subjected to hydrolysis in 1 M HCl first at room temperature for 20 min and then at 60°C for 8 min, and thereafter stained in Schiff's reagent for 20 min. After rinsing thoroughly in distilled water, the slides were additionally stained with 4% Giemsa in Sorensen's phosphate buffer, pH 6.8, for 20 min. The slides were rinsed briefly in distilled water, air-dried and mounted in Entellan mounting medium (Merk, NJ, USA).

Conventional chromosome bandings

Banding techniques were used to investigate the karyotype of one of the studied species, *A. viridis*. The techniques applied included: silver-staining for the visualization of nucleolus organizing regions (NORs); C-banding for the detection of constitutive heterochromatic regions (C-heterochromatin); GC-specific CMA_3 staining and AT-specific DAPI staining to reveal the molecular composition of C-heterochromatic bands.

Ag-banding was performed using the technique described in Howell & Black (1980). Slides were treated with a mixture of gelatin solution/$AgNO_3$ solution (50%) at 65°C for 6–8 min in a moist chamber, rinsed three times with distilled water, air dried and stained with 4% Giemsa.

C-banding was performed according to Sumner (1972). Slides were treated at 30°C with a saturated solution of $Ba(OH)_2$ for periods ranging from 10 to 30 min. After being washed in distilled water, slides were incubated in 2× SSC at 60°C for 60 min, and then stained in a 5% Giemsa solution.

Treatment using DAPI (4'-6'-diamidino-2-phenylindole) and CMA_3 (chromomycin A_3) was done according to Schweizer (1976) and Donlon & Magenis (1983), respectively, with minor modifications. First, C-banding pretreatment was performed using 0.2 M HCl at room temperature for 30 min, followed by 7–8 min treatment in saturated $Ba(OH)_2$ at room temperature and then an incubation in 2× SSC at 60°C for 1 h. Thereafter, the preparations (omitting Giemsa treatment) were stained first with CMA_3 (0.4 µg/ml) for 25 min and then with DAPI (0.4 µg/ml) for 5 min. After staining, the preparations were rinsed in McIlvaine buffer, pH 7, and mounted in an antifade medium (700 µl of glycerol, 300 µl of 10 mM McIlvaine buffer, pH 7, and 10 mg of N-propyl gallate).

Fluorescence in situ hybridization (FISH)

To study the karyotypes of *A. albostriella* (from *Castanea sativa* and *Alnus* sp.), *A. wahlbergi*, *A. viridis*, *A. coryli*, and Taxon 1, FISH with 18S rDNA and $(TTAGG)_n$ telomeric probes was used.

DNA isolation, PCR amplification, probe generation

Genomic DNA from a male *Pyrrhocoris apterus* (L., 1758) (Heteroptera: Pyrrhocoridae) was isolated using a Chelex-100 extraction method. FISH with 18S rRNA gene and telomeric $(TTAGG)_n$ probes was performed on chromosomes of all of five *Alebra* species examined. The 18S rRNA probe was PCR amplified (see Grozeva et al., 2014, for details of the primers and PCR conditions used) from the genomic DNA of *P. apterus*, and labelled by PCR with biotin. The telomere probe was PCR amplified and labelled with Rhodamine-5-dUTP (GeneCraft, Köln, Germany) (see Grozeva et al., 2014).

Table 1. Leafhoppers studied and cytogenetic techniques used.

