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Karyotype diversity of pseudoscorpions of the genus *Chthonius* (Pseudoscorpiones, Chthoniidae) in the Alps

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Abstract

Pseudoscorpions are found in almost all terrestrial habitats. However, their uniform appearance presents a challenge for morphology-based taxonomy, which may underestimate the diversity of this order. We performed cytogenetic analyses on 11 pseudoscorpion species of the genus *Chthonius* C. L. Koch, 1843 from the Alps, including three subgenera: *Chthonius* (*Chthonius*) C. L. Koch, 1843, *C.* (*Ephippiochthonius*) Beier, 1930 and *C.* (*Globochthonius*) Beier, 1931 inhabiting this region. The results show that the male diploid number of chromosomes ranges from 21–35. The sex chromosome system X0 has been detected in all male specimens. The X sex chromosome is always metacentric and represents the largest chromosome in the nucleus. Achiasmatic meiosis, already known from the family Chthoniidae, was further confirmed in males of *Chthonius.* C-banding corroborated the localization of constitutive heterochromatin in the centromere region, which corresponds to heteropycnotic knobs on the standard chromosome preparations. Morphological types and size differentiation of chromosomes in the karyotype suggest that the main chromosomal rearrangements in the evolution of *Chthonius* are centric or tandem fusions resulting in a decrease in the number of chromosomes. Pericentric inversions, inducing the change of acrocentric chromosomes into biarmed chromosomes, could also be expected. Variability in chromosome morphology and number was detected in several species: *C.* (*C.*) *ischnocheles* (Hermann, 1804), *C.* (*C.*) *raridentatus*, *C.* (*C.*) *rhodochelatus* Hadži, 1930, and *C.* (*C.*) *tenuis* L. Koch, 1873. We discuss the intraspecific variability within these species and the potential existence of cryptic species.

Keywords

Karyotype evolution, chromosome fusion, sex chromosomes, X0 sex chromosome system, achiasmatic meiosis

Introduction

Pseudoscorpions are the fourth most numerous order of the class Arachnida, comprising 3385 described species currently classified into 439 genera and 26 families (Harvey 2013). However, the true diversity of the order might be underestimated due to the challenging morphology of the group in addition to the very small body size of individuals (usually not exceeding 2mm). The taxonomy of pseudoscorpions is often based on character states with poorly defined variability. For instance, the range of frequently used measurements and counts of specific setae may differ significantly depending on the number of specimens analysed per species. Furthermore, overlaps in species-specific character states complicate the exact identification of many species complexes (compare e.g. Beier 1963, Christophoryová et al. 2011, Gardini 2013, 2014). Molecular techniques have further revealed the limitations of traditional morphology-based classification of pseudoscorpions. Despite the absence of molecular techniques in formal species delimitation in this group, independent lineages that may correspond to cryptic species have been detected in number of cases (e.g. Wilcox et al. 1997, Moulds et al. 2007, Pfeiler et al. 2009, van Heerden et al. 2013, Harrison et al. 2014). Another useful method for detecting unaccounted diversity is karyotype analysis. Significant interspecific differences in karyotypes may reveal distinct lineages constituting cryptic species in some morphologically challenging groups (e.g. Řezáč et al. 2007, Lukhtanov et al. 2015). In pseudoscorpions, the use of cytogenetic methods has enabled the detection of interspecific variability (Troiano 1990, Šťáhlavský et al. 2006, 2013) and has led to the description of a new species (Zaragoza and Šťáhlavský 2008).

Currently, there is karyotype information for about 51 species belonging to 25 genera from eight families (Atemnidae, Garypinidae, Geogarypidae, Cheliferidae, Chernetidae, Chthoniidae, Neobisiidae, Olpiidae) (Šťáhlavský 2016). The chromosome number among pseudoscorpions ranges from 2n = 7–143 (Šťáhlavský 2016), but the variability of the chromosome number tends to be specific for each group. Despite the previous success of using karyological differences for species delimitation in pseudoscorpions (Zaragoza and Šťáhlavský 2008), this approach is limited due to the lack of cytogenetic data for comparison. Most of the available data belongs to both geographically and evolutionary distant lineages, where major differences in karyotype are not surprising. Often, only a few specimens were sampled per species/population, resulting in a lack of information about intraspecific variability (e.g. Šťáhlavský et al. 2006, 2009, 2012). In order to shed light on the karyotype differentiation among more closely related species and to enhance our knowledge on intraspecific karyotype variability, we focus on cytogenetic analyses of the genus *Chthonius* from the alpine region.

The genus *Chthonius* comprises 260 described species, mainly inhabiting leaf litter (Harvey 2013). In the Alps, *Chthonius* is represented by 44 species classified into three subgenera: *Ephippiochthonius*, *Chthonius* and *Globochthonius* (Gardini 2013, 2014). Several species have been newly described, or taken from the synonymy during the recent morphological revision of the genus (Gardini 2013, 2014). New faunistic data

also suggest that the distribution of species ranges in the Alps may be different from previously thought (Gardini 2000).

Due to altitudinal zonation, high mountain regions such as the Alps offer a wide range of habitats and generally present high species richness. High levels of diversity and endemism are traditionally explained by the geographic isolation of organisms with specific ecological preferences in relatively small areas and distribution range shifts during periods of glaciation (Schmitt 2009). These two factors may have significant effects on organisms with low dispersal potential, where geographic isolation may lead to karyotype differentiation that subsequently presents an effective reproductive barrier (e.g. King 1993, Kawakami et al. 2011). Sedentary organisms such as ground dwelling pseudoscorpions of the genus *Chthonius* are excellent models for cytogenetic studies within the context of this ecologically diverse region with a dynamic climatic history.

Material and methods

Individuals used in the present study were obtained from leaf litter sifting or were collected individually under stones. The collection data for the species used in this study are listed below. After the name of each species, the information is lined-up in brackets as following: total number of analysed specimens / total number of analysed cells / total number of measured cells.

Chthonius (*Chthonius*) *alpicola* Beier, 1951 (2/16/5): Italy: Forni di Sotto (46.399 N, 12.689 E), 1 ♀; Italy: Santa Caterina (46.512 N, 13.395 E), 1 ♂.

Chthonius (*Chthonius*) *carinthiacus* Beier, 1951 (7/31/5): Italy: Lago di Ledro (45.866 N, 10.741 E), 1 ♂; Italy: Passo Cereda (46.194 N, 11.914 E), 1 ♂; Italy: Tarvisio (46.527 N, 13.545 E), 2 ♂; Italy: Tramonti di Sopra (46.353 N, 12.783 E), 1 ♂; Italy: Vittorio Veneto (45.983 N, 12.283 E), 1 ♂; Slovenia: Bohinjska Bistrica (46.279 N, 13.962 E), 1 ♂.

Chthonius (*Chthonius*) *ischnocheles* (Hermann, 1804), cytotype I (2/27/5): France: Glère (47.342 N, 06.971 E), 1 ♂; Switzerland: Bieane (47.123 N, 07.208 E), 1 ♂.

Chthonius (*Chthonius*) *ischnocheles* (Hermann, 1804), cytotype II (13/120/10): Switzerland: Valangin (47.016 N, 06.908 E), 1 ♂; Italy: Castello (46.027 N, 09.046 E), 1 ♂; Italy: Egna (46.313 N, 11.290 E), 2 ♂; Italy: Lebenberg (46.640 N, 11.135 E), 1 ♂; Italy: Lichtenberg (46.632 N, 10.564 E), 3 ♂; Italy: Pannone (45.871 N, 10.933 E), 1 ♂; Italy: Vermiglio (46.290 N, 10.678 E), 4 ♂.

Chthonius (*Chthonius*) *raridentatus* Hadži, 1930, cytotype I (35/169/129): Italy: Tramonti di Sopra (46.353 N, 12.783 E), 1 ♀; Slovenia: Kamnik (46.224 N, 14.614 E), 1 ♂; Slovenia: Kamniška Bistrica (46.310 N, 14.601 E), 1 ♂; Slovenia: over Bohinska Bistrica (46.276 N, 14.007 E), 1 ♂; Slovenia: Roče (46.108 N, 13.816 E), 31 ♂.

Chthonius (*Chthonius*) *raridentatus* Hadži, 1930, cytotype II (4/105/15): Austria: Barenhtal (46.482 N, 14.170 E), 1 ♂; Slovenia: Bohinjska Bistrica (46.279 N, 13.962 E), 2 ♂; Slovenia: Roče (46.108 N, 13.816 E), 1 ♂.

Chthonius (*Chthonius*) *rhodochelatus* Hadži, 1933, cytotype I (5/81/8): Italy: Lago di S. G. Sanzena (46.357 N, 11.069 E), 1 ♂; Italy: Loppio (45.859 N, 10.924 E), 1 ♂; Italy: Nuova Olomio (46.161 N, 09.433 E), 1 ♂; Italy: Puria (46.033 N, 09.049 E), 1 ♂; Italy: Sondrio (46.175 N, 09.857 E), 1 ♂.

Chthonius (*Chthonius*) *rhodochelatus* Hadži, 1933, cytotype II (1/32/6): 1 ♂; Italy: Lago di S. G. Sanzena (46.357 N, 11.069 E), 1 ♂.

Chthonius (*Chthonius*) *tenuis* L. Koch, 1873, cytotype I (37/514/10): Italy: Buisson (45.837 N, 07.605 E), 2 ♂; Italy: Cannobio (46.059 N, 08.699 E), 1 ♂; Italy: Carona (46.017 N, 09.780 E), 3 ♂; Italy: Dezzo di Scalve (45.974 N, 10.104 E), 5 ♂; Italy: Forte di Bard (45.606 N, 07.744 E), 1 ♂; Italy: Imperia (43.939 N, 07.829 E), 2 ♂; Italy: Isoladi Fondra (45.966 N, 09.734 E), 1 ♂; Italy: Loreglia (45.902 N, 08.370 E), 2 ♂; Italy: Melle (44.560 N, 07.314 E), 1 ♂; Italy: Noli (44.200 N, 08.405 E), 2 ♂, 1♀; Italy: Pont-Saint-Martin (45.607 N, 07.810 E), 1 ♂; Italy: Puria (46.033 N, 09.049 E), 1 ♂; Italy: Sondrio (46.175 N, 09.857 E), 2 ♂; Italy: Trarego Viggiona (46.042 N, 08.652 E), 3 ♂; Italy: Vermiglio (46.290 N, 10.678 E), 1 ♂; Italy: Zambla (45.877 N, 09.777 E), 2 ♂; Switzerland: Engelberg (46.828 N, 08.413 E), 4 ♂; Switzerland: Mauracker (46.279 N, 07.813 E), 2 ♂.

Chthonius (*Chthonius*) *tenuis* L. Koch, 1873, cytotype II (1/34/8): Slovenia: over Bohinska Bistrica (46.276 N, 14.007 E), 1 ♂.

Chthonius (*Chthonius*) *tenuis* L. Koch, 1873, cytotype III (1/19/8): Austria: Altfinkenstein (46.548 N, 13.876 E), 1 ♂.

Chthonius (*Chthonius*) *tenuis* L. Koch, 1873, cytotype IV (1/8/8): Italy: Pont-Saint-Martin (45.607 N, 07.810 E), 1 ♂.

Chthonius (*Chthonius*) *tenuis* L. Koch, 1873, cytotype V (2/17/8): Italy: Noli (44.200 N, 08.405 E), 2 ♂.

Chthonius (*Ephippiochthonius*) *boldorii* Beier, 1934 (11/120/10): Austria: Altfinkenstein (46.548 N, 13.876 E), 2 ♂; Austria: Saak (46.592 N, 13.626 E), 2 ♂; Austria: Tscheppachslucht (46.503 N, 14.284 E), 2 ♂; Switzerland: Somazzo (45.884 N, 08.992 E), 1 ♂; Italy: Loppio (45.859 N, 10.924 E), 1 ♂; Italy: Mezzoldo (46.015 N, 09.665 E), 1 ♂; Italy: Puria (46.033 N, 09.049 E), 1 ♂; Italy: Vittorio Veneto (45.983 N, 12.283 E), 1 ♂.

Chthonius (*Ephippiochthonius*) *fuscimanus* Simon, 1900 (2/26/8): Italy: Selva di Cerda (46.445 N, 12.024 E), 2 ♂.

Chthonius (*Ephippiochthonius*) *nanus* Beier, 1953 (3/21/6): Italy: Imperia (43.939 N, 07.829 E), 3 ♂.

Chthonius (*Ephippiochthonius*) *tetrachelatus* (Preyssler, 1790) (3/67/14): Austria: Altfinkenstein (46.548 N, 13.876 E), 1 ♂; Austria: Vittorio Veneto (45.983 N, 12.283 E), 1 ♂; Slovenia: Srpenica (46.295 N, 13.493 E), 1 ♂.

Chthonius (*Globochthonius*) *poeninus* Mahnert, 1979 (1/44/6): Italy: Castello (46.027 N, 09.046 E), 1 ♂.

Chromosome preparations were obtained by the "plate spreading" method (Traut 1976), which has been successfully applied on the genus *Chthonius* (Šťáhlavský and

Král 2004). Male gonads used in this protocol were immersed into hypotonic solution of 0.075 M KCL for 20 min and subsequently fixated in methanol: acetic acid (3:1) solution for 20 min. Fixed tissue was transferred onto a microscope slide, the cell dissociated and spread in a drop of 60% acetic acid on the histological plate (40-45 °C). Chromosome preparations were stained in a 5% Giemsa solution in Sörensen phosphate buffer for 30 min (Šťáhlavský and Král 2004). Constitutive heterochromatin was visualised by C-banding, following the standard protocol (Sumner 1972) on selected preparations of seven males of *Chthonius raridentatus* and one male of *Chthonius tetrachelatus*. Chromosome preparations were observed in Olympus AX70 Provis microscope and documented with an Olympus DP71 camera. Frequently, the centromeres were indistinct during the mitotic metaphases, thus cells at pachytene, postpachytene, metaphase I or metaphase II with a clearly distinct centromere position were used for karyotype analyses. Photographed chromosomes were checked for standard karyotype characteristics such as number, relative size, and morphology of the chromosomes using LEVAN plugin (Sacamato and Zacaro 2009) for IMAGEJ 1.47 program (http://imagej.nih.gov/ij/), which allows a direct classification of chromosomal types and their relative size calculation. Morphology of the chromosomes was determined following standard classification (Levan et al. 1964). Relative length of chromosomes (RCL) was calculated for a haploid set including the X sex chromosome. In *Chthonius raridentatus* cytotype I from Roče (Slovenia), given the abundance of dividing cells, we applied t-test using software STATISTICA 9.0 (www.statsoft.com) to determine whether the measurements of chromosomes are significantly different (the threshold chosen for statistical significance $\alpha = 0.05$) during distinct spiralization of chromosomes of various meiotic (postpachytene, metaphase I, metaphase II) and mitotic (mitotic metaphase) stages (Suppl. material 1).

Results

Karyology data were obtained for 11 species of pseudoscorpions from the genus *Chthonius* (Chthoniidae) (Table 1) comprising *Chthonius* (*Chthonius*), *Chthonius* (*Ephippiochthonius*) and *Chthonius* (*Globochthonius*) subgenera.

Chthonius (*Chthonius*) *alpicola* Beier, 1951

The diploid set consists of 21 chromosomes in male (Fig. 1a) and 22 chromosomes in female. The male karyotype comprises ten pairs of acrocentric autosomes and one metacentric X sex chromosome. The first three acrocentric pairs of autosomes are significantly longer (RCLs 13.23%, 12.49% and 10.92%) than the remaining autosome pairs that gradually decrease in RCL from 7.98% to 2.97%. The X represents the largest chromosome in the karyotype reaching the length of 27.36% of the haploid set.

Table 1. Summary of the cytogenetic data for the genus *Chthonius*.

		Sex	Morphology of autosomes					
Species	**2n**	**chrom.**	**M**	**SM**	**ST**	**A**	**Country**	**References**
C. (*C.*) *alpicola*	21	X0				20	IT	present study
C. (*C.*) *carinthiacus*	35	X0				34	CZ, IT	Šťáhlavský and Král 2004, present study
C. (*C.*) *heterodactylus*	33	X0		4		28	RO	Šťáhlavský and Král 2004
C. (*C.*) *ischnocheles*, cytotype I	31	X0	4			26	CH, FR	present study
C. (*C.*) *ischnocheles*, cytotype II	35	X0	4	2		28	CH, IT	present study
C. (*C.*) *litoralis*	35	X0			2	32	GR	Šťáhlavský and Král 2004
C. (*C.*) *orthodactylus*	33	X0		2		30	CZ	Šťáhlavský and Král 2004
C. (*C.*) *raridentatus*, cytotype I	29	X0	2		2	24	SI	present study
C. (*C.*) *raridentatus*, cytotype II	29	X0	4			24	SI	present study
C. (*C.*) *rhodochelatus*, cytotype I	35	X0	4			30	IT	present study
C. (*C.*) *rhodochelatus*, cytotype II	35	X0	2	2		30	IT	present study
C. (*C.*) *tenuis*, cytotype I	35	X0	2			32	CH, IT	present study
C. (*C.*) *tenuis*, cytotype II	33	X0	2			30	SI	present study
C. (*C.*) *tenuis*, cytotype III	33	X0	4		2	26	AT	present study
C. (*C.*) *tenuis*, cytotype IV	33	X0				32	IT	present study
C. (*C.*) *tenuis*, cytotype V	21	X0	6			14	IT	present study
C. (*E.*) *boldorii*	35	X0			2	32	AT, IT	present study
C. (*E.*) *fuscimanus*	35	X0				34	CZ, IT	Šťáhlavský and Král 2004, present study
C. (*E.*) *tetrachelatus*, cytotype I	35	X0		2		32	CZ	Šťáhlavský and Král 2004
C. (*E.*) *tetrachelatus*, cytotype II	35	X0		2	2	30	SI	present study
C. (*E.*) *nanus*	25	X0	2			22	IT	present study
C. (*E.*) *sp. 1*	29	X0	2	4		22	GR	Šťáhlavský and Král 2004
C. (*E.*) *sp. 2*	21	X0	4	2	2	12	GR	Šťáhlavský and Král 2004
C. (*G.*) *poeninus*	25	X0	2			22	IT	present study

Abbreviations: A – acrocentric, AT – Austria, CH – Switzerland, CZ – Czech Republic, FR – France, GR – Greece, IT – Italy, M – metacentric, RO – Romania, SI – Slovenia, SM – submetacentric, ST – subtelocentric

Chthonius (*Chthonius*) *carinthiacus* Beier, 1951

Seven individuals from central and eastern parts of the Alps displayed 2n = 35 in all cases (Fig. 1b). The karyotype of this species comprises 17 pairs of acrocentric autosomes and one metacentric X sex chromosome. The RCL of autosomes gradually decreases from 7.29% to 2.34%. The RCL of the X chromosome is 23.30%.

Chthonius (*Chthonius*) *ischnocheles* (Hermann, 1804)

Variability in chromosome number and morphology was detected in this species; two different cytotypes were distinguished. Cytotype I was detected only in two males from two geographically proximate localities in Switzerland and France. The diploid set of this cytotype comprises 31 chromosomes (Fig. 1c). There are 13 pairs

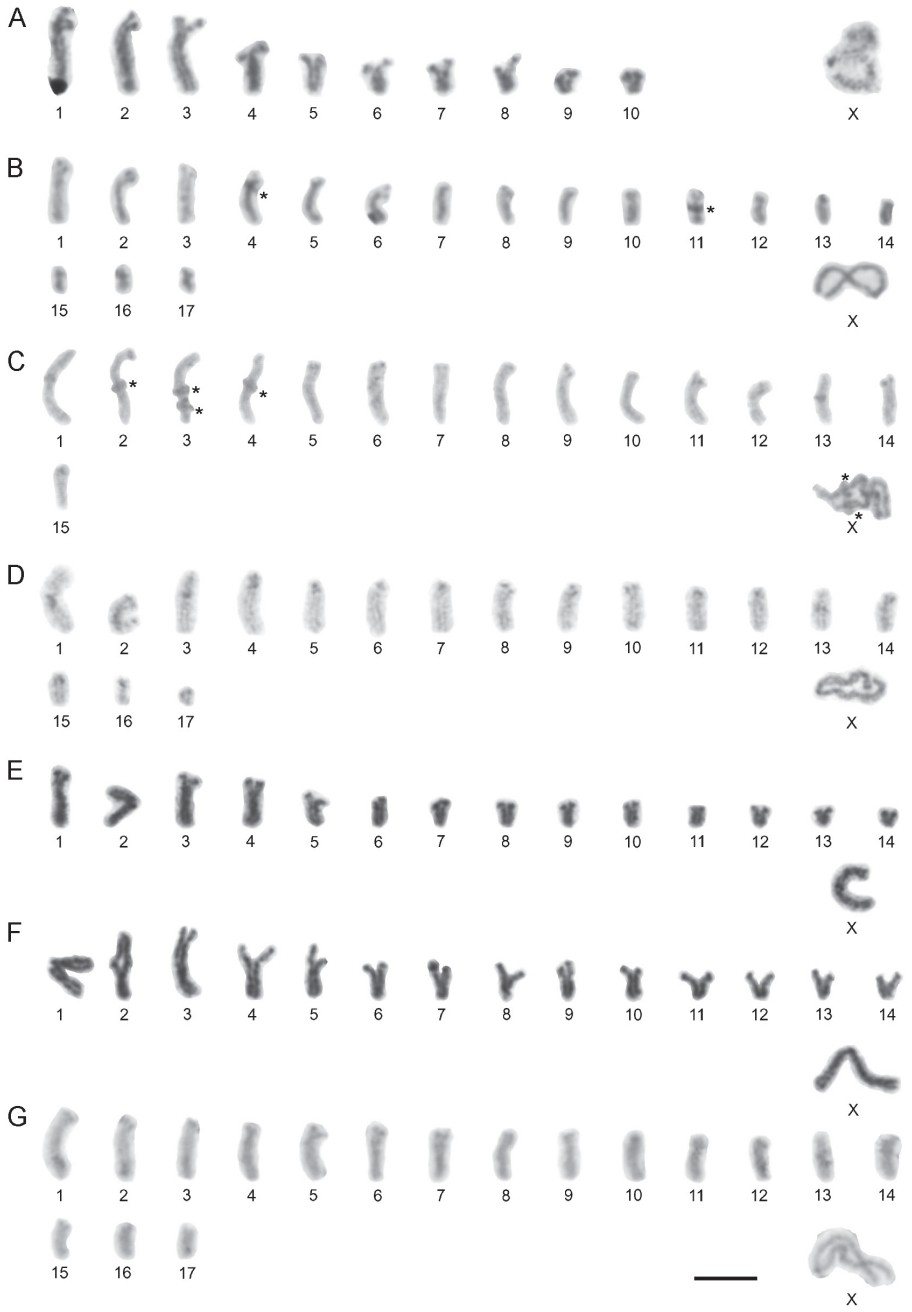

Figure 1. Karyotypes of *Chthonius* (*Chthonius*) males based on postpachytene and metaphase I. **A** *C.* (*C.*) *alpicola* (2n = 21, X0) (large black spot on the first autosome pair represents overlap with the sperm) **B** *C.* (*C.*) *carinthiacus* (2n = 35, X0) **C** *C.* (*C.*) *ischnocheles*, cytotype I (2n = 31, X0) **D** *C.* (*C.*) *ischnocheles*, cytotype II (2n = 35, X0) **E** *C.* (*C.*) *raridentatus*, cytotype I (2n = 29, X0) **F** *C.* (*C.*) *raridentatus*, cytotype II (2n = 29, X0) **G** *C.* (*C.*) *rhodochelatus*, cytotype II (2n = 35, X0). Asterisks indicate chromosome overlaps. Scale bar = 10 μm.

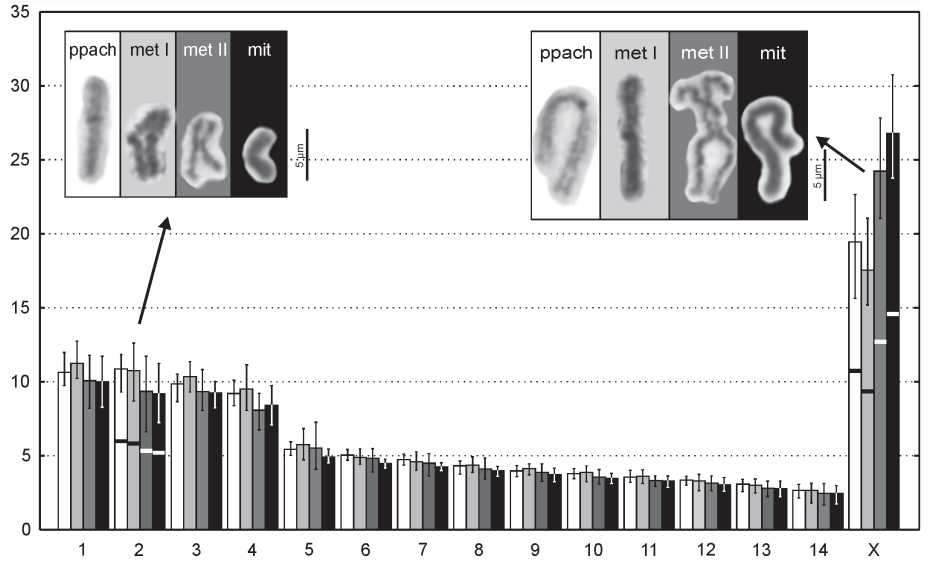

Figure 2. Ideograms of *Chthonius* (*Chthonius*) *raridentatus* cytotype I from Roče (y axis - % of the chromosome length of the haploid set). Comparison of different meiotic (ppach - postpachytene (white), met I - metaphase I (light grey), met II - metaphase II (dark grey)) and mitotic (mit - mitotic metaphase (black)) stages with examples of chromosomes 2 and X. Ideograms include min. - max. values and the centromeres are indicated only in metacentric chromosomes (all other chromosomes are acrocentrics).

of acrocentric and two pairs of metacentric autosomes (pairs No. 1 and 13) and one metacentric X chromosome. Autosome RCLs decrease gradually from 8.47% to 3.66%. The RCL of the X is 17.85%. Cytotype II was detected from seven different localities. The diploid number of chromosomes of this cytotype is 35 (Fig. 1d). The karyotype comprises 14 pairs of acrocentric autosome pairs, two metacentric pairs (pairs No. 1 and 13), and one submetacentric pair (pair No. 3), with the X chromosome metacentric. The RCLs of the autosomes decrease gradually from 7.47% to 1.45% and the last autosome is significantly shorter than the previous pair. The RCL of the X is 17.12%.

Chthonius (*Chthonius*) *raridentatus* Hadži, 1930

The diploid number of chromosomes in all analysed individuals was 29 (Fig. 1e). Detailed analyses detected the existence of two cytotypes within this species. Cytotype I was found in most of the males, comprising 12 acrocentric autosome pairs, one metacentric pair (pair No. 2), and one subtelocentric pair (pair No. 5), and one metacentric X chromosome. Cytotype II was detected only in two individuals from different localities in northwest Slovenia. The karyotype is composed of 12 pairs of acrocentric and two pairs of metacentric autosomes (pairs No. 1 and 2), and one metacentric X (Fig. 1f). The karyotypes in both cytotypes showed a length differentiation of the auto-

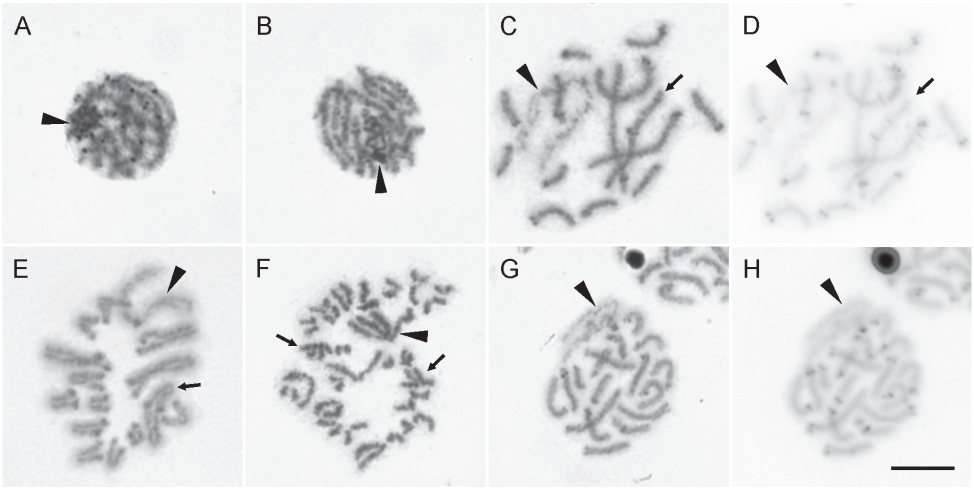

Figure 3. Meiotic chromosomes of *Chthonius* (*Chthonius*) *raridentatus*, cytotype I (**A–F**) and *Chthonius* (*Ephippiochthonius*) *tetrachelatus* (**G, H**). **A** zygotene **B** pachytene **C, D** postpachytene **E** metaphase I **F** metaphase II **G, H** postpachytene. Standard chromosomes stained with Giemsa (**A–C, E–G**) and the chromosomes after C-banding stained with DAPI (inverted) (**D, H**). Arrowheads indicate X sex chromosomes, arrows indicate metacentric autosomes. Scale bar = 10 μm.

somes; the first three pairs of autosomes are longer (roughly 3–3.5 ×) than the remaining chromosomes in the nucleus. In cytotype I, the first three autosomes are also considerably longer (RCLs 10.91%, 10.12% and 9.84%) than the remaining autosomes that gradually decrease in size, and X sex chromosome is the longest chromosome in the karyotype (Fig. 2). In cytotype II, RCLs of the three longest autosomes is 10.91%, 9.55% and 9.39% of the haploid set. The RCLs of the remaining autosomes gradually decrease from 7.39% to 2.90%, and the RCL of the X is 20.01%.

In cytotype I, we tested differences of the chromosome lengths and also arm ratio in biarmed chromosomes during distinct spiralization of several mitotic (mitotic metaphase (N = 44)) and meiotic stages (postpachytene (N = 14), metaphase I (N = 42), metaphase II (N = 29). We detected significant differences in two thirds of the comparisons among chromosomes (Suppl. material 1). The most considerable difference is noticed in the X chromosome during different stages (Fig. 2). It is probably an effect of different spiralization states of the X during meiosis and mitosis, also visible as different degrees of heteropycnosis (see paragraph below). The arm ratio of biarmed chromosomes (pair No. 2 and X) is significantly different only in few cases (Suppl. material 1) and the metacentric morphology has been detected during all analyzed stages.

During the meiosis X chromosome undergoes changes in condensation. During early prophase (leptotene-zygotene), the X forms a prominent spherical body and exhibits positive heteropycnosis (Fig. 3a). The body starts expanding during pachytene. The individual positively heteropycnotic arms of the X chromosome become visible, however they are still connected by their ends (Fig. 3b). During postpachytene, the sex chromosome becomes slightly negatively heteropycnotic (Fig. 3c). During metaphase I, all the chromosomes be-

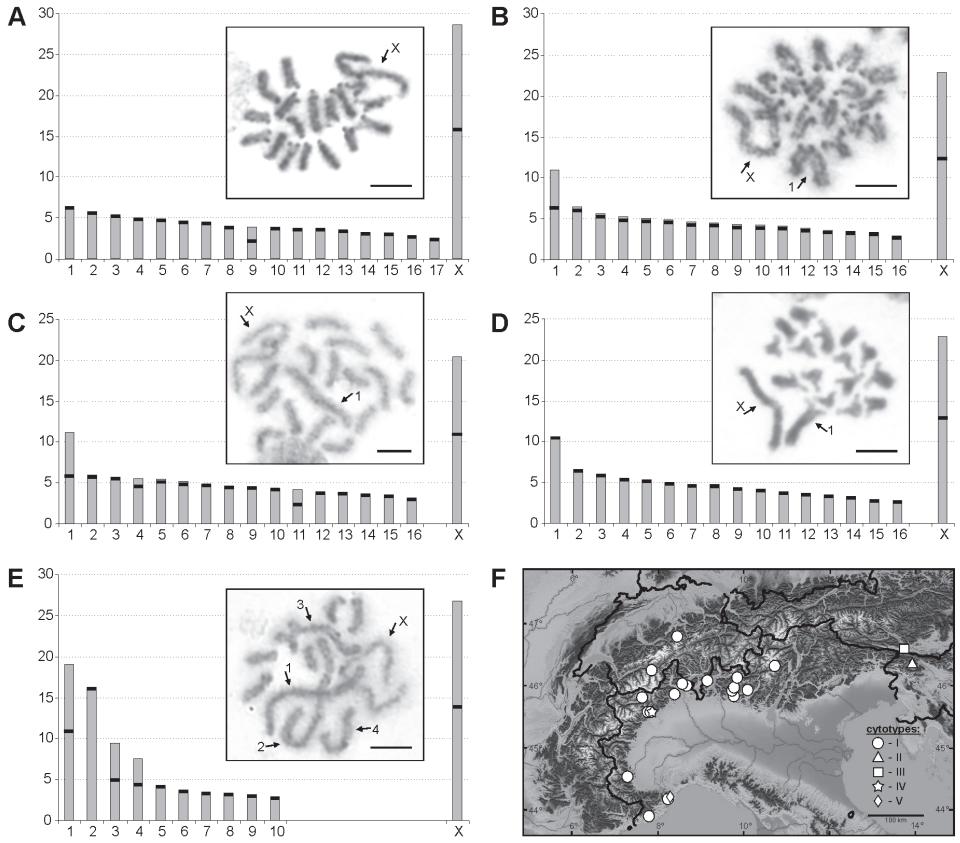

Figure 4. Ideograms of *Chthonius* (*Chthonius*) *tenuis* cytotypes (y axis - % of the chromosome length of the haploid set) and examples of chromosomes in postpachytene and metaphase I. **A** cytotype I (2n = 35, X0) **B** cytotype II (2n = 33, X0) **C** cytotype III (2n = 33, X0) **D** cytotype IV (2n = 33, X0) **E** cytotype V (2n = 21, X0) **F** distribution of cytotypes. Arrows indicate X sex chromosome and extra-large autosomes.

come isopycnotic except for prominent knobs in centromeric regions (Fig. 3e). These knobs are visible on the chromosomes from pachytene to metaphase I, but cannot be detected during metaphase II (Fig. 3f) and anaphase II. C-banding and analysis of cells at metaphase II of this species confirmed that constitutive heterochromatin is exclusively localized in these prominent knobs that correspond to the centromere regions (cf. Fig. 3c and 3d).

Chthonius (*Chthonius*) *rhodochelatus* Hadži, 1933

Variability in chromosome morphology was detected in this species, resulting in two distinguishable cytotypes. The diploid number of chromosomes in cytotype I is 35. The karyotype comprises 15 acrocentric and two metacentric autosome pairs (pairs No. 2 and 13), and one metacentric X chromosome. No significant length differentiation of autosomes

was detected. The RCLs of the autosomes gradually decrease from 6.25% to 3.24%. The RCL of the X is 22.43%. Cytotype II was detected in one male from a locality in the central Alps. This individual had a diploid set of 35 chromosomes (Fig. 1g). The karyotype comprises 15 acrocentric pairs, one metacentric pair (pair No. 13), and one submetacentric pair of autosomes (pair No. 14), with the X chromosome metacentric. The RCLs of the autosomes gradually decrease from 6.73% to 2.92%. The RCL of the X is 20.38%.

Chthonius (*Chthonius*) *tenuis* L. Koch, 1873

Variability in chromosome number and morphology was detected in this species, resulting in five distinguishable cytotypes (Fig. 4). Most individuals displayed cytotype I: 2n = 35 with 16 pairs of acrocentric and one pair of metacentric autosomes (pair No. 9), and one metacentric X chromosome (Fig. 4a). The RCLs of the autosomes gradually decrease from 6.41% to 2.65%. The RCL of X sex chromosome is 28.66%. Cytotypes II–IV have 2n = 33 and differ among themselves in the morphology of some autosome pairs. Cytotype II comprises 15 pairs of acrocentric and one pair of metacentric autosomes (pair No. 1) (Fig. 4b). The first autosomes pair (RCL 10.95%) is roughly two times longer than the rest. The RCLs of the remaining autosomes gradually decrease from 6.46% to 2.90%. Cytotype III comprises 13 pairs of acrocentric, two pairs of metacentric (pairs No. 1 and 11), and one pair of subtelocentric autosomes (pair No. 4) (Fig. 4c). The first pair of autosomes is significantly longer (RCL 11.18%) than the remaining autosomes whose RCLs gradually decrease from 5.95% to 3.13%. Cytotype IV comprises 16 acrocentric autosome pairs (Fig. 4d). The first autosome pair is significantly longer (RCL 10.61%) than the remaining autosomes whose RCLs gradually decrease from 6.59% to 2.72%. In all cytotypes with 2n = 33, the X is metacentric and the largest chromosome in the karyotype, with RCLs 22.87%, 20.44%, and 22.86% for cytotype II, III, and IV, respectively. Two male individuals of *C.* (*C.*) *tenuis* from Liguria showed a different chromosome number in the karyotype, namely 2n = 21. This cytotype V comprises seven pairs of acrocentric and three pairs of metacentric autosomes (pair No. 1, 3, and 4), and one metacentric X chromosome (Fig. 4e). In cytotype V, the two chromosomes of the first and the second pair are almost five times longer (RCLs 19.06% and 16.28%) than the other pairs. The two following autosome pairs are of medium size (RCLs 9.44% and 7.56%), whereas the remaining autosomes gradually decrease in RCL from 4.27% to 2.88% of the haploid set. The RCL of the X chromosome is 26.78%.

Chthonius (*Ephippiochthonius*) *boldorii* Beier, 1934

All examined individuals displayed 35 chromosomes in the diploid set (Fig. 5a). The karyotype of this species comprises 16 pairs of acrocentric and one pair of subtelocentric autosomes (pair No. 2), and one metacentric X sex chromosome. The autosomes gradually decrease in RCL from 7.38% to 2.43%. The RCL of the X is 19.99%.

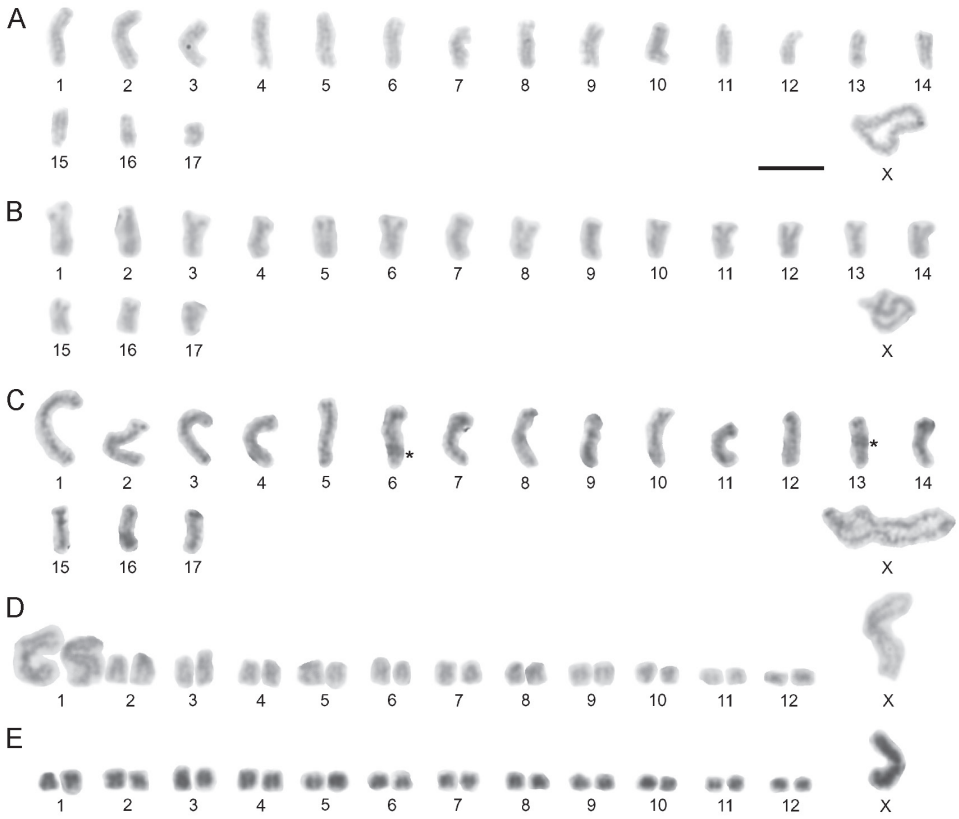

Figure 5. Karyotypes of *Chthonius* (*Ephippiochthonius*) and *Chthonius* (*Globochthonius*) males. **A** *C.* (*E.*) *boldorii* (2n = 35, X0), postpachytene **B** *C.* (*E.*) *fuscimanus* (2n = 35, X0), metaphase I **C** *C.* (*E.*) *tetrachelatus* (2n = 35, X0), postpachytene **D** *C.* (*E.*) *nanus* (2n = 25, X0), sister metaphases II **E** *C.* (*G.*) *poeninus* (2n = 25, X0), mitotic metaphase. Asterisks indicate overlaps of chromosomes. Scale bar = 10 μm.

Chthonius (*Ephippiochthonius*) *fuscimanus* Simon, 1900

The diploid set of this species consists of 35 chromosomes (Fig. 5b). The karyotype comprises 17 acrocentric autosomes pairs and one metacentric X chromosome. The autosomes gradually decrease in RCL from 6.70% to 3.33%, and the RCL of the X is 18.86%.

Chthonius (*Ephippiochthonius*) *tetrachelatus* Preyssler, 1790

The diploid set of this species comprises 35 chromosomes (Fig. 5c). They are 15 pairs of acrocentric, one pair of subtelocentric (pair No. 7), and one pair of submetacentric autosomes (pair No. 9); the X chromosome is metacentric. The autosomes gradually decrease in RCL from 7.45% to 2.96%, and the RCL of the X is 19.59%. C-banding confirmed the exclusive localization of constitutive heterochromatin in the centromere region (cf. Fig. 3g and 3h).