Species	Sampling locality	Sampling date	Food plant	No. ♂/♀ studied	Technique	Figures
A. albostriella (Fallen, 1826)	Portugal, Portoalegre (Ammaia) 525 m a.s.l.	27.vii.2008	*Alnus* sp.	4/0	Standard chromosome staining and FISH	2c
	Portugal, Chaves (Langarelhos) 800 m a.s.l.	27.vii.2008	*Castanea sativa*	3/0	Standard chromosome staining and FISH	2d
A. wahlbergi (Boheman, 1845)	Greece, Peloponnese (Kastanitsa) 900 m a.s.l.	10.vii.1989	*Castanea sativa*	0/1	Standard chromosome staining	1l
	Greece, Florina (Caries) 1100 m a.s.l.	27.vii.2009	*Acer opalus*	3/0	FISH	2f
A. coryli Le Quesne, 1977	Greece, Karpenisi (Agios Nicolaos) 980 m a.s.l.	8.vii.1991	*Quercus frainetto*	1/0	Standard chromosome staining	1j, k
	Greece, Florina (Agia Triada) 1200 m a.s.l.	28.vii.2009	*Corylus avellana*	1/0	FISH	2e
A. viridis Rey, 1894	Greece, Karpenisi (Agios Nicolaos) 1050 m a.s.l.	1.viii.1991	*Castanea sativa*	1/0	Standard chromosome staining	1c
	Portugal, Madeira Il. (Curral das Freiras-Eira do Serrado) 1000 m a.s.l.	15.–30. vii.2010	*Castanea sativa*	14/1	Standard chromosome staining	1a, b
	Spain, Salamanca (La Alberca) 1050 m a.s.l.	24.vii.2008	*Castanea sativa*	7/0	AgNOR-, C-, DAPI/CMA_3-bandings and FISH	1d–i, 2a, b
Taxon 1	Portugal, Beira Interior (Guarda) 875 m a.s.l.	23.vii.2008	*Quercus* sp.	4/0	Standard chromosome staining and FISH	1m, 2g

FISH procedure

This was performed as described by Schwarzacher & Heslop-Harrison (2000) with modifications (Grozeva et al., 2014). Chromosome preparations were dehydrated through 70/80/96% ethanol at RT (room temperature) and treated with 100 µg/ml RNaseA (Sigma-Aldrich, St. Louis, MO, USA) for 60 min at 37°C in a humid chamber; washed three times in 2× SSC (5 min each) at RT; dehydrated through 70/80/96% ethanol at RT; incubated in 5 mg/ml pepsin in 0.01 M HCl for 15 min at 37°C; washed sequentially in 1× PBS, in 1× PBS/0.05 M $MgCl_2$ for 5 min each, in 1% PFA in 1× PBS/0.05 M $MgCl_2$ for 10 min, in 1× PBS for 5 min, in 1× PBS/0.05 M $MgCl_2$ for 5 min at RT each; dehydrated through 70/80/96% ethanol at RT or ice cold and finally, dried. After pretreatment, a hybridization mixture containing about 100 ng of labelled probe, 50% formamide, 2× SSC, 10% (w/v) dextran sulfate, 1% (w/v) Tween 20 and 10 µg salmon-sperm DNA was added on preparations. Slides were mounted using glass coverslips and rubber cement. The slides were denatured for 5 min at 75°C, and then incubated for 42–44 h at 37°C. Following hybridization, they were washed in 2× SSC for 3 min at 45°C, then in 50% formamide in 2× SSC for 10 min at 45°C, twice in 2× SSC (10 min each) at 45°C, and blocked in 1.5% (w/v) BSA/4× SSC/0.1% Tween 20 for 30 min at 37°C in a humid chamber. The 18S rRNA gene probe was detected with 5µg/ml Avidin-Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA). Detection was performed in 1.5% BSA/4× SSC/0.1% Tween 20 for 1 h at 37°C. Slides were washed three times in 4× SSC/0.02% Tween 20 (10 min each) at 45°C and dehydrated through 70/80/96% ethanol at RT. Chromosomes were mounted in an antifade medium (ProLong Gold antifade reagent with DAPI, Invitrogen) and covered with a glass coverslip.

Microscopy and imaging

Images were taken using a Leica DM 6000 B 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

Testes and ovaries

In each of the *Alebra* species examined, testes in adult males were located ventrally in the anterior end of the abdomen and composed of five small roundish and transparent testicular lobes (follicles) each. Ovaries in mature females contained six small roundish telotrophic ovarioles each.