Chthonius (*Ephippiochthonius*) *nanus* Beier, 1953

The diploid set consists of 25 chromosomes (Fig. 5d). The karyotype possesses 11 acrocentric autosome pairs and one metacentric pair (pair No. 1), and one metacentric X chromosome. The metacentric pair of autosomes is significantly longer (RCL 20.91%) than the remaining autosomes whose RCLs decrease from 6.26% to 2.96%. The RCL of the X is 31.03%.

Chthonius (*Globochthonius*) *poeninus* Mahnert, 1979

The diploid set of this species consists of 25 chromosomes (Fig. 5e). The karyotype comprises of 11 pairs of acrocentric and one pair of metacentric autosomes (pair No. 2), and one metacentric X sex chromosome. The autosomes gradually decrease in RCL from 8.04% to 3.37%, and the RCL of the X is 33.74%.

Discussion

Chromosomal characteristics of the genus *Chthonius*

Only 11 species of Chthoniidae have been studied so far, eight of them belonging to the genus *Chthonius* from Romania, Czech Republic, and Greece (Šťáhlavský and Král 2004) (Table 1). Analyses of 11 species from the Alps fully confirm previously detected cytogenetic characteristics of the genus. Pseudoscorpions from the Alps present monocentric chromosomes, similar to other representatives of the genus as well as all other pseudoscorpion taxa that have been cytogenetically analysed (e.g. Šťáhlavský and Král 2004, Šťáhlavský et al. 2006). Achiasmatic meiosis has been confirmed in males of the genus *Chthonius*, which is probably characteristic for the entire family Chthoniidae (Šťáhlavský and Král 2004). This meiosis type is otherwise known within the class Arachnida only in scorpions (e.g. Schneider et al. 2009), spiders from the families Dysderidae and Segestriidae (Benavente and Wettstein 1980) and mites from the superfamily Hydrachnellae (Oliver 1977). Given that groups presenting achiasmatic meiosis are not closely related (e.g. Sharma et al. 2014), multiple independent origins of achiasmatic meiosis within arachnids could be assumed. Another characteristic specific to the genus *Chthonius* is the sex chromosome system. The typical sex chromosome system in the family Chthoniidae is X0 (Šťáhlavský and Král 2004), which was detected in all species analysed in the present study. The X sex chromosome is always metacentric and is the longest chromosome in the karyotype for all species in the genus *Chthonius*. The sex chromosome system with a large metacentric chromosome X has been detected in most pseudoscorpion families that have been cytogenetically analysed (Chernetidae, Geogarypidae, Garypinidae, Olpiidae, Atemnidae) (Šťáhlavský et al. 2005, 2006, 2012). Metacentric morphology and large size of X sex chromosomes have been documented within arachnids also in different species from different groups

of spiders (e.g. Král et al. 2006, 2013). Furthermore, extremely large metacentric X sex chromosomes is also known in some beetles from the family Chrysomelidae (Insecta, Coleoptera) (e.g. Almeida et al. 2009). It is evident that this type of X chromosome would have originated independently by different evolutionary mechanisms. However, there are only few exceptions to the morphology of the X chromosome in pseudoscorpions, namely one population of the species *Olpium pallipes* (Lucas, 1849) (Olpiidae) (Šťáhlavský et al. 2006) and two neotropic species *Semeiochernes armiger* (Balzan, 1892) and *Cordylochernes scorpioides* (Linnaeus, 1758) (Chernetidae) (Šťáhlavský et al. 2009). This evidence supports the assumption that the X0 sex chromosome system with large metacentric X is the plesiomorphic state in pseudoscorpions (e.g. Troiano 1990, 1997, Šťáhlavský et al. 2012).

The C-banding analyses performed in this study represent the first time that this procedure is applied in pseudoscorpions. Constitutive heterochromatin was only detected in the centromere regions. Blocks of heterochromatin located on different parts on the chromosome, known from some araneomorph spiders (Král et al. 2006), have not been detected. The concentration of constitutive heterochromatin in the centromere region could represent an ancestral state in this group, similar to that hypothesized in spiders (Rodríguez-Gil et al. 2007). The C-banding also confirmed the hypothesis that the prominent heteropycnotic blocks on chromosomes, visible in the early stages of meiosis, correspond to centromeres (Šťáhlavský et al. 2006). The results also indicate that the X is formed mainly of euchromatin outside of the centromere region, and the positive heteropycnosis during the early phases of meiosis is caused by intensive condensation, similarly as reported in wolf spiders (Dolejš et al. 2010).

Karyotype evolution of the genus *Chthonius*

Overall, the pseudoscorpions are represented by a great variety of chromosome numbers from 7 in Olpiidae to 143 in Atemnidae (Šťáhlavský et al. 2012). However, there is a much narrower range within individual families. For example, the typical range of the diploid number for a specific family is: 2n = 7–23 in Olpiidae, 2n = 15–23 in Geogarypidae, 2n = 16–67 in Neobisiidae, 2n = 47–73 in Chernetidae, and 2n = 65–143 in Atemnidae (see Šťáhlavský 2016). The results of this study confirm that the variability in chromosome number in the genus *Chthonius* is in agreement with previous findings (Šťáhlavský and Král 2004). The diploid number in males ranged from 21–35 (most frequently 35) and the acrocentric chromosomes are the most common morphology in the karyotype (see Table 1). Šťáhlavský and Král (2004) suggested that this chromosome number, acrocentric morphology of the chromosomes, and their gradual decrease in size are the ancestral conditions for species of this genus. The findings of this study confirm the assumption in *C.* (*C.*) *carinthiacus* and *C.* (*E.*) *fuscimanus* with 2n = 35 species presenting only acrocentric chromosomes. In other species with a diploid number 2n = 35 and a majority of acrocentric chromosomes, the presence of different morphological types of autosomes has also been detected, probably as a result of pericentric inversions.

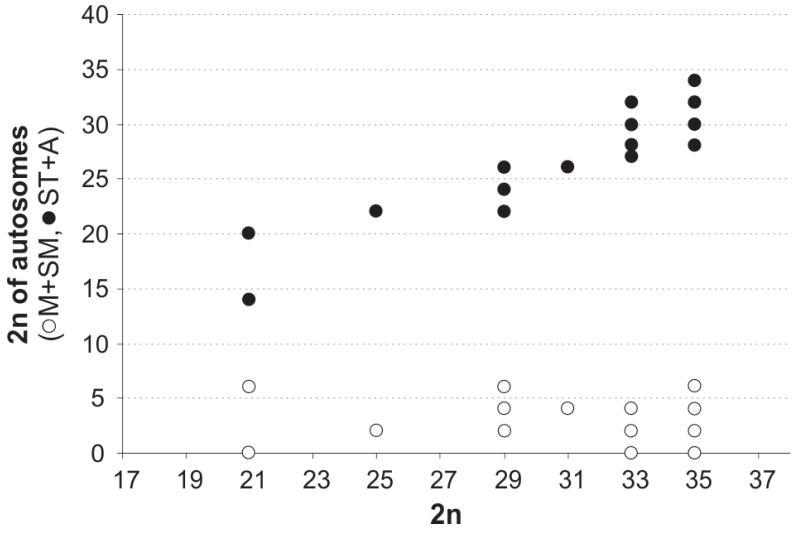

Figure 6. The proportion of biarmed (metacentric and submetacentric) and one-armed (subtelocentric and acrocentric) autosomes in karyotypes of the genus *Chthonius*. Data from Table 1.

Šťáhlavský and Král (2004) also hypothesized that centric and tandem fusions play an important role in the karyotype evolution of the genus *Chthonius,* leading to a decrease in the number of chromosomes and a change of their morphology from uniarmed to biarmed. The exact mechanism and direction of the karyotype evolution of pseudoscorpions remain unknown. However, the abundant frequency of karyotypes with 35 chromosomes (Table 1) could indicate that this number corresponds to the ancestral state. Similar changes of the number, morphology, and size of the chromosomes linked to both centric and tandem fusions can be observed in the different cytotypes of *C.* (*C.*) *tenuis* (Fig. 4a–e). We detected several cytotypes (II, III and IV) with the same diploid number of 33 in this species. The number presumably decreased from the hypothetic ancestral state of 2n = 35 (cytotype I, Fig. 4a) by means of centric (cytotype II, III, Fig. 4b, c) or tandem (cytotype IV, Fig. 4d) fusions. Independent (centric or tandem) fusions or subsequent pericentric inversions may produce a different morphology of extra large autosomes. Accumulation of chromosome fusions occurred in cytotype V decreasing the number to 2n = 21 and leading to differentiation of autosomes into three categories: large, medium, and small (Fig. 4e). In *C.* (*C.*) *alpicola* species, with a chromosome number lowered to 2n = 21 and presence of only acrocentric chromosomes in the karyotype (Fig. 1a), multiple tandem fusions presumably played a key role in the process of lowering the chromosome number. From the comparison of the frequency of morphologic types of chromosomes in the karyotypes of the genus *Chthonius* (Fig. 6), it is apparent that the frequency of metacentric and submetacentric chromosomes does not vary considerably, but the number of acrocentric and subtelocentric autosomes decreases in karyotypes with lower chromosome numbers. This phenomenon could be explained because of tandem fusions, or potentially as a result of a centric fusion subsequently accompanied by a pericentric inversion. However, for precise understanding of these mechanisms,

the use of additional cytogenetic tools would be needed in for the exact identification of homologous segments of the chromosomes and detection of particular chromosome rearrangements leading to changes in both chromosome morphology and size (e.g. Nie et al. 2012). Unfortunately, the exact direction of karyotype evolution within the genus *Chthonius* is also not possible to determine without the knowledge of the phylogenetic relationships among the *Chthonius* species, which are currently unknown.

Cryptic species or intraspecific variability of karyotypes?

Karyotypes of pseudoscorpions show considerable differences among species within all analysed families (see Šťáhlavský 2016), and therefore cytogenetic data have great potential for taxonomic application in the order. The usefulness of karyology has been demonstrated in the genus *Roncus* L. Koch, 1873. This mainly European group, with more than 140 described species (Harvey 2013), usually exhibits similar external morphology (e.g. Gardini 1991). However, the karyotypes of very morphologically similar species differ in diploid number, chromosome morphology, and sex chromosome systems (X0 and XY) (e.g. Troiano 1990). Substantial differences in karyotypes were used to distinguish *R. montsenyensis* Zaragoza and Šťáhlavský, 2008 (2n = 16) from *R. cadinensis* Zaragoza, 2007 (2n = 38), which are morphologically very similar (Zaragoza and Šťáhlavský 2008). Unfortunately, the use of cytogenetics in cryptic species detection in pseudoscorpions is complicated due to the limited amount of data available for comparison among different species. Furthermore, different degrees of intraspecific karyotype variability can represent a problem to determine and distinguish the existence of cryptic or isomorphic species. These challenges, observed in the genus *Chthonius* from the Alps, are also known from various arachnid groups (e.g. Tsurusaki 1985, Řezáč et al. 2007, Schneider et al. 2009), and many other organisms (e.g. Duffy et al. 2008, Severns and Liston 2008, Dincă et al. 2011, Sadílek et al. 2013, Sember et al. 2015).

Our data completely agree with described karyotypes of *C.* (*E.*) *fuscimanus* and *C.* (*C.*) *carinthiacus* (misidentified see Christophoryová et al. 2012) from Central Europe (both species 2n = 35, only acrocentric autosomes) (Šťáhlavský and Král 2004). Small differences between karyotypes of *C.* (*E.*) *tetrachelatus* from Central Europe (Šťáhlavský and Král 2004) and from the Alps (present study) may be an artefact of the quality of the preparations. Furthermore, the precision of visualizing the centromere position, using C-banding in this study, was likely better. Karyotype similarity from distant localities within these three species suggests that the use of these approaches may also contribute to characterize pseudoscorpion species very well.

In contrast to these findings, we identified different cytotypes in more abundant material of four species of the subgenus *Chthonius* from the Alps (Table 1). Only in *C.* (*C.*) *ischnocheles* and *C.* (*C.*) *rhodochelatus,* different cytotypes were complemented by visible morphological differences in surface granularity and pedipalp size. These particular morphological differences would potentially allow us to treat the cytotypes as new cryptic species. Nevertheless, standard morphological characteristics overlap substantially

among the cytotypes in both *C.* (*C.*) *raridentatus* and *C.* (*C.*) *tenuis*. The interpretation of the variability in this case is not trivial. The *Chthonius* (*C.*) *tenuis* cytotypes may present both intraspecific variability and a mix of different levels of speciation. Specific cytotypes from different parts of the Alps have been reported in species with large distribution (e.g. Capanna and Riscassi 1978, Zima et al. 1996), but the difference between the highly derived cytotype V (2n = 21) and the remaining cytotypes most likely represents a strong reproductive barrier between them. Thus, cytotype V may represent a cryptic species without evident morphological differentiation. The fact that cytotype I spatially overlaps cytotype V (Fig. 4f) coupled with the fact that no anomalies during meiosis have been detected in the studied material, suggests an absence of hybridization between them and further supports the cryptic species status of cytotype V (e.g. King 1993). It should be noted that material used in the present study is probably not substantial enough to rule out the existence of hybrids and more investigation is needed before any taxonomic changes can be made. The putative chromosomal speciation of *Chthonius* in the Alps may be owed to the dispersal limitation of the genus. Chromosomal rearrangements may be fixed in certain areas with limited gene flow and consequently cause hybrid sterility among different cytotypes. Alternatively, the environments of the Alps may influence chromosome rearrangement that favours higher fitness for specific environmental conditions (see e.g. Faria and Navarro 2010). Additional sampling, comprehension of phylogenetic relationships among the analysed species, and knowledge of the genetic structure of their populations are needed for the full understanding of karyotype variability and its role in the speciation of the genus *Chthonius* in the Alps.

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Supplementary material 1

Table S1

Authors: Jana Kotrbová, Vera Opatova, Giulio Gardini, František Šťáhlavský

Data type: karyometric data

Explanation note: Comparison of karyometric data of particular chromosomes using t-test: t values (above the diagonal), p-values (below the diagonal), significant differences ($\alpha = 0.05$) in bold.

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Karyotype stability in the family Issidae (Hemiptera, Auchenorrhyncha) revealed by chromosome techniques and FISH with telomeric (TTAGG)*n* and 18S rDNA probes

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Abstract

We report several chromosomal traits in 11 species from 8 genera of the planthopper family Issidae, the tribes Issini, Parahiraciini and Hemisphaeriini. All species present a 2n = 27, X(0) chromosome complement known to be ancestral for the family. The karyotype is conserved in structure and consists of a pair of very large autosomes; the remaining chromosomes gradually decrease in size and the X chromosome is one of the smallest in the complement. For selected species, analyses based on C-, AgNOR- and CMA_3-banding techniques were also carried out. By fluorescence *in situ* hybridization, the $(TTAGG)_n$ probe identified telomeres in all species, and the major rDNA loci were detected on the largest pair of autosomes. In most species, ribosomal loci were found in an interstitial position while in two species they were located in telomeric regions suggesting that chromosomal rearrangements involving the rDNA segments occurred in the evolution of the family Issidae. Furthermore, for 8 species the number of testicular follicles is provided for the first time.

Keywords

Fulgoroidea, Issidae, karyotypes, C-banding, NORs, fluorochrome staining, FISH, $(TTAGG)_n$, 18S rDNA

Introduction

During the last decades, the worldwide planthopper family Issidae was comprehensively revised based on morphological features (Emeljanov 1999, Gnezdilov 2003a, b, 2007, 2012a, b, 2013a, b, c, Gnezdilov and Wilson 2006, Gnezdilov et al. 2014). Several groups treated previously as Issidae subfamilies were upgraded to the family rank (Caliscelidae and Acanaloniidae). The subfamilies Trienopinae and Tonginae were transferred as tribes to the families Tropiduchidae and Nogodinidae respectively, while the tribes Adenissini and Colpopterini were transferred to the Caliscelidae and Nogodinidae, respectively. The term "issidoid group" has been suggested for grouping the families Issidae, Caliscelidae, Acanaloniidae, Tropiduchidae and Nogodinidae (Gnezdilov 2013b, Gnezdilov et al. 2015).

As a result of these changes, the family Issidae *sensu stricto* is now considered to comprise more than 1000 species and subspecies with around 170 genera classified within the only nominatypical subfamily Issinae, including three tribes, Issini Spinola, 1839, Hemisphaeriini Melichar, 1906 and Parahiraciini Cheng & Yang, 1991 (Gnezdilov 2013a, Bourgoin 2016). The largest tribe Issini exhibits worldwide distribution while the two other tribes are mainly endemics of the Oriental Region (Gnezdilov 2013a, Gnezdilov et al. 2014).

Recent molecular data on the Issidae *sensu lato* using a partial sequence of the 18S rDNA and the *wingless* gene (Sun et al. 2015) are not congruent in all cases with the above classification resulted from morphological data. However, the monophyly of the Issidae *s. str.* and the existence of three distinct phylogenetic lineages (tribes) were confirmed. The phylogenetic position of another tribe, the Tongini, might be an artifact (Gnezdilov et al. 2015). Thus in our current paper we follow the morphology-based classification.

Up to now, studies on the Issidae *s. str*. karyotypes were performed on 36 species (20 genera), all being from the tribe Issini (Maryańska-Nadachowska et al. 2006, Kuznetsova et al. 2010). Pioneering karyological studies (Parida and Dalua 1981, Tian et al. 2004) and later comparisons based on standard (Schiff-Giemsa) and differential (Ag-NOR and DAPI/CMA_3) staining techniques (Maryańska-Nadachowska et al. 2006, Kuznetsova et al. 2009, 2010) showed that issids are characterized by a pronounced karyotypic conservatism. They have strikingly similar karyotypes with only three male diploid chromosome numbers: 27, 26 and 25. The most common karyotype of 2n = 27 (26 + X) is considered as phylogenetically ancestral in the family (Kuznetsova et al. 2010) and appears similar in structure among the species studied. It consists of a pair of very large autosomes; the remaining chromosomes gradually decrease in size, and the X chromosome is among the small chromosomes of the set. As revealed by CMA_3 staining and silver nitrate impregnation (AgNOR staining), the largest autosomal pair bears nucleolus organizer regions (NORs) in all studied species. In contrast to the above chromosome techniques, C-banding revealed differences between species in the amount and distribution of heterochromatin, and its staining

affinity using DAPI and CMA_3 (Kuznetsova et al. 2009, 2010). Thus, despite the vast variation within the Issidae, the cytogenetics of this group remains poorly explored and no molecular cytogenetic techniques have previously been applied.

Recent publications dealing with karyotypes of the Issidae have additionally reported some data on internal reproductive organs, mainly on the number of testicular follicles (Maryańska-Nadachowska et al. 2006, Kuznetsova et al. 2010). Issids were shown to be characterized by testes with rather numerous follicles, ranging from 4 (*Palmallorcus punctulatus*) to 30 (*Zopherisca tendinosa*) per testis, with a predominant number of 10.

In this paper we report karyotypes of 11 species in 8 genera of the tribes Issini, Parahiraciini and Hemisphaeriini, studied by several chromosome techniques, including fluorescence *in situ* hybridization (FISH) with $(TTAGG)_n$ telomeric and 18S rDNA probes. We particularly focused on whether karyotypes with the same chromosome number show different patterns if new molecular cytogenetic markers are applied. In addition, we present, for the first time, the number of testicular follicles for 8 species, including first observations on members of the tribes Parahiraciini and Hemisphaeriini. All currently available data on the family Issidae are summarized and tabulated.

Material and methods

Details on the material analyzed, including the geographical location, number of specimens, information about the authorship of the noted specific names, diploid (2n) chromosome number, sex-determining mechanism in males, cytogenetic methods used in karyotyping and the number of testicular follicles are given in Table 1. Moreover, Table 1 summarizes all species studied so far in respect to karyotype and reproductive system in the family Issidae.

Insects

All specimens were identified by V.M. Gnezdilov. Several species were identified only to the genus level because of taxonomic difficulties in these genera. Only males were used for chromosome analyses. In the field, males were collected with an insect net, fixed alive in 3:1 fixative (96% ethanol: glacial acetic acid) and stored at +4 °C.

Slide preparation

Gonads of adult males were used for chromosome analysis. Testes were dissected in a drop of 45% acetic acid and squashed. The coverslips were removed using dry ice. Prior to staining, the preparations were examined by phase contrast microscopy.

Table 1. List of the Issidae species studied in respect to karyotype and testis structure[1]

Taxa	Collection locality	No. of males (m) and females (f) studied	2n♂	Number of follicles	Method	Gaps/AgNORs/ rDNA FISH location on the largest pair of autosomes	Source
Issidae Spinola **Issinae Spinola** **Issini Spinola**							
Agalmatium bilobum (Fieber, 1877)	Russia, Greece	? m, ? f	-	11/11 8/8	-	-	Emeljanov and Kuznetsova 1983
	Italy, Gemona del Friuli, Alps, ca. 25 km north from Udine, Udine prov., 07.07.2005, leg. A. Maryańska-Nadachowska	3m 1f	26+X	11/11 8/8	Schiff	Interstitial gap	Maryańska-Nadachowska et al. 2006
	Bulgaria, Krupnik, S from Simitli, Struma River valley, 9.05.2007, leg. A. Maryańska-Nadachowska	2m	-"-	11/11	rDNA FISH	Interstitial cluster	Present data **Fig. 11**
A. flavescens (Olivier, 1791)	Spain, Sierra d'Alhamilla, Almeria prov., 3.06.2006, leg. A. Maryańska-Nadachowska	2m	26+X	11/11	Schiff	-	Maryańska-Nadachowska et al. 2006
	-"-	1m	-"-	-"-	rDNA FISH	Interstitial cluster	Present data **Fig. 12**
Bergevinium ?malagense (Matsumura, 1910)	Spain, El Burgo, Malaga prov., 11.06.2005, leg. A. Maryańska-Nadachowska	2m	26+X	9/9	Schiff	-	Maryańska-Nadachowska et al. 2006
Brahmaloka sp.	India	?m	24+X	-	-	-	Parida and Dalua 1981
Bubastia obsoleta (Fieber, 1877)	Greece, Litohoro, eastern slopes of Mt. Olympus, Pieria District, 17.05.2007, leg. A. Maryańska-Nadachowska	4m	26+X	10/10	Schiff, C-banding, DAPI	-	Kuznetsova et al. 2010
B. saskia Dlabola, 1984	Greece, Varvara, Stratoniko Range (600-800 m a.s.l.), Halkidiki District, 11.06.2007, leg. A. Maryańska-Nadachowska	4m	26+X	10/10	Schiff, C-banding, DAPI	-	Kuznetsova et al. 2010
B. taurica (Kusnezov, 1926)	Russia, Krasnodar Territory, near Gelendzhik, 30.08.2004, leg. V. Gnezdilov	1m	26+X	10/10	Schiff	-	Maryańska-Nadachowska et al. 2006

Taxa	Collection locality	No. of males (m) and females (f) studied	2n♂	Number of follicles	Method	Gaps/AgNORs/ rDNA FISH location on the largest pair of autosomes	Source
Conosimus coelatus Mulsant & Rey, 1855	France, prov. Vaison-la-Romaine, 1.06.2010, leg. A. Maryańska-Nadachowska	2m	26+X	9/9	Schiff, AgNOR	Interstitial gap Interstitial NOR	Present data **Fig. 1a, b**
Corymbius tekirdagicus (Dlabola, 1982)	Greece, Litohoro eastern slopes of Mt. Olympus, Pieria District, 17.05.2007, leg. A. Maryańska-Nadachowska	2m	26+X	10/10	Schiff	-	Kuznetsova et al. 2010 as: *Kervillea tekirdagica*
Dentatissus damnosus (Chou & Lu, 1985)	China	?m	26+X	-	Phenol fuchsine	-	Tian et al. 2004 as *Sivaloka damnosa*
	-"-	?m ?f	-	18/18 9/9		-	Meng et al. 2010 as *Sivaloka damnosa*
Falcidius doriae (Ferrari, 1884)	Italy, Caltabellotta, alt. ca. 900 m a.s.l., ca. 30 km north from Sciacca, southern Sicily, 22.05.2006, leg. A. Maryańska-Nadachowska	3m	26+X	10/10	C-banding	-	Kuznetsova et al. 2010
F. limbatus (A. Costa, 1864)	Italy, Chiaramonte, ca. 15 km north from Ragusa, southern Sicily, 16.05.2006, leg. A. Maryańska-Nadachowska	4m	24+XY	-	C-banding	Interstitial gap	Kuznetsova et al. 2010
Hysteropterum albaceticum Dlabola, 1983	Spain, Soria prov., 07.2005, leg. A. Maryańska-Nadachowska	3m	26+X	10/10	Schiff	-	Maryańska-Nadachowska et al. 2006
H. dolichotum Gnezdilov & Mazzoni, 2004	Spain, Segovia prov., 07.2005, leg. A. Maryańska-Nadachowska	2m	26+X	-	Schiff	-	Maryańska-Nadachowska et al. 2006
H. vasconicum Gnezdilov, 2003	Spain, Soria prov., 07.2005, leg. A. Maryańska-Nadachowska	3m	26+X	10/10	Schiff	-	Maryańska-Nadachowska et al. 2006
Issus coleoptratus (Fabricius, 1781)	Spain, near Almonte, 26.06.2004 (south Spain), leg. A. Maryańska-Nadachowska	2m	26+X	13/13	Schiff	-	Maryańska-Nadachowska et al., 2006
I. lauri Ahrens, 1814	Italy (Sicily), Acireale, east Sicily, 2.06.2006, leg. A. Maryańska-Nadachowska	2m 1f	26+X -	13/13 13/12	Schiff	Terminal gap	Maryańska-Nadachowska et al. 2006
	-"-	1m	-"-	13/13	rDNA FISH	Terminal cluster	Present data **Fig. 13**

Taxa	Collection locality	No. of males (m) and females (f) studied	2n♂	Number of follicles	Method	Gaps/AgNORs/ rDNA FISH location on the largest pair of autosomes	Source
Kervillea basiniger (Dlabola, 1982)	Greece, Litohoro, eastern slopes of Mt. Olympus, Pieria District, 17.05.2007, leg. A. Maryańska-Nadachowska	2m	26+X	10/10	Schiff	-	Kuznetsova et al. 2010
	-"-	1m	-"-	-"-	rDNA FISH	Interstitial cluster	Present data **Fig.14**
K. scoleogramma (Fieber, 1877)	Turkey, Gülcük, (1100m a.s.l., Boz Dağlar ca. 18 km north from Edemis, prov. Izmir, 3.06.2010, leg. A. Maryańska-Nadachowska	3m	26+X	12/12	Schiff	Interstitial gap	Present data **Fig. 2 a, b**
Latematium latifrons (Fieber, 1877)	Bulgaria, Central Rodopy Mts., 2010, leg. A. Maryańska-Nadachowska	3m	26+X	12/12	Schiff	-	Present data **Fig. 3**
Latilica maculipes (Melichar, 1906)	Italy, Gemona del Friuli, Alps, ca. 25 km north from Udine, Udine prov., 07.07.2005, leg. A. Maryańska-Nadachowska	2m	24+X	10/10	Schiff	-	Maryańska-Nadachowska et al. 2006
Latissus dilatatus (Fourcroy, 1785)	Italy, Lagonegro, ca.15 km north from Lauria, 11.06.2006, leg. A. Maryańska-Nadachowska	5m	26+X	12/12	Schiff, C-banding, AgNOR, DAPI	Subtelomeric gaps, NORs	Kuznetsova et al. 2010
Mulsantereum abruzicum (Dlabola, 1983)	Italy, Sicily, Nébrodi Mountains, western part, surroundings of di Luminaria (1260 m), dell Obolo Pass (1503 m), 27.05.2006, leg. A. Maryańska-Nadachowska	2m	26+X	10/10	Schiff	-	Kuznetsova et al. 2010
Mycterodus (*Mycterodus*) *drosopoulosi* Dlabola, 1982	Greece, near Athens, Parnitha Mt., 05.05.2015, leg. V. Gnezdilov	2m	26+X	10/13, 7/18	Schiff	-	Present data **Fig. 4**
		1f	-	15/15			
M. (*M.*) *etruscus* Dlabola, 1980	Italy, Passo del Muraglione, 907 m a.s.l., ca. 50 km north-east from Firenze, 14.06.2006, leg. A. Maryańska-Nadachowska	1m	26+X	16/16	Schiff	-	Kuznetsova et al. 2010
M. (*M.*) *intricatus* Stål, 1861	Crimea, Chatyr-Dag, 1000 m a.s.l., 06.2008, leg. A. Maryańska-Nadachowska	1m	26+X	20/20	Schiff, C-banding	-	Kuznetsova et al. 2010

Taxa	Collection locality	No. of males (m) and females (f) studied	2n♂	Number of follicles	Method	Gaps/AgNORs/ rDNA FISH location on the largest pair of autosomes	Source
M. (*Semirodus*) *colossicus* (Dlabola, 1987)	Greece, Varvara, Stratoniko Range (600-800 m a.s.l), Halkidiki District, 11.06.2007, leg. A. Maryańska-Nadachowska	3m	26+X	18/18	Schiff, C-banding, AgNOR, DAPI	Interstitial gaps	Kuznetsova et al. 2010
M. (S.) pallens (Stål, 1861)	Greece, leg. S. Drosopoulos	1m 1f	26+X -	18/18 9/9	Schiff	-	Maryańska-Nadachowska et al. 2006
	-"-	1m	-"-	-"-	rDNA FISH	Interstitial (?) clusters	Present data **Fig. 15**
Mycterodus (Semirodus) sp.	Turkey, Kayacidad Mts (700-800 m a.s.l), south from Canakale, 06.2010, leg. A. Maryańska-Nadachowska	2m	26+X	-	Schiff, C-banding	-	Present data **Fig. 5a, b**
Palaeolithium distinguendum (Kirschbaum, 1868)	Spain, Goñar, Almeria prov., 07.2005, leg. A. Maryańska-Nadachowska	5m	26+X	7/13, 8/8, 8/11 9/9, 9/11	Schiff	-	Maryańska-Nadachowska et al. 2006
	-"-	1m	-"-	8/8	rDNA FISH	Interstitial clusters	Present data **Fig. 16**
Palmallorcus balearicus (Dlabola, 1982)	Spain, Mazagón, Huelva prov, 14.06.2005, leg. A. Maryańska-Nadachowska	3m	26+X	9/10, 10/10, 11/11	Schiff	-	Maryańska-Nadachowska et al. 2006
P. nevadensis (Linnavuori, 1957)	Spain, Sierra de la Nieves, Malaga prov., 4.06.2005, leg. A. Maryańska-Nadachowska	2m	26+X	10/10	Schiff	Interstitial gaps	Maryańska-Nadachowska et al. 2006
P. punctulatus (Rumbur, 1840)	Spain, Avila prov., 07.2005, leg. A. Maryańska-Nadachowska	1m	26+X	?4/4	Schiff	Interstitial gaps	Maryańska-Nadachowska et al. 2006
Sarnus sp.	Chile, La Campana, 2014, leg. A. Emeljanov	4m	26+X	6/6	Schiff, AgNOR,	? ?	Present data **Fig. 6a, b**
Scorlupaster asiaticum (Lethierry, 1878)	Kazakhstan, 42°50´20.724´´N 71°10´12.900´´E, 29.07.2006, leg. V.Gnezdilov	2m	26+X	9/9	Schiff	-	Kuznetsova et al. 2010

Taxa	Collection locality	No. of males (m) and females (f) studied	2n♂	Number of follicles	Method	Gaps/AgNORs/ rDNA FISH location on the largest pair of autosomes	Source
Scorlupella discolor (Germar, 1821)	Crimea, Chatyr-Dag, 1000 m a.s.l., 06.2008, leg. A. Maryańska-Nadachowska	1m	26+X	6/6	Schiff	-	Kuznetsova et al. 2010
	-"-	1m	-"-	-"-	rDNA FISH	Interstitial ? clusters	Present data **Fig. 17**
Thionia obtusa Melichar, 1906	Southern Mexico, 11. 2012, leg. A. Maryańska-Nadachowska,	1m	26+X	-	Schiff	Interstitial gaps	Present data **Fig. 7**
Tingissus tangirus (Matsumura, 1910)	Spain, El Burgo, Malaga prov. 20.06.2006, leg. A. Maryańska-Nadachowska	4m 1f	26+X -	10/10 6/6	Schiff	-	Maryańska-Nadachowska et al. 2006
Tshurtshurnella pythia Dlabola, 1979	Greece, 2003, leg. S. Drosopoulos	3m 1f	26+X	12/12 7/7	Schiff	-	Maryańska-Nadachowska et al. 2006
Zopherisca penelopae (Dlabola, 1974)	Greece, 2003, leg. S. Drosopoulos	3m	26+X	24/24	Schiff	-	Maryańska-Nadachowska et al. 2006
	Greece, Myrsini, ca 20 km W from Githio, Lakonia distr., Peloponessus, 2007.05. 24, leg. A. Maryańska-Nadachowska	1m	-"-	-"-	rDNA FISH	Interstitial clusters	Present data **Fig. 19**
Z. skaloula Gnezdilov & Drosopoulos, 2006	Greece, Skaloula village, 2003, leg. S. Drosopoulos	1m	26+X	30/30	Schiff	-	Maryańska-Nadachowska et al. 2006
Z. tendinosa (Spinola, 1839)	Greece, Achladokambos, ca. 20 km E from Tripoli, Arkadia District, Peloponessus, 23.05.2007, leg. A. Maryańska-Nadachowska	3m	26+X	28/28	Schiff, C-banding, DAPI	-	Kuznetsova et al. 2010
	-"-	1m	-"-	-"-	rDNA FISH	Terminal clusters	Present data **Fig. 20**
Parahiraciini							
Thabena sp.	Vietnam, Dak Lak Prov, Yok Don Nat. Park, 20.06.2014. leg. V. Gnezdilov	1m	26+X	11/11	Schiff, DAPI/CMA_3 rDNA FISH	Interstitial clusters	Present data **Fig. 8a, b** **Fig. 18**

Taxa	Collection locality	No. of males (m) and females (f) studied	2n♂	Number of follicles	Method	Gaps/AgNORs/ rDNA FISH location on the largest pair of autosomes	Source
Hemisphaeriini							
Hemisphaerius interclusus Noualhier, 1896	South Vietnam, Cat Tien, Nat. Res., 2012, leg. V. M. Gnezdilov	2m	26+X	8/11, 12/12	Schiff	Interstitial gaps	Present data **Fig. 9**
Hemisphaerius sp.	Indonesia, 2011, leg. D.A. Gapon	4m	26+X	8/9, 11/11, 12/12, 12/12	Schiff rDNA FISH	- Interstitial clusters	Present data **Figs 10, 21a, b**

[1] With several exceptions (Tian et al. 2004, Meng et al. 2010, Parida and Dalua 1981), all species were identified by V.M. Gnezdilov.

Conventional chromosome staining methods

All the conventional staining techniques used herein were described in detail by Kuznetsova et al. (2009, 2010) for other issid species, i.e., Schiff-Giemsa staining, C-banding, AgNOR-banding and CMA_3-banding. All species were studied using the standard Schiff-Giemsa technique by Grozeva and Nokkala (1996), whereas the other techniques were used only for selected species (Table 1).

Chromosome banding techniques contribute to the identification of specific chromosomes within karyotypes. AgNOR-banding reveals chromosomal nucleolus organizing regions (NORs) representing the sites for the tandemly arranged 18S and 28S ribosomal RNA genes. The AgNOR-banding presumably differentiates only those NORs which were metabolically active during the preceding interphase (Howell and Black 1982). Some chromosome banding techniques, including C-banding and fluorochrome banding, are strongly dependent on the amount of heterochromatin and its distribution in chromosomes. Chromomycin A_3 (CMA_3) reveals the presence of GC-rich heterochromatin, which is usually associated with NOR regions, and thus differentiates NORs regardless of their prior metabolic activity.

Fluorescence *in situ* hybridization (FISH)

This method was applied for the first time in the family Issidae. We used FISH with a $(TTAGG)_n$ and 18S rDNA probes in 11 species from 8 genera; 9 species from Issini tribe while that one species of the Parahiraciini and Hemisphaeriini tribes (Table 1). FISH with both probes was applied as previously reported (Maryańska-Nadachowska et al. 2013, Golub et al. 2014, Kuznetsova et al. 2015b, c). In brief, chromosome preparations were treated with 100 μg/ml RNase A, and 5 mg/ml Pepsin solution was used to remove excess RNA and proteins. Chromosomes were denatured on a slide in a hybridization mixture with biotinylated 18S rDNA probe from the genomic DNA of *Pyrrhocoris apterus* (Linnaeus, 1758) and rhodaminated $(TTAGG)_n$ probe with addition of salmon sperm DNA and then hybridized for 36 h. Hybridization signals were detected with NeutrAvidin-FITC.

Chromosomes were mounted in antifade medium (ProLong Gold antifade reagent with DAPI; Invitrogen) and covered with a glass coverslip. Chromosome slides were analyzed under a Leica DM 6000 B microscope. Images were taken with a Leica DFC 345 FX camera using Leica Application Suite 3.7 software with an Image Overlay module.

Results

Testicular and ovarian follicles

The testicular follicles were counted in 8 species (Table 1). The follicles were tubular and their number ranged from 6 to 18 among the species studied (here and elsewhere

numbers are given per testis) and occasionally varied among males and in different testes of the same male, e.g. in *Mycterodus drosopoulosi* (2 males: 10/13, 17/18), *Hemisphaerius* sp. (2 males: 11/8, 12/12) and *H. interclusus* (4 males: 9/8, 11/11, 12/12, 12/12). In the only studied female of *M. drosopoulosi*, about 15 ovarian follicles were counted in each gonad.

Conventional and differential chromosome techniques

Chromosome data on 10 species from 8 genera were obtained for the first time, including first observations on members of the tribes Parahiraciini and Hemisphaeriini (Table 1). Representative photographs of standard and sometimes also differentially stained meiotic karyotypes are presented in Figs 1–10. All species showed holokinetic chromosomes and the same chromosome number in males. In meiotic cells (diakinesis, metaphase I), there were 13 autosomal bivalents and a univalent X chromosome, i.e., 2n = 26 + X. Also, the karyotype structure seemed to be uniform with a pair of very large autosomes, 12 bivalents more or less gradually decreasing in size and the X chromosome as one of the smaller chromosomes of the set. The largest bivalent had a very large "secondary" constriction (a gap) in each homologue (Figs 1a, 2a, 7, 8a, 9). This constriction divided the chromosome into two unequal parts, however it was not always visible, especially when the chromosomes were more condensed (Figs 2b, 3, 4, 5a, 6a, 10). The silver staining technique used in *Conosimus coelatus* and *Sarnus* sp. produced a precipitation of silver at these regions suggesting that they harbor NORs (Figs 1b, 6b). In *Thabena* sp., the CMA_3/DAPI staining showed homogeneous DAPI staining (results not shown) and distinct patterns of GC-rich blocks (CMA_3-positive) in the NORs (Fig. 8b). The C-banded karyotype of *Mycterodus* sp. showed prominent telomeric C-bands in the largest and one of the medium-sized bivalents (Fig. 5a, b).

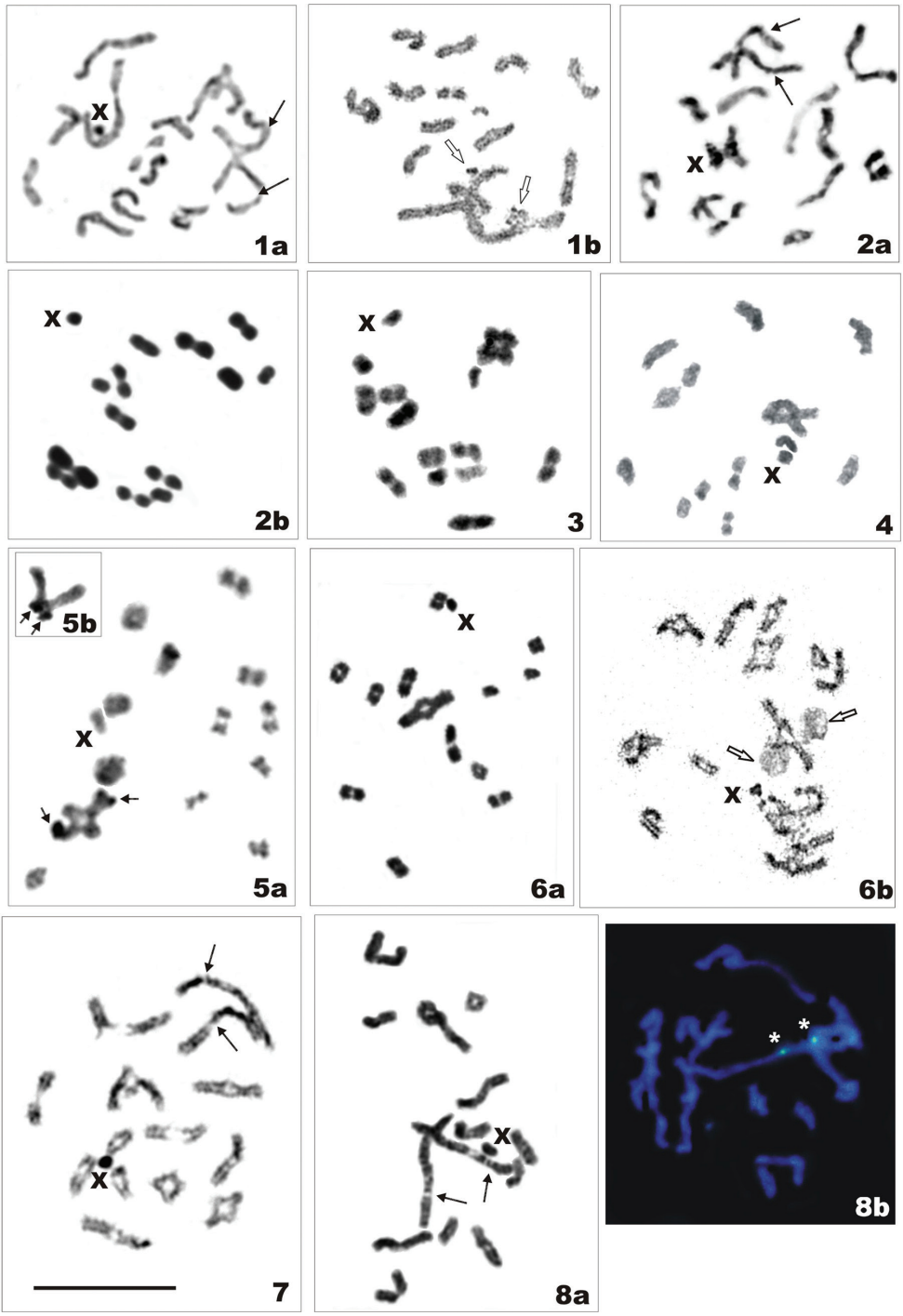
Fluorescence *in situ* hybridization (FISH)

Detection of a tandem telomeric repeat sequence by FISH with a $(TTAGG)_n$ probe.