Chromosome complements

Representative mitotic and/or meiotic images of *A. viridis*, *A. wahlbergi*, *A. coryli* and Taxon 1 subjected to the different techniques applied (except FISH) are shown in Fig. 1a–m. Mitotic and meiotic images of *A. viridis* and meiotic ones for *A. coryli*, *A. wahlbergi*, Taxon 1 and *A. albostriella* (from *Castanea sativa* and *Alnus* sp., respectively) subjected to FISH with the 18S rDNA and TTAGG probes are shown in Fig. 2a–g. The karyotypes of *A. coryli*, *A. viridis*, Taxon I and *A. albostriella* collected from *Alnus* sp. were studied here for the first time. All the studied species were found to display the same chromosome complement, 2n = 22 + XX in females and 2n = 22 + X in males. The chromosomes possessed no primary constrictions, i.e. centromeres (see Figs 1a, b, d–f and 2a). In every species examined, the male karyotype consisted of one very large pair and ten medium pairs of autosomes, whilst the X chromosome was similar in size to the larger chromosomes within this group (see Fig. 1a, b, f). In male cells during the diakinesis and metaphase I (MI) stages of meiosis, 11 bivalents and a univalent X chromosome were present, n = 12 (11AA + X) (Figs 1c, g, h–j, m and 2b–g). Occasionally,