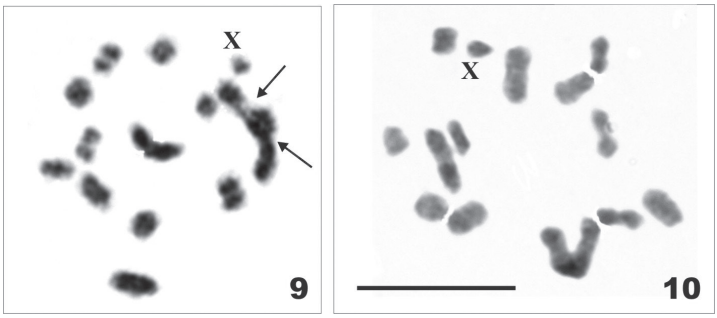
The telomeric probe identified $(TTAGG)_n$ repeats on the chromosomal ends in the nine species analyzed (Table 1), but not all telomeres were distinctly labeled in each chromosome spread (Figs 11–21). Some chromosomes showed only faint hybridization signals.

Detection of ribosomal genes revealed by FISH with an 18S rDNA probe

In all species, the major rDNA loci were located in the largest autosomal pair. In the majority of species, the rDNA clusters were found in the interstitial position; however


X
1a
1b
X
2a
X
2b
X
3
X
4
5b
X
5a
X
6a
X
6b
X
7
X
8a
*
*
8b

Figures 1–8. Meiotic analyses of species of the tribes Issini (Figures 1–7) and Parahiraciini (Figure 8), (n = 13 bivalents + X) with different cytogenetic techniques. **1** *Conosimus coelatus*, **a** diakinesis with standard and **b** AgNOR-staining. Arrows point to "secondary" constrictions (gaps) (**a**) and empty arrows NORs (**b**) point to the largest autosomal pair **2** *Kervillea scoleogramma*, **a** diakinesis and **b** metaphase I with standard staining. Arrows point to "secondary" constrictions on the largest autosomal pair (**a**) **3** *Latematium latifrons*, metaphase I with standard staining **4** *Mycterodus* (*Mycterodus*) *drosopoulosi*, diakinesis with standard staining **5** *Mycterodus* (*Semirodus*) sp., diakinesis with C-banding. **a** Arrows point to C-bands on the largest and medium-sized bivalents. In the largest bivalent, C-bands are located at the terminal or **b** at the proximal (chiasmate) parts of chromosomes. Short arrows point to C-bands **6** *Sarnus* sp., **a** metaphase I with standard staining and **b** diakinesis with AgNOR-banding. Arrows point to NORs on the largest autosomal pair **7** *Thionia obtusa*, diakinesis with standard staining. Arrows point to "secondary" constrictions on the largest autosomal pair **8** *Thabena* sp. **a** diakinesis with standard staining, and **b** diplotene with CMA_3-banding. Arrows point to "secondary" constrictions (**a**) and asterisks mark CMA_3-positive, GC-rich regions (**b**) of the largest autosomal pair. Scale bar =10 μm.

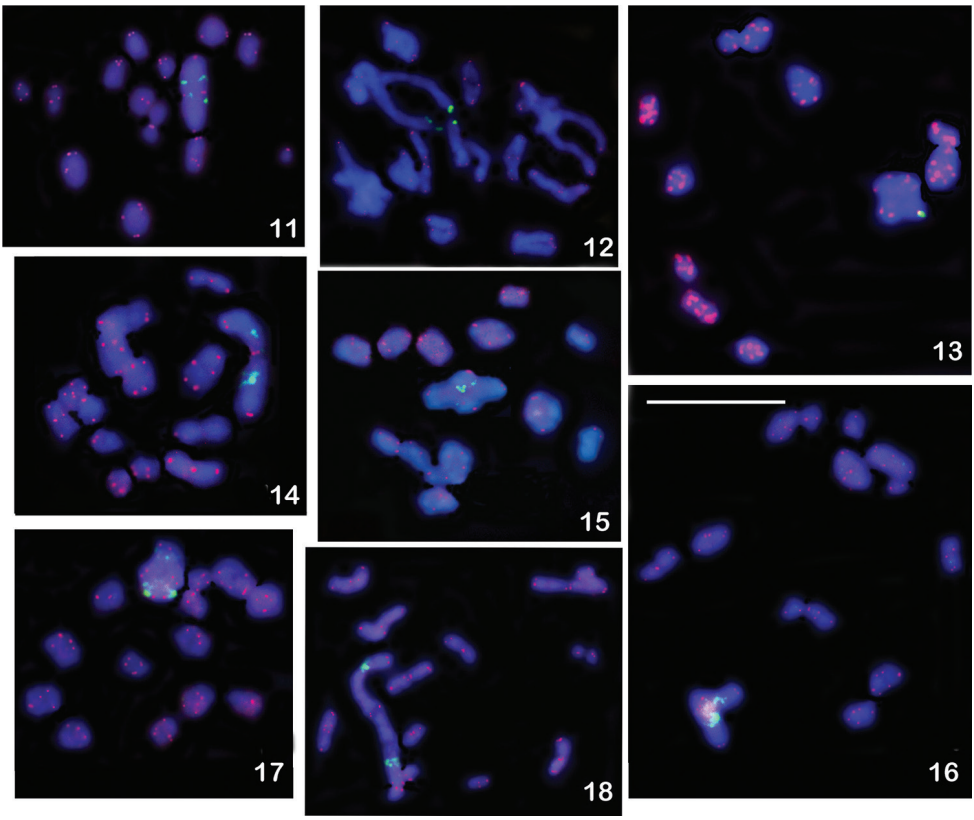


Figures 9–10. Conventionally stained meiotic karyotypes of two species of the tribe Hemisphaeriini (n = 13 bivalents + X). **9** *Hemisphaerius interclusus*, metaphase I with standard staining. Arrows point to "secondary" constrictions in the largest autosomal pair **10** *Hemisphaerius* sp., metaphase I with standard staining. Scale bar = 10 μm.

in *Issus lauri* and *Zopherisca tendinosa* they were clearly seen in the terminal regions (Figs 13, 20). In some species, rDNA FISH revealed heteromorphism in size of rDNA clusters (Figs 14, 13, 20).

Compilation of data on karyotypes and testis structure

We made a thorough compilation of all data reported so far in the family Issidae, including the tribes Issini, Parahiraciini and Hemisphaeriini. Table 1 covers information on a total of 44 species from 27 genera studied in respect to karyotypes and on 40 species from 26 genera studied in respect to the number of testicular follicles.

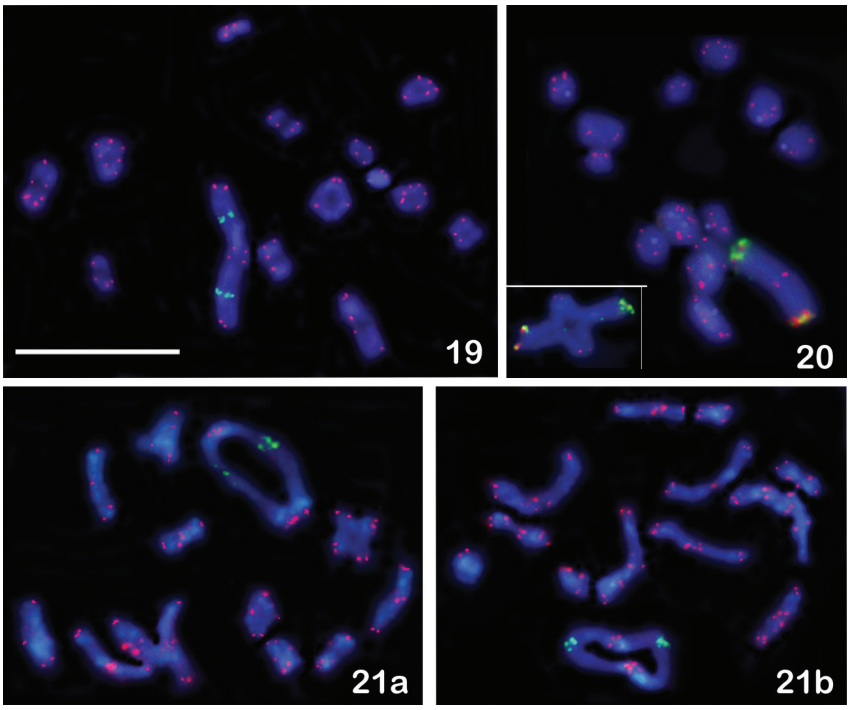


Figures 11–18. FISH with rDNA (green signals) and telomeric $(TTAGG)_n$ (red signals) probes on male meiotic karyotypes of eleven Issidae species (n = 13 bivalents + X). The rDNA clusters are seen on the largest autosomal pair, located interstitially in all species with the exception of *Issus lauri* (Figure 13) and *Zopherisca tendinosa* (Figure 20) with the terminal location of these clusters. **11** *Agalmatium bilobum*, metaphase I **12** *A. flavescens*, diplotene-diakinesis transition **13** *Issus lauri*, metaphase I **14** *Kervillea basinger*, metaphase I **15** *Mycterodus (Semirodus) pallens*, metaphase I **16** *Palaeolithium distinguendum*, metaphase I **17** *Scorlupella discolor*, metaphase I **18** *Thabena* sp., metaphase I. Scale bar = 10 μm.

Discussion

Follicle number

The number of testicular follicles per testis, counted here in males of eight species, ranged from 6 to 30, being the lowest in *Sarnus* sp. and the highest in *Zopherisca tendinosa* (both from Issini). In some species, the number of follicles varies among males of the same species and between testes of the same male. Specifically, variation was observed in *Mycterodus drosopoulosi* in which three examined males had testes with 17 and 18; 10 and 13; and 15 and 15 follicles, respectively. As in other planthopper families, in Issidae testicular follicles are of tubular shape. D'Urso et al. (2005) pointed out that Fulgoromorpha are differentiated by this pattern from Cicadomorpha in which follicles are lobular.



Figures 19–21. FISH with rDNA (green signals) and telomeric $(TTAGG)_n$ (red signals) probes on male meiotic karyotypes of eleven Issidae species (n = 13 bivalents + X). The rDNA clusters are seen on the largest autosomal pair, located interstitially in all species with the exception of *Issus lauri* (Figure 13) and *Zopherisca tendinosa* (Figure 20) with the terminal location of these clusters. **19** *Zopherisca penelopae*, diakinesis-metaphase I transition **20** *Z. tendinosa*, metaphase I **21** *Hemisphaerius* sp., **a** and **b** diakinesis. Scale bar = 10 μm.

The evolutionary trends and the phylogenetic importance of the number of follicles in Auchenorrhyncha were repeatedly discussed in the literature (e.g., Emeljanov and Kuznetsova 1983, Kirillova 1989, D'Urso et al. 2005, Kuznetsova et al. 2009, Gnezdilov 2013b). In some groups variation in this character agrees with their taxonomy and phylogeny. For instance, the number of follicles is conserved at the level of tribes and/or subfamilies within the planthopper families Delphacidae and Dictyopharidae, with changes in this pattern correlated with their overall morphological evolution (Kirillova 1989, Kuznetsova et al. 2009). However, studies of testis structure in the Issidae documented the lability of the follicle number (Maryańska-Nadachowska et al. 2006, Kuznetsova et al. 2010). In 40 species studied so far, a wide range of follicle numbers have been reported, from four (in *Palmallorcus punctulatus*; but *P. balearicus* and *P. nevadense* have higher numbers, 10 or 11) and six (in *Scorlupella discolor* and *Sarnus* sp.) to 30 (in *Zopherisca skaloula*). Noteworthy are the unusually high numbers (24, 28 and 30) found in the three studied species of the genus *Zopherisca* Emeljanov, 2001. Interestingly, the number of follicles varies between closely related species (in the genera *Kervillea* Bergevin, 1918, *Mycterodus* Spinola, 1839, *Palmallorcus* Gnezdilov, 2003, *Zopherisca*) and even within the same species (e.g. *Palaeolithium*

distinguendum, *Palmallorcus balearicus* and *Mycterodus drosopoulosi*) suggesting that evolutionary changes in the follicle number can be relatively rapid in the Issidae. In the opinion of Gnezdilov (2013b), the polymerization of seminal follicles inherent in the Issidae and also in other higher fulgoroid families, such as Nogodinidae, Recaniidae and Flatidae (Kuznetsova et al. 1998), indicates that these families are relatively young in terms of evolution and that the testis structure has not yet been stabilized within their supraspecific taxa.

Although numbers between 9 and 18 and especially 10 (observed in one third of the species) seem to be more typical for the Issidae, there is still no conclusive evidence of the most characteristic number in this group. This problem can be resolved primarily through improved taxon sampling.

Karyotypes

The nine species of the Issidae studied here for the first time have broadly similar karyotypes having the male diploid number (2n) of 27 chromosomes, including 13 autosomal pairs and an X(0) sex determination system. The karyotype includes a relatively small X chromosome, one pair of very long autosomes and the remaining autosomes which gradually decrease in size. Issidae, like other Auchenorrhyncha and Hemiptera, have holokinetic chromosomes. The largest bivalent is always NOR-bearing, and NORs are interstitial in the majority of species. The exceptions are *Issus lauri* and *Zopherisca tendinosa,* in which the 18S rDNA cluster is located terminally; this particular pattern probably resulted from inversions. GC-rich DNA segments labeled by CMA_3 are associated with nucleolus organizer regions.

Our study confirms that Issidae are a group characterized by the high karyotypic conservatism, with the basic karyotype of 2n = 27 (26 + X) (Maryańska-Nadachowska et al. 2006, Kuznetsova et al. 2010). At present, data on karyotypes are available for 44 species (around 4.5 % of the described species) and 27 genera (around 16 % of the recognized genera) in the three currently accepted tribes, Issini, Parahiraciini and Hemisphaeriini. With the exception of *Latilica maculipes* and *Brahmaloka* sp., both with 2n = 24 + X, and *Falcidius limbatus* with 2n = 24 + XY (the Issini), all species have 2n = 27 (26 + X). This makes the monophyletic origin of the latter karyotype an attractive hypothesis and, indeed, the ancestrality of this pattern has been inferred (Maryańska-Nadachowska et al. 2006, Kuznetsova et al. 2010). Every other karyotype could thus have arisen by a single tandem fusion, either between two pairs of autosomes (*L. maculipes* and *Brahmaloka* sp., 2n = 24 + X) or between an autosome and the X chromosome (*F. limbatus*, 2n = 24 + XY), respectively. Thus, the chromosome number decreased at least three times in the evolution of the family Issidae. Sex chromosomes of *F. limbatus* are most likely of the neo-XY type. Notably, neo-sex chromosome systems derived via autosome-sex chromosome fusion have been frequently reported in Auchenorrhyncha (see Kuznetsova and Aguin-Pombo 2015). This mechanism, necessarily resulting in reduced chromosome numbers, was clearly involved in sex chromosome diversification of the genus *Falcidius* Stål, 1866, in which the other studied species, *F. doriae*, has the basic chromosome complement of 2n = 27 (26 + X).

The basic karyotype appears conservative in structure within the Issidae, at least as regards the very large pair of autosomes, present in all the studied species. Based on a variety of observations (Giemsa-negative "secondary" constrictions, CMA_3, AgNOR and rDNA FISH patterns), the largest chromosomes are the NOR-bearing pair in the issid karyotypes.

C- banding has revealed unsuspected patterns of variation in the amount and distribution of constitutive heterochromatin in auchenorrhynchan karyotypes (see Kuznetsova and Aguin-Pombo 2015), and this is also true of Issidae. For example, *Hysteropterum albaceticum* was shown to have several bivalents easily distinguishable in meiotic cells by characteristic banding patterns (Kuznetsova et al. 2009). In the same paper, *Agalmatium bilobum* was shown to have C-bands on the largest and three medium-sized bivalents. Closely related species occasionally share the same or similar patterns as in *Mycterodus colossicus* and *Mycterodus* sp. (present study), both having telomeric C-bands on the largest and one of the medium-sized bivalents. On the other hand, *Falcidium doriae* and *F. limbatus* were demonstrated to differ extensively in their C-band pattern (Kuznetsova et al. 2010). Some additional examples can be found in Kuznetsova et al. (2010). Based on the data obtained, it can be stated that the gain and loss of heterochromatin is an important source of karyotype diversification in the Issidae.

Chromosomal mapping of repeated DNAs by fluorescence *in situ* hybridization (FISH)

Over the past decades, the FISH technique revolutionized the cytogenetic analysis providing significant advances on evolution of different insect groups with holokinetic chromosomes. At present, telomeres and the major rDNA loci are the most widely documented chromosomal regions in insects, including the order Hemiptera (e.g. Blackman et al. 2000, Manicardi et al. 2002, Monti et al. 2011a, b, Grozeva et al. 2011, 2014, Panzera et al. 2012, Chirino et al. 2013, Maryańska-Nadachowska et al. 2013, Pita et al. 2013, Bardella et al. 2013, Golub et al. 2014, 2015, Kuznetsova et al. 2012, 2015a). In addition, recent publications have shown that the number and chromosomal locations of the major rDNA multigene families are useful for the study of karyotype evolution in other insect groups (e.g. Grzywacz et al. 2011: Orthoptera; Gokhman et al. 2014: Hymenoptera; Vershinina et al. 2015: Lepidoptera; Lachowska-Cierlik et al. 2015: Mantophasmatodea; Mora et al. 2015: Coleoptera).

In Auchenorrhyncha, most cytogenetic studies were carried out by standard staining and conventional chromosome banding techniques. In this large hemipteran (= homopteran) group, FISH with rDNA and conserved insect telomeric $(TTAGG)_n$ repeats has so far been applied to 25 species, including 8 species of the genus *Philaenus* Stål, 1864 from the froghopper family Aphrophoridae (Maryańska-Nadachowska et al. 2013, Kuznetsova et al. 2015c); *Mapuchea chilensis* (Nielson, 1996) from the leafhopper family Myerslopiidae (Golub et al. 2014); 5 species of the genus *Alebra* Fieber, 1872 from the leafhopper family Cicadellidae (Kuznetsova et al. 2015b); and

11 species of the planthopper family Issidae (present paper). In addition, Frydrychová et al. (2004) reported on telomeric sequences in *Calligypona pellucida* (Fabricius, 1794) from the planthopper family Delphacidae. In all examined species, including those studied here, the presence of the $(TTAGG)_n$ telomeric repeat, known as the ancestral insect DNA motif of telomeres (Frydrychová et al. 2004), was detected.

The major rDNA loci were shown to vary in number (1 or 2 per haploid set) and chromosome location (autosomes, sex chromosomes or both; terminally or interstitially) in different species of Auchenorrhyncha. For example, in *Mapuchea chilensis* (2n = 16 +XY), the 18S rDNA clusters were present on a medium-sized pair of autosomes. In the karyotypically uniform genus *Alebra* (2n = 22 + X), they seem conserved and located on the largest pair of autosomes. In the genus *Philaenus*, which includes species with different chromosome numbers and karyotype structure, variation in number (1 or 2 per haploid set) and location (autosomes, sex chromosomes or both) of ribosomal genes was observed suggesting plasticity of the genomic organization within the genus. In the all species (11) of the Issidae from 8 genera and the three tribes, the 18S rDNA clusters were only detected in the largest autosomal pair. Basically, rDNA loci were located in an interstitial position, while in *Issus lauri* and *Zopherisca tendinosa* they were found at chromosomal ends suggesting that chromosomal rearrangements involving rDNA sequences occurred in the evolution of these unrelated species. In several karyotypes, FISH demonstrated size heteromorphism of rDNA clusters, suggesting that it can be attributed to differences in the number of ribosomal cistrons.

A brief comparison between families of the "issidoid group"

Among the families Caliscelidae, Acanaloniidae, Tropiduchidae and Nogodinidae, which are phylogenetically related to the Issidae, data on karyotypes and the number of follicles are still very scarce (Kuznetsova et al. 1998, 2010, Maryańska-Nadachowska et al. 2006), while molecular cytogenetic data are not yet available.

Follicle number

The "issidoid" families Caliscelidae, Acanaloniidae, Tropiduchidae and Nogodinidae taken together have currently only 11 species with known testis structure (Kuznetsova et al. 1998, Maryańska-Nadachowska et al. 2006). The relatively high and variable follicle numbers of the Issidae resemble the situation in the families Nogodinidae and Acanaloniidae, but not in the families Caliscelidae and Tropiduchidae, which share low and relatively stable numbers. In the four studied Nogodinidae species, numbers 5, 9 and 24 were observed, with the latter value found in two unrelated species, *Biolleyana pictifrons* Stål, 1864 and *Pisacha* sp. (Kuznetsova et al. 1998), whereas in the family Acanaloniidae, the only examined species, *Acanolonia bivittata* (Say, 1825), has 13 follicles per testis (Maryańska-Nadachowska et al. 2006).

In the Tropiduchidae, the three studied species have either 6 or 3 follicles (Kuznetsova et al. 1998), while each of the three studied species of Caliscelidae has 6 follicles per testis (Maryańska-Nadachowska et al. 2006).

Karyotype

The currently available data on the families Tropiduchidae, Nogodinidae, Caliscelidae and Acanaloniidae concern just 13 species (Kuznetsova et al. 1998, 2010, Maryańska-Nadachowska et al. 2006). The 2n = 26 + X and secondarily derived 2n = 24 + XY chromosome complements are shared by Issidae and Nogodinidae. In the latter family, *Bladina magnifrons* Walker, 1858 and *Biolleyana pictifrons* have 2n = 26 + X, whereas *Mindura subfasciata kotoshonis* Matsumura, 1941 and *Pisacha* sp. share 2n = 24 + XY (Kuznetsova et al. 1998). In the Tropiduchidae, *Achilorma* ?*bicincta* Spinola, 1838 was found to have 2n = 26 + X, whereas the three other studied species have different karyotypes, i.e., 2n = 24 + X in *Tambinia bizonata* (Matsumura, 1914) and *Barunoides albosignata* Distant, 1912, while 2n = 28 + X in *Varma distanti* Melichar, 1914 (Kuznetsova et al. 1998). Putative ancestral issid karyotype of 2n = 26 + X (Kuznetsova et al. 2010), has not yet been found in the families Caliscelidae and Acanaloniidae (Maryańska-Nadachowska et al. 2006).

Concluding remarks

Based on the currently available data, which are still highly insufficient, we can infer that Issidae are characterized by 10 follicles per testis as the most frequent number, the presence of canonical insect telomeric repeats $(TTAGG)_n$, a stable karyotype constitution with the predominant karyotype of 2n = 26 + X(0), and the major rRNA gene clusters located on the largest pair of autosomes. A much broader taxonomic coverage is necessary to discuss possible implications of the above characters for the taxonomy and phylogeny of the Issidae.

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Karyotype and sex chromosome differentiation in two *Nalassus* species (Coleoptera, Tenebrionidae)

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Abstract

Cytogenetic features of *Nalassus bozdagus* Nabozhenko & Keskin, 2010 and *Nalassus plebejus* Küster, 1850 were analysed using conventional and differential staining. Mitotic and meiotic chromosomal analysis revealed the diploid number as 2n = 20 (9+X_{y_p}) in both species. Besides the general resemblance of two *Nalassus* Mulsant, 1854 karyotypes, important differences related to variations in the number of metacentric/submetacentric chromosomes, localization of highly impregnated regions which are considered as NOR and heterochromatin distribution are clearly observed. The most prominent difference between two species is found related to the X chromosome which is clearly larger in *N. bozdagus* and has a conspicuous secondary constriction on the long arm. As a result of silver staining, the existence of highly impregnated areas associated with X_{y_p} of *N. bozdagus* in both prophase I and metaphase I, suggests that NORs are seemingly located on sex chromosomes. On the other hand, the potential NORs of *N. plebejus* were observed only in prophase I nuclei. With the application of fluorescence dye DAPI, the AT rich chromosome regions and X_{y_p} which forms the parachute configuration were shown in both species.

Keywords

Karyotype, Tenebrionidae, Helopini, *Nalassus bozdagus*, *Nalassus plebejus*, sex chromosomes, heterochromatin, NOR, DAPI

Introduction

In the light of fossil and molecular dating analysis, the darkling beetles are dispersed and diversified over the last 180 million years prior to Gondwanan fragmentation. Tenebrionids represent a hyperdiverse family of Coleoptera with ca. 20000 recognized species worldwide. In consequence of undergoing multiple evolutionary radiations, tenebrionids show considerable morphological variations and several adaptations in life history traits such as feeding behaviour, habitat preferences, flight ability etc. Although higher level of tenebrionid phylogeny based on sequences from seven out of nine subfamilies shows well supported monophyly, the subfamilies Diaperinae, Pimeliinae and Tenebrioninae were recovered as paraphyletic or polyphyletic (Kergoat et al. 2014a, b).

The karyotypes of more than 250 darkling beetle species have been determined (Holecová et al. 2008a, Juan and Petitpierre 1991a, Blackmon and Jeffery 2015, Gregory 2016). Although most species present a karyotype with 2n = 20, the diploid number ranges from 2n = 14 to 2n = 38 (Juan and Petitpierre 1991a, Pons 2004, Holecová et al. 2008a, Lira-Neto et al. 2012). Chromosomal data are only available for several representatives of subfamilies Lagriinae, Tenebrioninae, Pimelinae, Allecullinae and Diaperinae mostly distributed in Mediterranean (Juan and Petitpierre 1991a).

The genus *Nalassus* Mulsant, 1854 (Tenebrioninae: Helopini) comprises 71 described taxa distributed mainly in the Western Palearctic, but with disjunctively isolated species in the Russian Far East and Northern China (Medvedev 1987, Nabozhenko 2001). Even though a significant part of the species is found in alpine and subalpine mountainous belts with high level of local endemism, some species that are adapted to lowlands have wider distribution. In the recent reviews of *Nalassus* species from the European part of CIS (Commonwealth of Independent States), Caucasus, Iran, Georgia, China and Turkey, several new species and combinations were also noted (Nabozhenko 2001, Nabozhenko 2007, Nabozhenko 2008, Keskin and Nabozhenko 2010, Nabozhenko 2011, Nabozhenko 2013, Nabozhenko 2014, Nabozhenko and Ivanov 2015). Therefore, the actual diversity is certainly higher than previously estimated and the monophyly of the genus *Nalassus* needs to be tested with several new additional characters. The chromosomes of *Nalassus* have not yet been studied. Furthermore, cytogenetic data concerning the tribe Helopini which provide no more than chromosome numbers and sex determination systems are only known for some *Nesotes* and *Probaticus* species (Juan and Petitpierre 1986, 1989, 1991a, 1991b, Palmer and Petitpierre 1997).

In this study, with the aim of providing first cytogenetic information about *Nalassus*, the mitotic and meiotic chromosomes of endemic *N. bozdagus* and widespread *N. plebejus* were analysed using conventional, DAPI fluorochrome staining and silver impregnation.

Material and methods

The meiotic and mitotic chromosomes of 12 male *N. plebejus* and 4 male *N. bozdagus* individuals from Western Anatolia were analysed. The specimens of *Nalas-*

sus plebejus were retrieved from Ballıkayalar Natural Park, Gebze (40°50'22.96"N / 29°30'56.11"E, 120m) and the specimens of *Nalassus bozdagus* were collected from Bozdağ, İzmir (38°15'17.49"N / 27°57'44.72"E, 2300m). Adult beetles were collected on the trunks of trees and on the ground at night when they are active.

The chromosome preparations were obtained from the gonads of male specimens using Murakami and Imai's (1974) splashing method with some modifications. Briefly, testes were carefully dissected and macerated with sterilized needles. Testes were treated with hypotonic solution (0.65% KCl) for 5 minutes and fixed in 3:1 ethanol: acetic acid at least for 1 h on ice.

We also applied a microspreading method (Chandley 1994) for obtaining prophase I nuclei. The slides were stained with 4% Giemsa in phosphate buffer pH 6.8, for 20 minutes for standard staining. The silver impregnation technique of Patkin and Sorokin (1984) was performed to determine the possible NOR regions. Briefly, slides were incubated in distilled water for 30 min. at room temperature and stained with $AgNO_3$ working solution (2:1, 50% $AgNO_3$: 2% gelatin containing 0.5% formic acid) in a humid chamber at 60 °C for 3-10 minutes. After a golden-brown color has developed, the reaction was stopped by rinsing with distilled water. Slides were then dehydrated, counterstained with 4% Giemsa in phosphate buffer pH 6.8.

For determining of heterochromatin distribution, the slides were mounted with antifade mounting medium with fluorochrome DAPI (Vectashield) specific to AT-rich chromosomal regions. The visualization of DAPI stained plates were carried out with Olympus BX50 fluorescent microscope.

The mitotic and meiotic plates were analysed and photographed with Zeiss Axio Scope light microscope using ZEN software. The chromosomal measurements were made with the LEVAN plugin (Sakamoto and Zacaro 2009) and the karyotypes and idiograms were created with the CHIAS plugin (Kato et al. 2011) of the programme IMAGE J (Rasband 1997-2015).

Results

Conventional Giemsa staining

Analysis of spermatogonial cells of *Nalassus bozdagus* and *Nalassus plebejus* revealed the diploid chromosome number as 2n = 20 ($9+X_yY_p$) (Fig. 1). In both species, most of the autosomes showed metacentric morphology, the X chromosomes were submetacentric and the y chromosomes were subtelocentric. In *N. bozdagus* the autosomal pairs 8 and 9 were submetacentric while in *N. plebejus* autosomal pairs 1, 5 and 8 were submetacentric. The smallest chromosome in both species was determined to be the y chromosome (~1 μm). The biggest chromosome of *Nalassus bozdagus* was the X chromosome (~4.315 μm), in *Nalassus plebejus* the biggest chromosome was the 1. chromosome with the length of 4.442 μm (Table 1).

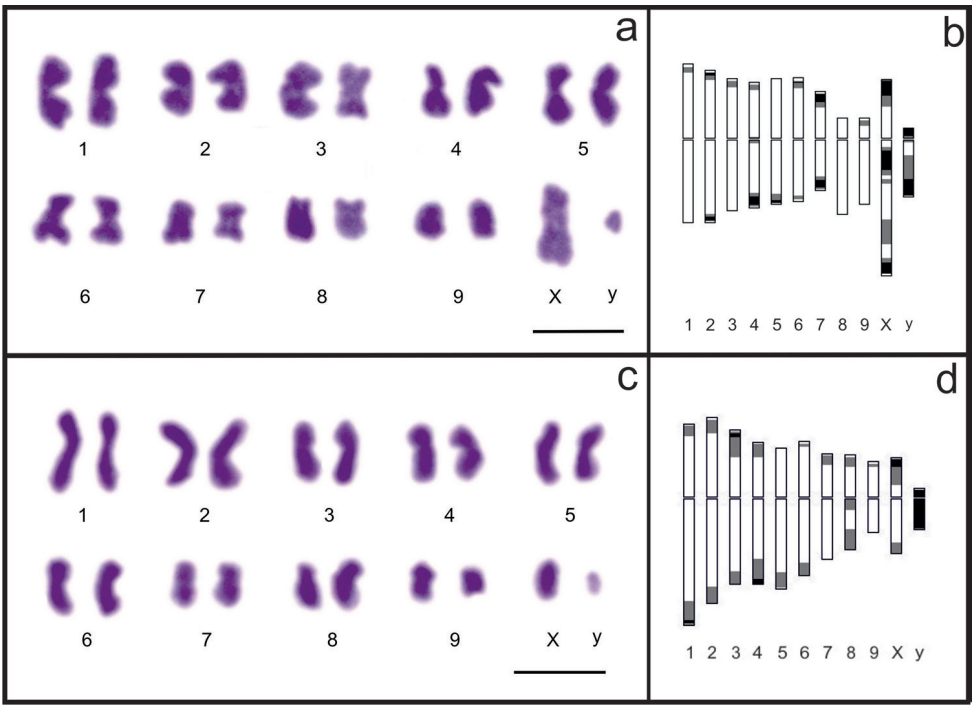

Figure 1. a–b *N. bozdagus* 2n = 20. **a** karyotype **b** idiogram **c–d** *N. plebejus* 2n = 20 **c** karyotype **d** idiogram. Bar = 5 μm.

Table 1. Chromosome morphologies and measurements of *N. bozdagus* and *N. plebejus.* CI: centromere index, RL: relative length, AR: arm ratio, *secondary constriction.

	N. bozdagus					*N. plebejus*				
Chromosome	**Length (μ)**	**CI**	**%RL**	**AR**	**Morphology**	**Length (μ)**	**CI**	**%RL**	**AR**	**Morphology**
1	3.895	46	12.4	1.20	m	4.442	35	14.26	1.8	sm
2	3.420	47	10.89	1.23	m	4.207	48	13.5	1.04	m
3	3.375	46	10.74	1.29	m	3.316	43	10.64	1.4	m
4	3.204	46	10.2	1.10	m	3.117	43	10	1.28	m
5	2.876	47	9.16	1.20	m	3.222	28	10.34	2.52	sm
6	2.715	48	8.64	1.06	m	3.040	44	9.76	1.27	m
7	2.204	48	7.02	1.07	m	2.439	45	7.83	1.18	m
8	2.162	33	6.88	2.13	sm	2.476	29	7.95	2.38	sm
9	2.149	32	6.84	2.01	sm	1.853	46	5.95	1.17	m
X	4.315	28	13.74	2.47	sm*	2.04	30	6.55	2.31	sm
y	1.097	20	3.5	3.88	st	1.010	18	3.24	1.17	st

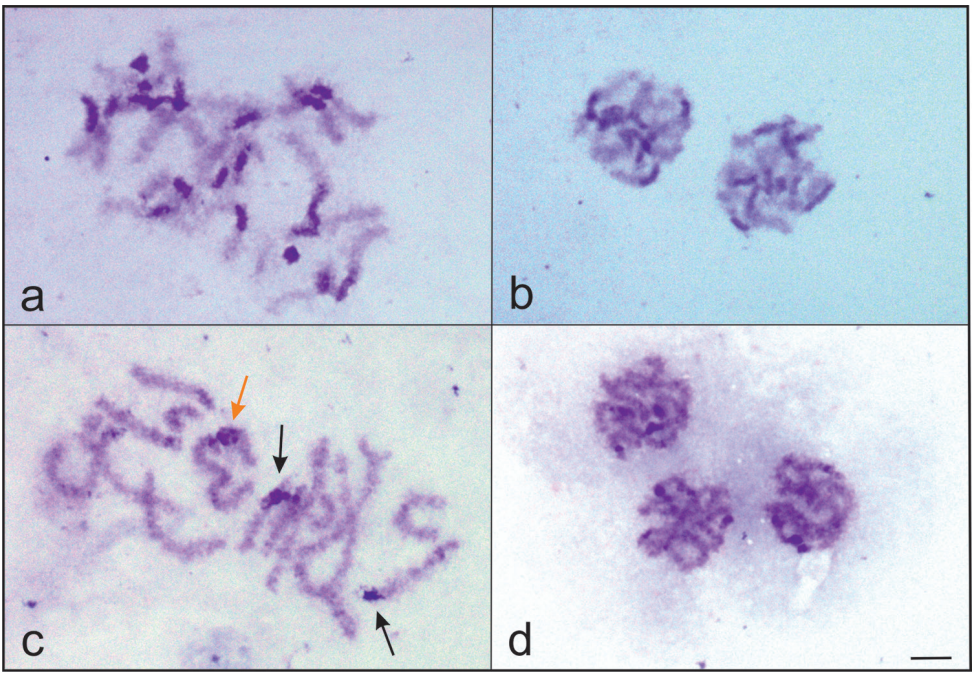

Figure 2. a–b *N. bozdagus* with dark heterochromatic blocks on all chromosomes. **a** leptotene–zygotene **b** pachytene **c–d** *N. plebejus* with two distinctive heterochromatic blocks (black arrows); **c** leptotene–zygotene **d** pachytene. Orange arrow indicates X_{y_p} sex bivalent, Bar = 5 μm.

In prophase I nuclei, all chromosomes of *Nalassus bozdagus* showed dark heterochromatic blocks mainly located in centromeric regions (Fig. 2a–b). But in *Nalassus plebejus,* while most of the chromosomes have relatively small amounts of heterochromatin dispersed throughout the whole length (Fig. 2c–d), only 2 chromosomes with distinctive heterochromatic blocks were observed.

In diplotene/diakinesis of *N. plebejus*, 5-6 rod-shaped (terminal chiasma), 2-3 ring-shaped (two terminal chiasmata) and 1-2 cross-shaped (interstitial chiasma) bivalents were observed (Fig. 3a). In diakinesis/metaphase I; most of the homologous chromosomes of both species formed rod shaped bivalents due to being monochiasmatic and 2-3 ring shaped bivalents due to being bichiasmatic (Fig. 3b–c). In metaphase I plates, the parachute formation of sex bivalents was clearly observed for both *Nalassus* species (Fig. 4a–b). In metaphase II plates, relatively small sized 10 chromosomes (Fig. 4c–d) were observed. However, the plates which possessed the minute y chromosome were seemed to have only 9 chromosomes in their haploid sets. (Fig. 4d).

Sex chromosomes of two species were differed from each other by the length of X chromosome. The X chromosome of *N. bozdagus* was determined to be almost twice the size of the X chromosome of *N. plebejus* (Fig. 1, Table 1).

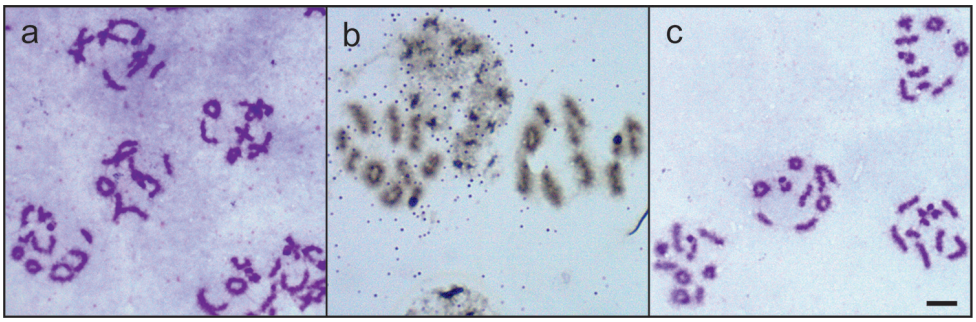

Figure 3. a diplotene–diakinesis in *N. plebejus* **b–c** diakinesis–metaphase I **b** *N. bozdagus* **c** *N. plebejus*. Bar = 5 μm.

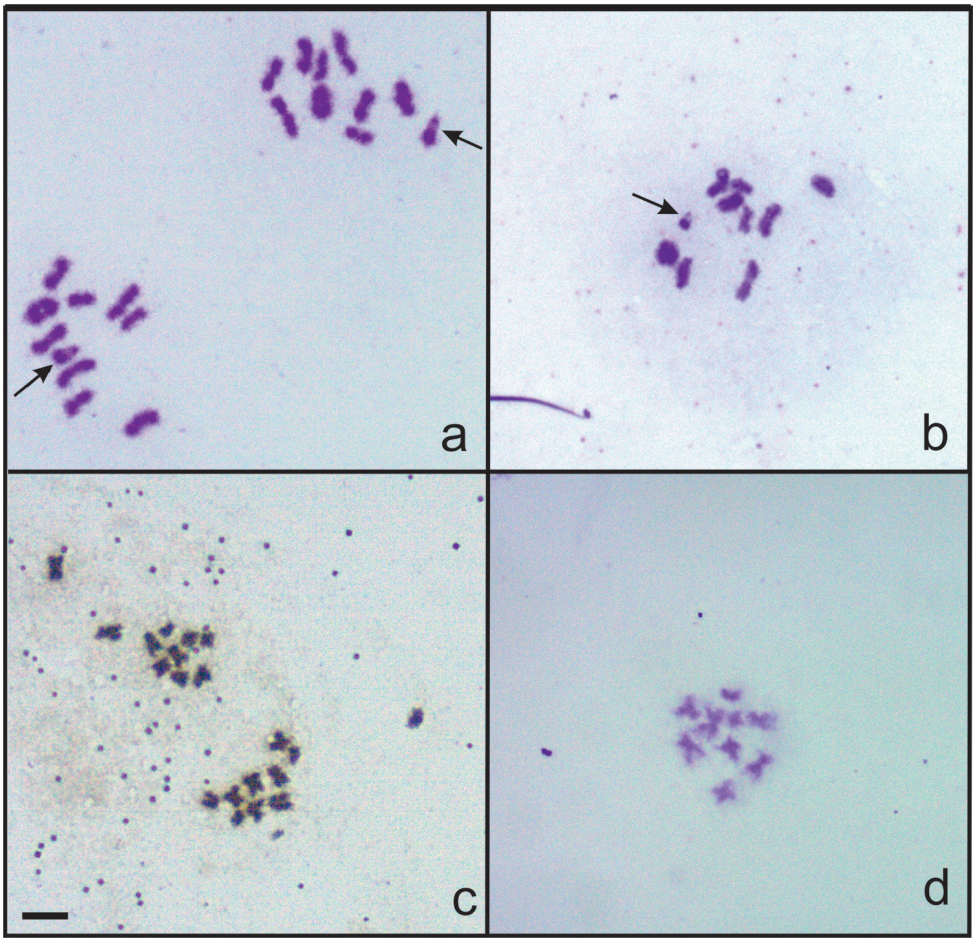

Figure 4. a–b metaphase I **a** *N. bozdagus* **b** *N. plebejus* **c–d** metaphase II **c** *N. bozdagus* **d** *N. plebejus*. Arrows show X_{y_p} sex bivalents, Bar = 5 μm.