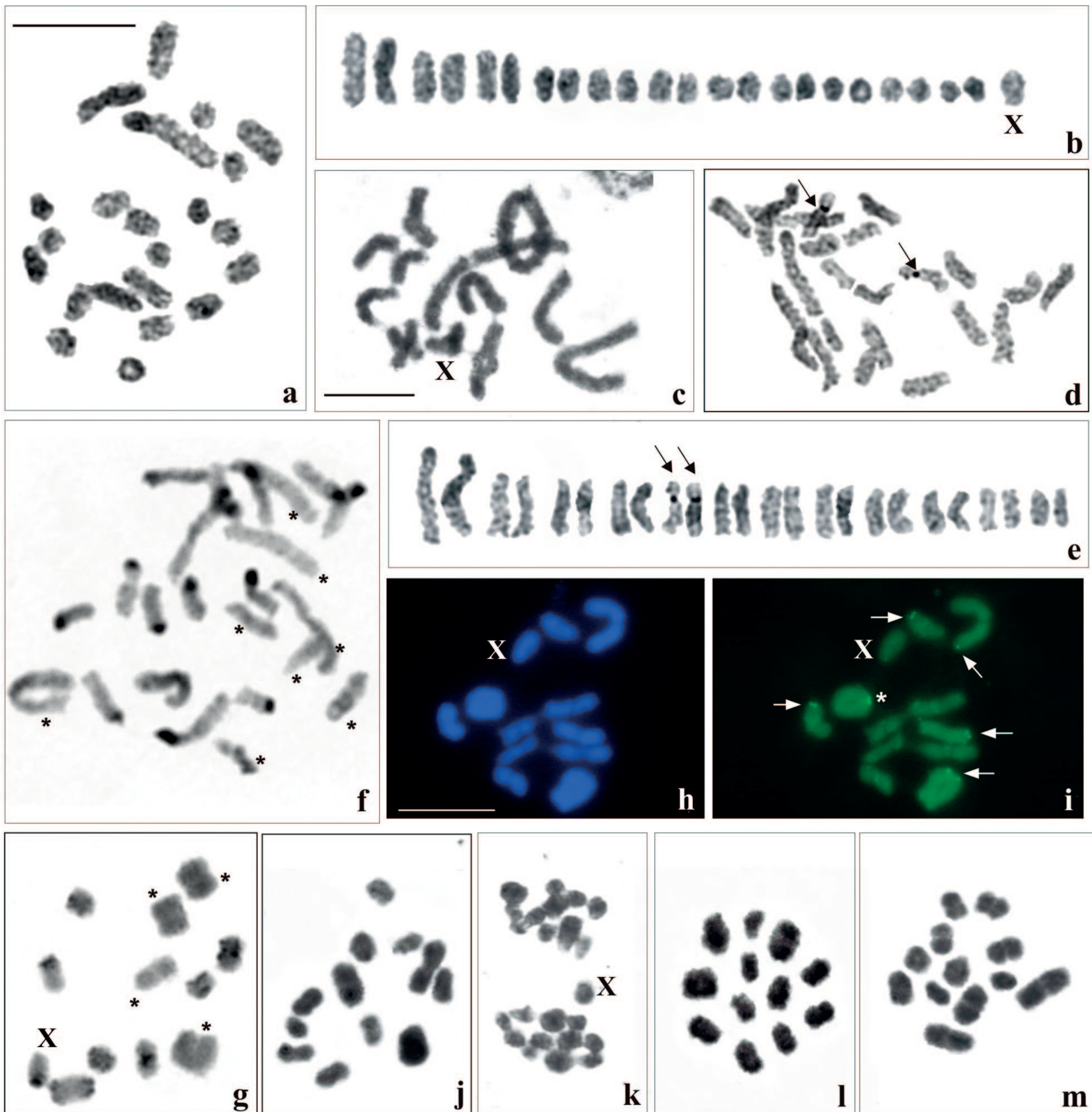

Fig. 1a–m. The karyotypes of *Alebra* spp: standard staining (a–c, j–m), silver staining (d, e), C-banding (f, g) and fluorochrome DAPI/CMA_3-staining (h, i). *A. viridis*. a, b – spermatogonial metaphase and karyogram, 2n = 22 + X; c – spermatocyte diplotene/diakinesis, n = 11AA + X, with bivalents displaying one to two chiasmata of terminal/subterminal or interstitial localizations; d, e – oogonial prometaphase and karyogram with NORs (arrows) located interstitially on a pair of medium-sized autosomes; f – spermatogonial prometaphase showing terminal C-bands at one end of all but 8 (asterisks) chromosomes; g – spermatocyte MI showing C-bands on all but 4 (asterisks) bivalents and on X chromosome; h, i – one and the same diakinesis after DAPI-staining (h) and CMA_3-staining (i). Signals are absent after DAPI and present after CMA_3 in at least 5 bivalents (arrows) including NOR-bivalent (asterisk). *A. coryli*. j – MI with n = 11AA + X (j); k – anaphase I with lagging X chromosome. *A. wahlbergi*. l – oogonial MI with n = 11AA + XX. Taxon I. m – MI with n = 11AA + X. Bar = 10 μm.

the number of chromosomes in the prophase/metaphase nuclei was higher or lower, most likely relating to some kind of meiotic disturbance (e.g. Fig. 2f, g). The majority of bivalents displayed a single subterminal/terminal or rarely interstitial chiasma, but two chiasmata were a common finding in larger bivalents (e.g. Figs 1c and 2c–e). At anaphase I (AI), autosomal bivalents and X-chromosome divided reductionally, with X chromosome frequently lagging behind autosomes, and the two daughter MII cells showed hence 11 (only autosomes) and 12 (plus X) chromosomes, respectively (e.g. Fig. 1k). The stage between two meiotic divisions, interkinesis, was absent, and the second division occurred immediately after the first one. In females, oogonial prometaphases showed 24 chromosomes (e.g. Fig. 1d, e). Additionally, it was possible to count the number of bivalents at MI in a single egg of *A. viridis* and