Differential staining

Silver nitrate staining of the chromosomes of *Nalassus bozdagus* revealed the presence of a highly impregnated nucleolus (NOR) associated with one of the long chromosomes in prophase I nuclei (Fig. 5a–b) and that X_{y_p} sex bivalent is strongly argyrophilic in metaphase I (Fig. 5c). In *Nalassus plebejus*, these strongly argyrophilic regions were observed only in pachytene nuclei (Fig. 5d). With base-specific (A-T) DAPI staining; metaphase I plates and prophase I nuclei were observed. In metaphase I stages there were no significant difference between species (Fig. 6a–b). Prophase I nuclei of *N. bozdagus* showed strong signals on pericentromeric heterochromatic blocks compared to other chromosomal regions (Fig. 6c). On the other hand, in *N. plebejus* only some centromeric regions showed slightly stronger fluorescence signals (Fig. 6d).

Discussion

The family Tenebrionidae is considered a karyologically conservative group due to the frequent occurrence of 2n = 20 formula (Juan and Petitpierre 1991a, Palmer and Petitpierre 1997). Heretofore, variation in the diploid chromosome numbers between

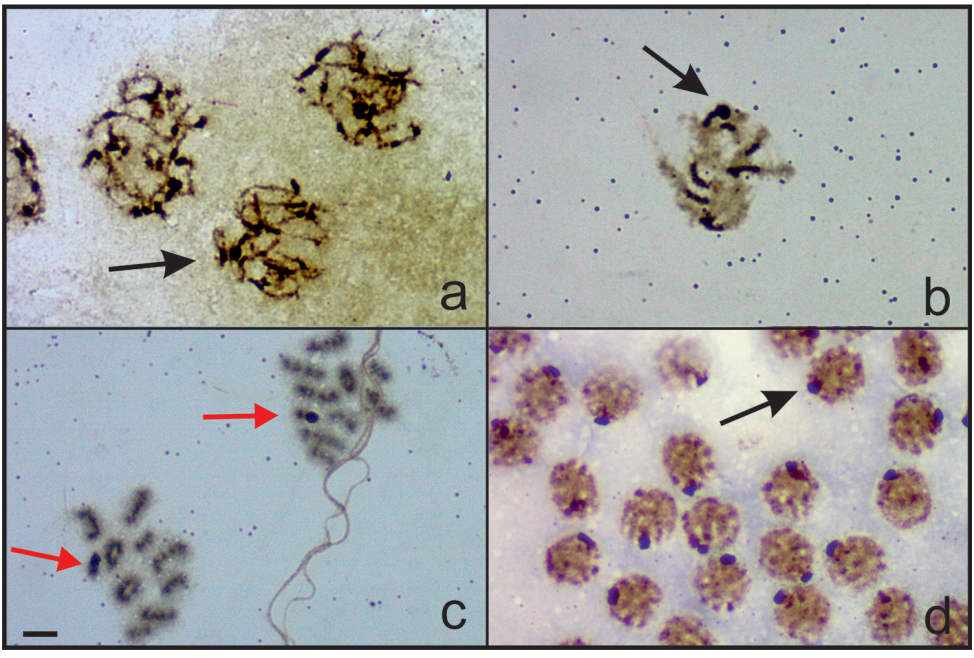

Figure 5. Silver nitrate staining **a–c** *N. bozdagus* **d** *N. plebejus.* Black arrows indicate NOR, red arrows indicate argyrophilic sex bivalents, Bar = 5 μm.

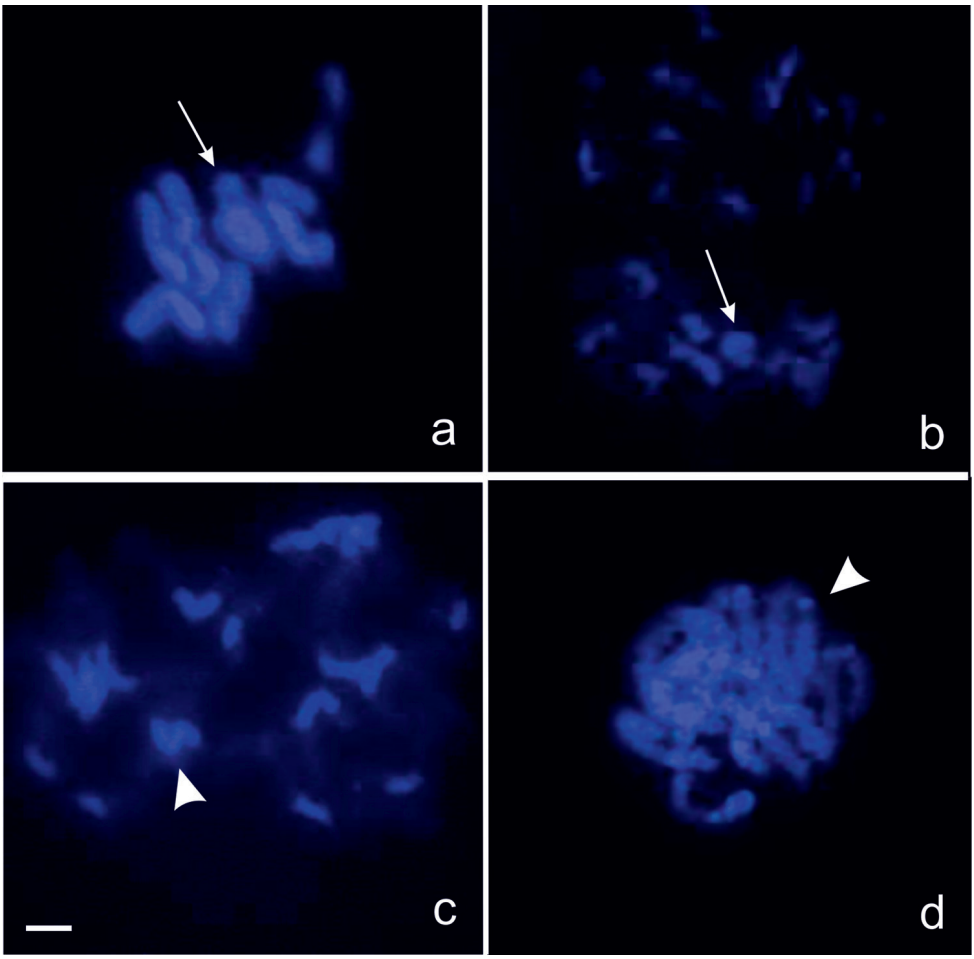

Figure 6. DAPI staining **a** metaphase I of *N. plebejus* **b** metaphase I of *N. bozdagus* **c** prophase I nucleus of *N. bozdagus* **d** prophase I nucleus of *N. plebejus*. Arrows show X_{y_p} sex bivalents and arrowheads indicate heterochromatic regions, Bars = 5 μm.

14–38 within the family are also noted (Juan and Petitpierre 1991a, Pons 2004, Holecová et al. 2008a, Lira-Neto et al. 2012). Although, tenebrionid karyotypes characterized with predominant presence of metacentric chromosomes (Guenin 1950, 1951a, b; Smith 1952, Yadav and Pillai 1974, Yadav et al. 1980, Juan and Petitpierre 1988, 1989, 1990, Juan et al. 1989), several species from different subfamilies have mostly subtelocentric/acrocentric sets (e.g. *Laena reiteri* Weise 1877, 2n = 18, Holecová et al. 2008a, *Palembus dermestoides* Fairmaire 1893, 2n = 20, Almeida et al. 2000). Furthermore, many tenebrionid beetles possess similar chromosome number but differ in karyotype structure, which reveal additional evidence for karyotype divergence through the intra-chromosomal rearrangements. The major patterns of karyological variations in tenebrionid beetles are mainly observed in sex determining systems, chromosome

morphology and distribution of heterochromatin (Juan and Petitpierre 1990, Petitpierre et al. 1991, Juan and Petitpierre 1991a-b, Juan et al. 1993, Bruvo-Madaric et al. 2007).

Tenebrionidae comprises 9 subfamilies but most of the cytogenetically studied species (96%) belong to the Pimeliinae and Tenebrioninae (Bouchard et al. 2005, Holecová et al. 2008a). The diploid number in Pimeliinae shows a decrease from 2n = 20 to 2n = 18 caused by fusion of an autosomal pair while in Tenebrioninae there is a tendency of increased diploid number probably caused by centric fissions (Juan and Petitpierre 1991a).

We showed here that the karyotypes of *Nalassus bozdagus* and *Nalassus plebejus* consist of 10 pairs of chromosomes (2n = 20) (Fig. 1), which is considered as modal chromosome number for Tenebrionidae (Juan and Petitpierre 1991a, Pons 2004, Holecová et al. 2008a, Lira-Neto et al. 2012). The presence of heteromorphic sex chromosomes for both species is confirmed by occurrence of a X_{y_p} configuration in both conventionally (Fig. 4a-b) and differentially (Fig. 5c, 6a–b) stained metaphase I plates. The X_{y_p} sex determining system is the most frequent type among Tenebrionidae as well as order Coleoptera (Smith and Virkki 1978, Juan and Petitpierre 1991a). However, sex chromosomes or sex determining systems are mentioned as one of the major chromosomal changes involved in tenebrionid divergence.

Besides the general resemblance of two *Nalassus* karyotypes, important differences related to X chromosomes, variations in the number of metacentric/submetacentric chromosomes (Fig. 1, Table 1), localization of highly impregnated regions which are considered as NOR (Fig. 5a–d) and heterochromatin distribution (Fig. 6c-d) are clearly observed. The most prominent difference between two species is found related to X chromosome which is clearly larger (13.74% of total complement) in *N. bozdagus* and has a conspicuous secondary constriction on the long arm (Fig. 1). It was also observed that metaphase I plates *of N. bozdagus* have relatively larger X_{y_p} (Fig. 4a–b). The increase in relative length of X which does not alter parachute configuration is named as giant X_{y_p} and generally thought to be derived from either heterochromatin amplification or translocation (Dutrillaux and Dutrillaux 2009). Difference in size and heterochromatin content of X chromosomes also observed in two closely related tenebrionid species of *Gonocephalum* Solier 1834 (Tenebrioninae) (Juan and Petitpierre 1989).

The differences found in chromosome morphology (1., 5. and 9. pairs) between these two *Nalassus* species are thought to be related to pericentromeric inversions that resulted in centromeric shift. Pericentromeric rearrangements are already known within several Coleopteran families such as Cicindelidae, Chrysomelidae, Meloidae, Scarabaeidae and Tenebrionidae (Serrano 1981, Petitpierre 1983, Juan et al 1990, Almeida et al 2000, Petitpierre and Garneria 2003, Wilson and Angus 2005, De Julio et al 2010, Petitpierre 2011).

The karyotypes of *N. bozdagus* and *N. plebejus* also show obvious differences, especially in distribution of heterochromatin. The presence of strong signals on pericentromeric heterochromatin blocks on all chromosomes of *N. bozdagus* (Fig. 2a–b) and only few chromosomes in *N. plebejus* (Fig. 2c–d) was supported with both conven-

tionally and differentially stained prophase I nuclei (Fig. 4g–h). Although, occurrence of heterochromatin observed mainly in the pericentromeric areas of the tenebrionid chromosomes, variability of heterochromatin localization and composition were also reported (Juan and Petitpierre 1989, 1991, Pons 2004, Rozek et al. 2004, Cabral-de-Mello et al. 2010, Schneider et al. 2007).

As a result of silver staining, the existence of highly impregnated areas associated with X_{y_p} of *N. bozdagus* in both prophase I and metaphase I, suggests that NORs are seemingly located on sex chromosomes (Fig. 5a–c). On the other hand, the potential NORs of *N. plebejus* were observed only in prophase I nuclei (Fig. 5d). Similar findings on argyrophilic X_{y_p} in metaphase I as well as prophase I were previously reported for several beetles such as *Zophobas* aff. *confusus* Gebien 1906 (Tenebrionidae) (Lira- Neto et al. 2012), *Lagria villosa* Fabricius 1781 (Tenebrionidae) (Goll et al. 2013), *Palembus dermestoides* (Tenebrionidae) and *Epicauta atomaria* Germar 1821 (Meloidae) (Almeida et al. 2000). Although, rDNA-FISH studies has shown that these strongly argyrophilic areas in prophase I bivalents are associated with NOR (Juan et al. 1993, Bruvo-Madaric et al. 2007), the existence of highly impregnated areas on sex chromosomes until metaphase I thought to be related to association and segregation of sex bivalents due to nucleolar material or distinctive heterochromatin (Juan et al. 1993, John and Lewis 1960). The association between sex chromosomes and nucleolar material is widely known for several animal groups from mammalians to insects (Smith and Virkki 1978, Virkki et al. 1991, Tres 2005), although autosomal localization of NORs by FISH using 18S rDNA probes were also reported for some tenebrionid species (Goll et al. 2003, Juan et al. 1993).

It was observed that bichiasmatic autosomes form ring bivalent while monochiasmatics form rod bivalents due to terminal chiasmata (Fig. 3b–c). Ring bivalents are frequent among Tenebrionidae, Scarabaeidae, Meloidae, Buprestidae, Curculionidae, Chrysomelidae and Cerambycidae (Petitpierre 1985, Bisoi and Patnaik 1988, Petitpierre and Garneria 2003, Karagyan et al. 2004, 2012, Lachowska et al. 2004, 2006a, 2006b, Rozek et al. 2004, Angus et al. 2007, Holecová et al. 2008b). During diplotendiakinesis of *N. plebejus*, in addition to ring and rod bivalents we also observed cross shaped bivalents (Fig. 3a) due to interstitial chiasmata.

The information acquired from metaphase I plates of only few *Nesotes* Allard 1876 species (Juan and Petitpierre 1986, 1989, 1991a, 1991b) and *Probaticus ebeninus* A. Villa and J. B. Villa, 1838 (Palmer and Petitpierre 1997) are the only cytogenetic data concerning the tribe Helopini. On the basis of metaphase I plates, it was only briefly noted that 5 *Nesotes* species have similar 2n = 20, 9+ X_{y_p} formula (Juan and Petitpierre 1986, 1989, 1991a) and *P. ebeninus* have 2n = 20, 9+XY (Palmer and Petitpierre 1997). Although, our findings for chromosome numbers correspond to previous cytogenetic data, comparative genomic analyses of Helopini require detailed descriptions of chromosome morphologies.

In conclusion, this study revealed that the cytogenetic features differed between endemic *N. bozdagus* and widespread *N. plebejus*. But, in the absence of molecular cytogenetic and phylogenetic approaches, it is not possible to make a strong conclusion

about the major forces underlying these chromosomal variations. For definitive testing of the general trends in both *Nalassus* and tenebrionid karyotype evolution, it is necessary to increase the taxa sampling for major tenebrionid lineages.

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RESEARCH ARTICLE

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Microchromosome polymorphism in the sand lizard, *Lacerta agilis* Linnaeus, 1758 (Reptilia, Squamata)

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Abstract

Most true lizards (Lacertidae) share a conservative karyotype, consisting of 18 pairs of macrochromosomes and one microchromosome pair. Homeologues of the microchromosome are present in other squamates and even in chickens. No structural autosomal microchromosome polymorphisms have been described previously in lizards. We found homozygous and heterozygous carriers of a microchromosome variant in a Siberian population of the sand lizard, *Lacerta agilis* Linnaeus, 1758. The variant microchromosome was almost twice as long as the standard one. In heterozygotes at pachytene, the microchromosomes firstly pair in proximal regions and the central part of the longer axial element undergoes foldback synapsis, then its distal region pairs with the distal region of the standard partner. At metaphase-I, the heteromorphic microchromosome bivalents have a proximal chiasma. The content of the additional segment was Ag-NOR, C-like DAPI, CMA3 negative. FISH with telomere PNA probe did not detect interstitial $(TTAGGG)_n$ sequences in the heteromorphic and any other bivalents. Both homo- and heterozygous carriers were phenotypically normal. The presence of homozygotes shows that heterozygotes are fertile. Reduction in the number of microchromosomes is a clear trend in squamate evolution, as a result of microchromosomes fusing together or with macrochromosomes. Our findings indicate that gaining additional DNA may lead to a transformation of microchromosomes into small macrochromosomes without fusion.

Keywords

Synaptonemal complex, Lacertidae, chromosome evolution

Introduction

Microchromosomes are considered as the part of the ancestral tetrapod genome (Uno et al. 2012). They have been conserved in different degrees in modern lineages. In amphibians, microchromosomes are present in primitive families of all three orders, independently disappearing in the more derived ones (Morescalchi 1980). Among amniotes, birds retain the most archaic karyotype with many microchromosomes, whereas in squamate reptiles the number of microchromosomes has decreased (Olmo 2008). They comprise a half or more of the chromosome sets in iguanids, agamids, snakes and varanids. However they are not present in crocodiles (Crocodilia) and geckos (Gekkota). It is believed that the independent and parallel loss of microchromosomes in these groups has been achieved by their fusion with each other and with macrochromosomes (Srikulnath et al. 2014, 2015). The true lizards (Lacertidae) also lost most of microchromosomes. Lacertidae are one of the most widespread reptile families. At the moment, 322 species are recognized within the family (Uetz, Hošek, The Reptile Database). Karyotypes of the true lizards are rather conservative. Most of them have 18 pairs of macrochromosomes and one pair of microchromosomes (2n=38) (Gorman 1969, Olmo and Signorino 2005). The microchromosome is usually much shorter than the smallest macrochromosome and can be unequivocally identified by size. Some species, such as *Zootoca vivipara* (Lichtenstein, 1823) and *Iberolacerta monticola* (Boulenger, 1905), do not have microchromosomes at all. Homeologues of this microchromosome have been found in snakes, varanids, agamids and even in chicken (Srikulnath et al. 2014).

Chromosome polymorphism in the lacertids is poorly known. We are aware of only one case of chromosome polytypism: variation in C-band distribution between subspecies and populations of Italian wall lizard *Podarcis siculus* (Rafinesque-Schmaltz, 1810) (Olmo et al. 1986). No examples of microchromosome morphology polymorphism have been described in true lizards.

In this paper we describe a long microchromosome variant which covers the gap between micro- and macrochromosomes. We found this variant in Siberian population of the sand lizard, *Lacerta agilis* Linnaeus, 1758, and examined its meiotic behavior in homo- and heterozygotes by fluorescent microscopy of synaptonemal complexes (SCs) and metaphase-I spreads using immunolocalization of SYCP3 (the major protein of the SC axial elements) and centromeres, and electron microscopy using Ag-NOR staining.

The SC analysis via electron microscopy and immunofluorescent staining is widely used in vertebrate cytogenetics (Lisachov et al. 2015; Wallace and Wallace 1995; Reed et al. 1990; Calderon and Pigozzi 2006; Basheva et al. 2014). At pachytene, the compactization of chromatin is much lower than at metaphase, and thus the SC analysis provides higher resolution than the conventional metaphase techniques. This is particularly useful in the microchromosome studies. The requirement of the homologous pairing at pachytene makes the SC analysis a perfect tool in studying any chromosomal heteromorphisms, both autosomal and gonosomal. Our work is the first in which the immunofluorescent staining is applied for reptiles.

Materials and methods

The lizards were caught near Berdsk (54°46.37'N, 83°5.77'E) (ten specimens) and Novosibirsk (54°50.78'N, 82°57.92'E) (four specimens), Novosibirsk region, Russia. Trapping, handling, and euthanasia of animals were performed according to the protocols approved by the Animal Care and Use Committee at the Institute of Cytology and Genetics of the Russian Academy of Sciences. All institutional and national guidelines for the care and use of laboratory animals were followed. No additional permits are required for research on this non-listed species in Russia. The specimens were deposited in the research collections of the Institute of Cytology and Genetics of the Russian Academy of Sciences.

The spreads of spermatocytes were prepared according to the protocol of Peters et al. (1997).

For electron microscopic examination the spreads were stained with silver nitrate (Howell and Black 1980) and covered with plastic film. The spreads, after light microscopic examination, were transferred to specimen grids and examined with electron microscope JEM-100 (JEOL, Japan) at 80 kV.