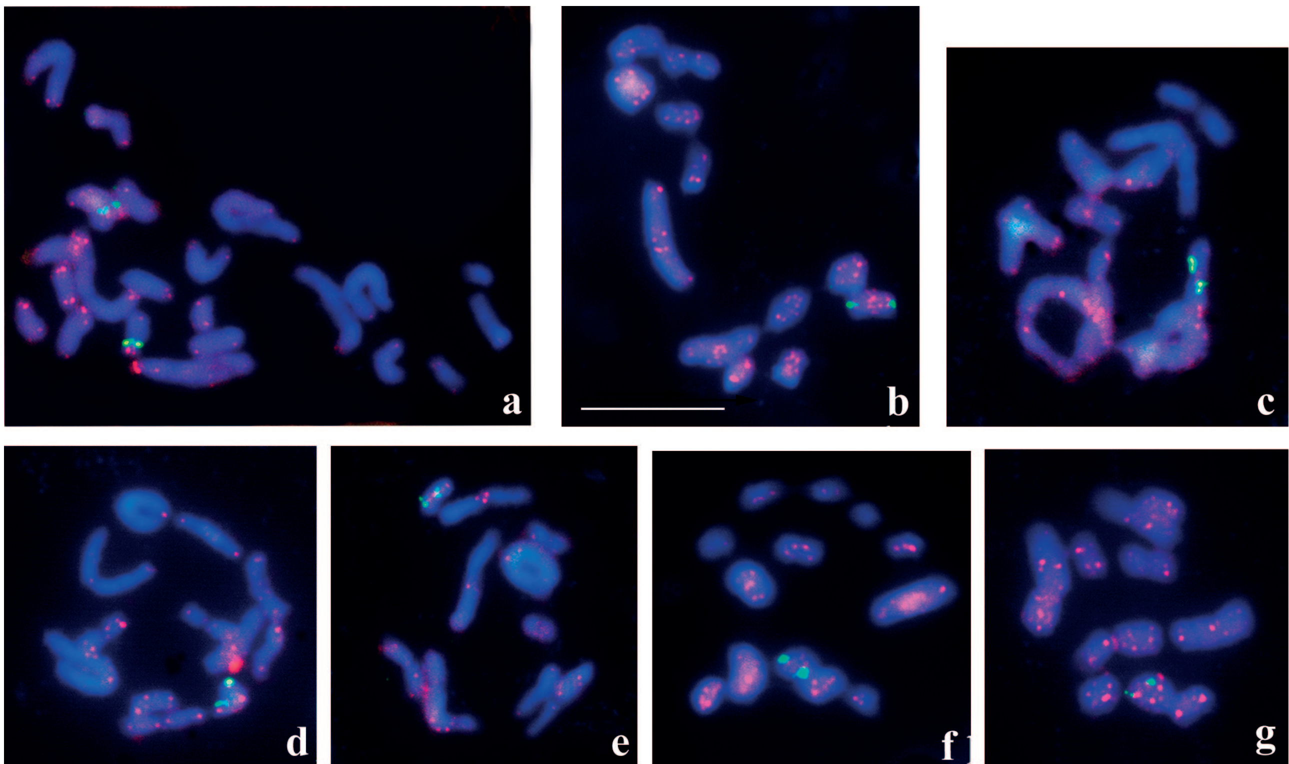

Fig. 2. Localization of rRNA gene clusters (green signals) and TTAGG telomeric repeats (red signals) on male mitotic (a) and meiotic (b–g) chromosomes of *Alebra* species. *A. viridis* – oogonial prometaphase (a) and diakinesis/MI (b); *A. albostriella* from *Alnus* sp. – diakinesis (c) and from *C. sativa* – diakinesis (d); *A. coryli* – diakinesis (e); *A. wahlbergi* – MI with one additional element (f); Taxon 1 – diakinesis/MI (g). Bar = 10 µm.

A. albostriella (from *Castanea sativa*), respectively, and in two eggs of *A. wahlbergi*. In every case, 12 bivalents were present, however, the sex chromosome bivalent could not be identified among them (e.g. Fig. 1l).

AgNOR-, C- and DAPI/CMA$_3$-banding

These techniques were applied to *A. viridis*. In a silver-stained oogonial prometaphase (Fig. 1d, e), argentum-positive sites, NORs, were present in a medium-sized pair of autosomes, presumably number 5, located at a distance well away from the end of every homologue. After C-banding, spermatogonial prometaphases showed heterochromatic regions (as C-bands) in all but eight chromosomes (Fig. 1f) whilst MI in all but four bivalents and in the X (Fig. 1g). In every case, C-bands were localized at only one telomere of a chromosome. C-negative autosomes were the three largest ones and one medium-sized pair marked with asterisks in Figs 1f and 1g. As indicated in Figs 1h and 1i, one and the same cell at diakinesis subjected to DAPI- and CMA$_3$-staining, respectively, showed an absence of DAPI-positive signals (Fig. 1h) and presence of CMA$_3$-positive signals on at least six bivalents (arrows), including that bearing the NOR (asterisk) (Fig. 1i).

Fluorescence in situ hybridization (FISH)

Chromosomal location of rDNA loci

In *A. viridis*, as expected from the results of both Ag-NOR- and CMA$_3$-staining (Fig. 1d, e, i), the 18S rDNA probe yielded specific hybridization signals at a subterminal region of every homologue of a medium-sized pair of autosomes (Fig. 2a, b; green spots). In other species examined, signals were likewise observed at subterminal regions of homologous chromosomes of a medium-sized bivalent (Fig. 2c–g; green spots). The occurrence of an rDNA-FISH signal at one end of each of the synapsed homologues of the NOR-bivalent allowed discrimination of the two chromosome ends of a homologue and hence allowed assessment of the kinetic behaviour of each chromosome end during the first meiotic division. At diakinesis/metaphase I, this bivalent could be seen oriented with homologous "kinetic" telomeres towards the opposite poles: the kinetic activity in both homologues could be present either at the chromosomal ends with signals (e.g. Fig. 2b, d) or at the ends without signals (e.g. Fig. c). As a whole, 29 cells were analyzed, and the frequencies of both orientations were similar (13 against 16).

The molecular structure of telomeres

In each of the *Alebra* species examined (Table 1), FISH using the TTAGG repeat as a probe showed the presence of hybridization signals at the ends of chromosomes (Fig. 2a–g; red spots). At diakinesis/MI, the bivalents most commonly revealed four signals, two in each of the homologous chromosomes of a bivalent (Fig. 2b–g).

DISCUSSION

As revealed in the present study, the leafhopper genus *Alebra* presents remarkable cytogenetic uniformity in the five species studied to date, despite the fact that some species were collected from different food plants (*A. albostri-*

ella from *Castanea sativa*, *Alnus* sp., *Fagus sylvatica* and *Quercus certis*; *A. wahlbergi* from *Acer opalus*, *Castanea sativa* and *Ulmus* sp.; *A. coryli* from *Quercus frainetto* and *Corylus avellana*) and also from distant geographical localities, i.e. Greece, Portugal and Spain (Kuznetsova et al., 2013; present paper). Like other hemipterans, the species studied are characterized by holokinetic chromosomes, i.e., chromosomes with non-localized centromeres. Both male and female karyotypes were identical in terms of chromosome number and structure, i.e. 2n = 22 + XX/X(0), with one very large pair of autosomes, the remaining pairs gradually decrease in size, and with the X chromosome close in size to the medium-sized autosomes. Among Cicadellidae, both X(0) and XY sex chromosome systems occur, the latter only occasionally, whilst the presently known chromosome numbers vary from 7 to 27 in male diploid complements, the most frequent numbers lying between 11 and 23 (Kirillova, 1988; Wei, 2010; Juan, 2011; Kuznetsova & Aguin-Pombo, in press). Thus, our results on the *Alebra* species here investigated fall within this range.

The uniformity in chromosome number in *Alebra* contrasts with the fairly variable number found in other genera of Typhlocybinae. This variability, although always within narrow limits, makes it difficult to establish the most characteristic number for each genus and for the subfamily as a whole. For example, the 19 species of the genus *Eurhadina* Haupt, 1929, which have been studied cytologically, vary broadly in chromosome number, with males showing karyotypes comprising 2n = 11 (1 species), 13 (6 species), 15 (1 species), 17 (7 species), and 19 (4 species) (Halkka, 1959; Juan, 2011). The genus *Empoasca* Walsh, 1864 is another group which seems to display a striking range in terms of chromosome number. This cosmopolitan genus with more than 1,000 described species is known to be diverse and by far the most species-rich genus within Cicadellidae (Southern & Dietrich, 2010). Apart from several parthenogenetic forms, most likely triploid (Aguin-Pombo et al., 2006), the 13 diploid bisexual species of *Empoasca* examined so far have 2n = 15 (4 species), 17 (3 species), 19 (4 species), and 21 (2 species) (Kirillova, 1988; Aguin-Pombo et al., 2006; Juan, 2011). It is argued by Southern & Dietrich (2010) that the genus requires comprehensive revision, and a cytogenetic approach might be important in the context of the systematics of this group. Theoretically, because of the holokinetic structure of the chromosomes, chromosome fusions and fissions can more easily operate in holokinetic groups, producing a variability of chromosome number (White, 1973). Simple fusion and fission of chromosomes are most probably associated with the divergence of the above mentioned genera. However, the genus *Alebra* with its conservative chromosome number, 2n = 23/24 (male/female) in the 5 studied species, seems to be an exception to this general rule, at least within Typhlocybinae.

The cytogenetic uniformity of this genus is further reinforced by the results of our FISH experiments, which detected 18S rRNA gene clusters, presumably, on the same pair of autosomes in every species examined. These findings contrast with those in the species of the genus *Philaenus* which differ from one another in the number and distribution of 18S rDNA (Maryańska-Nadachowska et al., 2013). In organisms with holokinetic chromosomes, rDNA sites tend to occupy terminal regions of chromosomes. For example, of the 92 species of heteropterans used for determining the number and location of rDNA sites, 86 species had these sites at a terminal position (reviewed in Grozeva et al., 2014). This suggests the existence of some limitations regarding the establishment of non-terminal rDNA sites in holokinetic chromosomes. Heckmann et al. (2011) hypothesized that the terminal location of rDNA might be a functional requirement to ensure chromosome stability in holokinetic karyotypes. According to their hypothesis, a secondary constriction (a site of NOR location) in the interstitial region would break the kinetochore plate along the holokinetic chromosome and result in a condition similar to an unstable dicentric chromosome, with consequent derangements in mitotic segregation. However, although there may be selection pressures which favor the terminal location of rDNA sites in holokinetic chromosomes (Roa & Guerra, 2012), data of some groups indicate that there are no strict restrictions to the functioning of rDNA clusters in other positions (e.g. Fenton et al., 1994; Kuznetsova et al., 2009; Nguyen et al., 2010; Maryańska-Nadachowska et al., 2013; Grozeva et al., 2014). In *Alebra* species, simultaneous labelling with telomeric and 18S rDNA probes (these results) showed that ribosomal genes were not located in the functional region of the telomere. Moreover, based on observation of meiotic stages, the FISH-rDNA sites could be referred to as terminal, however in silver-stained mitotic chromosomes of *A. viridis* active NORs were found positioned at a distance well away from the end of every homologue thereby suggesting that they were in fact of an interstitial location.

The molecular structure of telomeres in insects has, over the past decade or so, received special attention (e.g. Frydrychová et al., 2004; Vítková et al., 2005; Lukhtanov & Kuznetsova, 2010). Telomeres are specialized structures located at the ends of chromosomes that ensure their complete replication and protect them from fusion and abnormal recombinations. In most eukaryotic species, telomeric DNA is marked by the presence of long stretches of conserved short repeats that are synthesized by RNA-depended polymerases (telomerases) (Kipling, 1995). Telomeres of insect species are predominantly composed of a pentanucleotide sequence repeat $(TTAGG)_n$, which is thought to be an ancestral sequence of telomeres in this class (Sahara et al., 1999; Frydrychová et al., 2004; Lukhtanov & Kuznetsova, 2010). However, in some higher-level insect taxa this telomeric motif has been lost during evolution (reviewed in Frydrychová et al., 2004). It seems that this repeat has been lost in entire orders of insects, e.g. true flies, Diptera (Sahara et al., 1999), or within major lineages of an order, e.g. in the true bugs, heteropteran Hemiptera (Kuznetsova et al., 2012) and the parasitic Hymenoptera (Gokhman et al., 2014). Even so, it can also be missed at a much finer taxonomic scale, usually in a repeated man-

ner, as in the huge beetle order Coleoptera (Frydrychová & Marec, 2002).

There is a tendency to overgeneralize about the telomeric structure in a group from observations of a very few species and further work may reveal a far greater variety of patterns than previously thought (see e.g. Mravinac et al., 2011). Despite the suborder of the Hemiptera, the Auchenorrhyncha, comprises more than 42,000 valid species (Deitz, 2008), investigations of telomeric DNA sequences have to date been done on only 15 species belonging to 4 genera and 4 families in this group. Frydrychová et al. (2004) were the first to perform Southern hybridization with the "classical" insect telomeric repeat $(TTAGG)_n$ in *Calligypona pellucida* F. (Delphacidae). Maryańska-Nadachowska et al. (2013) applied FISH for the first time using the TTAGG repeats to eight species of the spittlebug genus *Philaenus* (Aphrophoridae), whilst recently Golub et al. (2014) reported FISH telomeric sequences for *Mapuchea chilensis* (Myerslopiidae). Lastly, as here presented, we provide data on five leafhopper species of the genus *Alebra* (Cicadellidae). All the species studied to date share TTAGG-type telomere repeats. Since the species investigated are from two phylogenetically-distant superfamilies, the Fulgoroidea (Delphacidae) and Membracoidea (Aphrophoridae, Myerslopiidae and Cicadellidae), we tentatively conclude that these large superfamilies and perhaps even the hemipteran suborder Auchenorrhyncha as a whole have retained the "classical" insect telomeric motif, $(TTAGG)_n$. Ultimately though, further empirical work is required to validate this hypothesis.

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