Immunostaining was performed according to the protocol described by Anderson et al. (1999) with modifications. SCs were detected by rabbit polyclonal antibodies to the SC axial element protein SYCP3 (1:500, Abcam, Cambridge) and goat anti-rabbit Cy3-conjugated secondary antibodies (1:500, Jackson, West Grove). Centromeres were detected by human anti-centromere antibodies (ACA) (1:100, Sigma-Aldrich) and goat anti-human FITC conjugated secondary antibodies (1:100, Vector Laboratories). All antibodies were diluted in PBT (3% bovine serum albumin and 0.05% Tween 20 in 1xPBS). A solution of 10% PBT was used for blocking unspecific antibody binding. Primary antibody incubation was performed overnight in a humid chamber at 37 °C, and secondary antibody incubation was performed for 1 h at 37 °C. Finally, slides were mounted in Vectashield with DAPI (Vector Laboratories) to stain DNA and reduce fluorescence fading. The spreads were photographed using an Axioplan 2 Imaging (Carl Zeiss) microscope with CCD camera (CV M300, JAI Corporation, Japan), CHROMA filter sets, and ISIS4 image processing package (MetaSystems GmbH, Germany). The length of each bivalent in the spread was measured using MicroMeasure 3.3 software. Statistica 6.0 software package (StatSoft, Tulsa, OK, USA) was used for descriptive statistics.

The heterochromatic regions were visualized by a previously described C-like DAPI staining technique (Lisachov 2013). The coverslips were carefully removed after the photographs were taken. The preparations were washed in 2×SSC for 5 min to remove the antifade solution and then dehydrated in ethanol series 70%, 80% and 100% for 3 minutes in each. The preparations were then air-dried and kept in 0.2 N HCl at room temperature for 20 min to 30 min. The slides were transferred to saturated barium hydroxide solution at 55 °C for 1 min to 10 min. The preparations were then incubated in 2×SSC at 55 °C to 60 °C for 60 min. The preparations were re-mounted in the antifade solution with DAPI.

For chromomycin A_3 (CMA_3) staining, we used the solution of 0.4 mg/ml CMA_3 and 0.01 M $MgCl_2$ in PBS. After preparing, the solution was left to stabilize at +4 °C for two days. Then 25 µl of the solution was put onto the slide already subjected to immunostaining, and covered by the coverslip. After 1 h, the slide was washed in PBS for 5 min and then mounted in the antifade solution with DAPI. After staining, the slide was again left to stabilize at the room temperature in the dark for three days.

The telomeric $(TTAGGG)_n$ sequences were detected with a commercial FITC-conjugated PNA probe (LifeTechnologies) according to the manufacturer's protocol.

Results

We examined 14 male lizards, ten from Berdsk and four from Novosibirsk. In all of them, 19 acrocentric bivalents were seen at synaptonemal complex spreads and at metaphase I plates (2n=38). The mean total length of the SCs was 178±21 µm. The macroSCs formed a gradually decreasing set. In five individuals from Berdsk and the four from Novosibirsk, the microchromosome (SC 19) was significantly smaller than the smallest macrochromosome (SC 18). Their mean sizes, relative to the total SC length, were 1.68±0.14% and 3.14±0.31% respectively (P <0.001)). The microchromosome was thus easily identifiable at SC spreads, as well as at meiotic metaphase I plates (Figs 1a, 2a, 3a, 5a–c).

In one Berdsk individual (#3) the difference between SC 18 and SC 19 was less pronounced although still significant, 3.24±0.24% and 2.78±0.14% respectively (P <0.001) (Figs 1b, 2b, 5d–f).

In four Berdsk individuals ##1, 7, 9, 10 we detected a heteromorphic SC 19 with one axial element element significantly longer than the other (P=0.03). The relative length of the longer element was 3.08±0.53% of the total macrochromosomal SC length plus the long element 19. The shorter element was 1.77±0.33% of the total macrochromosomal SC length plus the short element 19 (Figs 1c, 2c, 4).

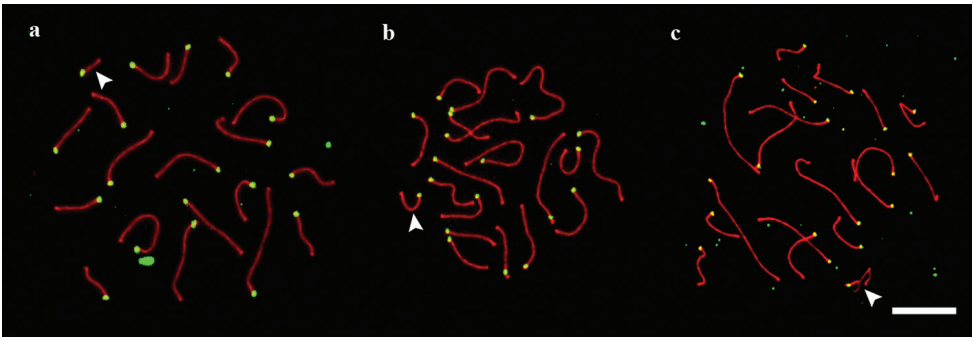

Figure 1. SC spreads of sand lizards. **a** standard karyotype **b** homozygote for the long variant of SC 19 **c** heterozygote for the long variant of SC 19. Arrowheads indicate SC 19. Red: SYCP3. Green: ACA. Scale bar: 10 µm.

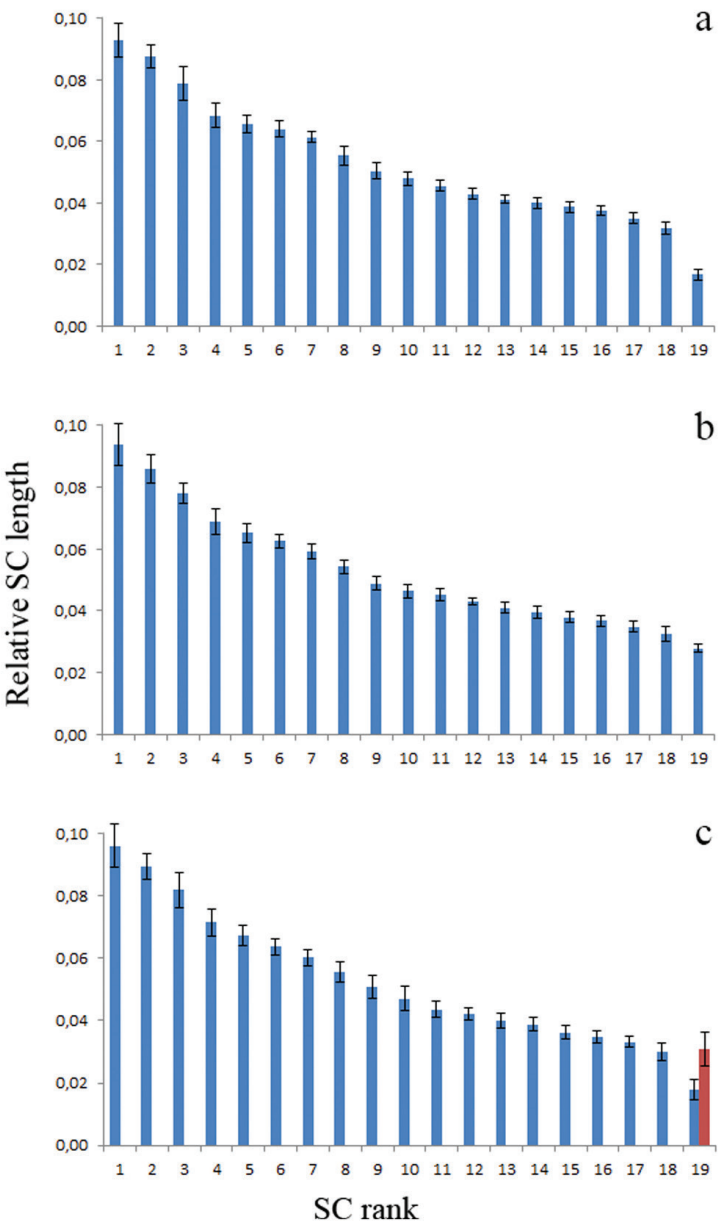

Figure 2. Relative lengths of the SCs in three sand lizards. **a** standard karyotype (21 spreads) **b** homozygote for the long variant of SC 19 (22 spreads) **c** heterozygote for the long variant of SC 19 (18 spreads). Red column: the long variant in the heterozygote. Bars show standard deviation.

Fig. 4 shows variability of the synaptic configurations of the heteromorphic SC 19, which probably reflects the sequence of conjugation. At early stage of conjugation the proximal parts of the elements were synapsed while the distal ones remained unpaired

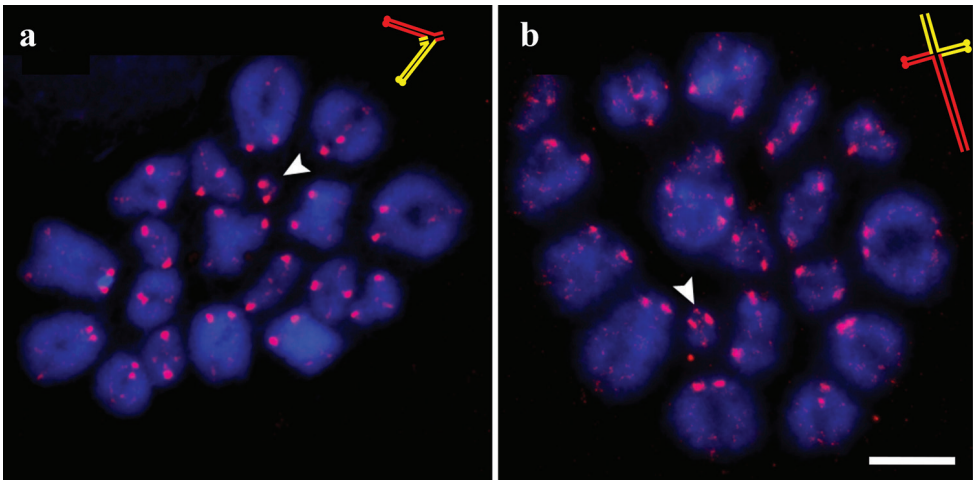

Figure 3. Meiotic metaphase I in sand lizards. **a** standard karyotype **b** heterozygote for the long variant of SC 19. Arrowheads and schematic inserts show bivalent 19. In schematic inserts red and yellow colors show the homologues. Red: SYCP3. Blue: DAPI. Scale bar: 10 μm.

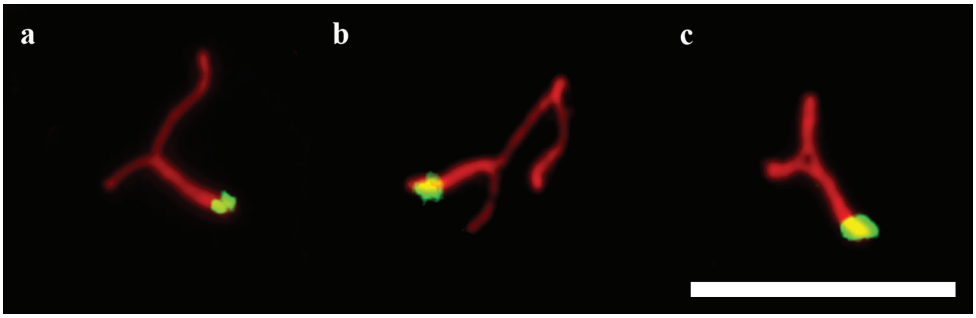

Figure 4. Synaptic configurations of the heteromorphic SC 19. **a** synapsis in the proximal regions **b** the longer element showing foldback self-synapsis **c** completely paired forming a T-shaped configuration. Scale bar: 5 μm.

(Fig. 4a). At later stages, the longer element formed a foldback in the middle (Fig. 4b). Finally, the distal ends of the elements became synapsed, forming a T-like structure (Fig. 4c).

An interesting feature of the metaphase I bivalents of the sand lizard is that they retain some traces of SYCP3 (Fig. 3), which is more pronounced at the centromeres. A strong SYCP3 signal is usually co-localized with the centromere signal (Suppl. material 1). We found five good metaphase I plates in a specimen with heteromorphic bivalent 19. In all of them the smallest bivalent had one proximal chiasma and asymmetric distal ends (Fig. 3b). This may indicate that recombination in the heteromorphic bivalent usually occurs in the proximal region.

Insertions or/and amplifications of C-positive chromatin have been suggested as common causes of an increase in chromosome size (Agulnik et al. 1993). To test this,

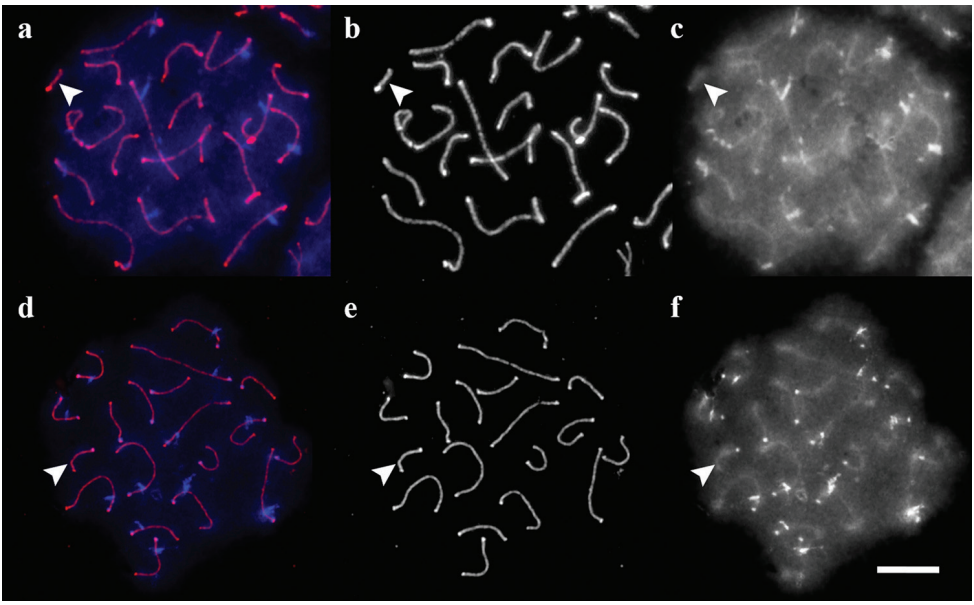

Figure 5. SC spreads of the sand lizards after C-like DAPI staining. **a–c** standard homozygote **d–f** homozygote for the long variant of chromosome 19 **a, d** merged images **b,e** SYCP3 **c, f** DAPI. Arrowheads show SC 19. Scale bar: 10 μm.

we carried out C-like DAPI staining (Lisachov 2013) in the carriers of the enlarged variant of the chromosome 19. The centromeres of all chromosomes were C-positive. We also detected pericentromeric C-bands in 12 large chromosomes. A similar pattern has been observed in the Iberian rock lizard *I. monticola* (Giovannotti et al. 2014). However, we did not find an interstitial C-band in either long or standard variants of the chromosome 19 (Fig. 5).

To test whether there is an accumulation of GC-rich sequences in the long variant of the chromosome 19, we used CMA_3 staining. This fluorochrome mostly gave a uniform fluorescence along all the chromosomes, including the enlarged chromosome 19 (Fig. 6). When present, the differential staining repeated the DAPI staining pattern.

The telomeric sequences are known to extensively accumulate at the W chromosome of *L. agilis* (Matsubara et al. 2015). To test whether this sequence is responsible for the enlargement of the chromosome 19, we carried out FISH with the FITC-labeled PNA probe after immunostaining. The telomeric signals occurred only at the terminal parts of the chromosomes, and no extensive accumulation was present at the enlarged variant of the chromosome 19 (Suppl. material 2). The nucleolus organizer (NOR) amplification or transposition might be the cause of chromosome elongation (Woznicki et al. 1998). Ag-NOR staining revealed a single NOR distally at one of the medium chromosomes (1st to 6th in the set), but nothing at chromosome 19 (Fig. 7) This NOR location is consistent with the previous findings (Vujošević and Blagojević 1999).

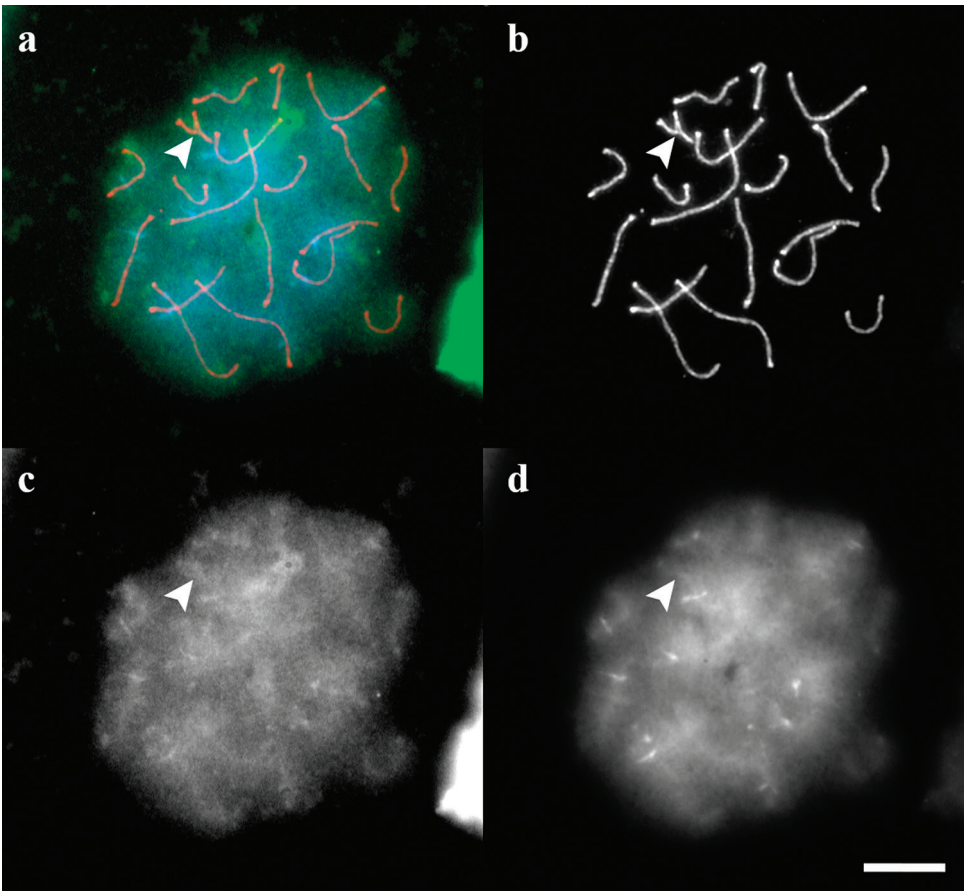

Figure 6. CMA3 and DAPI staining of the SC spread from a heterozygous individual. **a** merged image **b** SYCP3 **c** CMA3 **d** DAPI. Arrowhead shows SC 19. Scale bar: 10 μm.

Discussion

We have concluded that the carriers of the heteromorphic SC 19 (specimens ##1, 7, 9, 10) were heterozygotes for the long variant of the chromosome 19, while the specimen #3 was homozygous for this variant, and all other individuals were homozygous for the standard variant described for this species previously. Since similar karyotypes consisting of 18 macrochromosome pairs and 1 microchromosome pair are characteristic for most other lacertid species (Olmo and Signorino 2005), the short microchromosome is presumably ancestral, and the long variant described here originated from it by a chromosomal rearrangement.

Based on the synaptic configurations observed in the heterozygote, we suggest that the long variant of the microchromosome probably contains a palindromic sequence in its median region. This sequence shows foldback self-synapsis (Figs 1c, 4b-c, 7). Foldback synapsis is not necessarily connected with palindromes. For example, in the iguanian lizard *Sceloporus graciosus* Baird & Girard, 1852, which has sex chromosomes

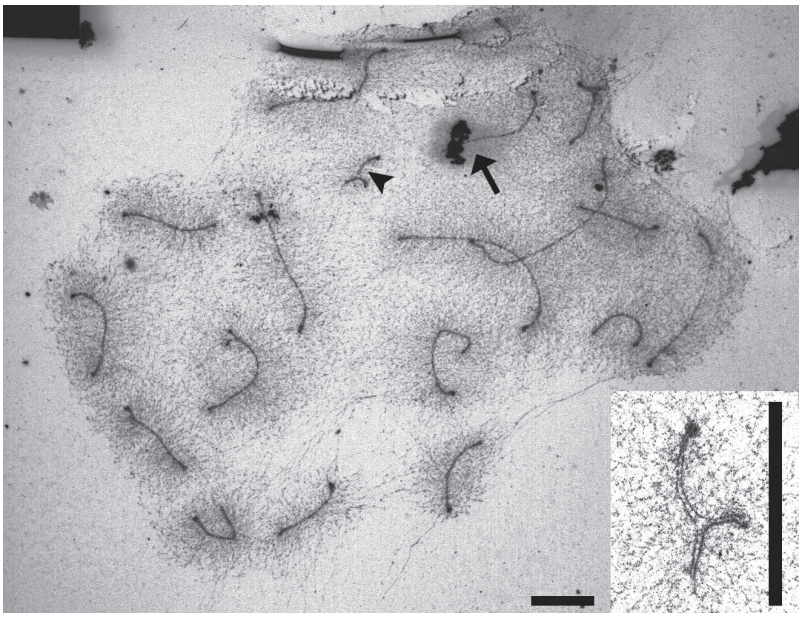

Figure 7. Ag-NOR stained SC spread of the sand lizard heterozygous for the long variant of chromosome 19. The arrowhead and insert show heteromorphic SC 19. The arrow shows NOR at a macrochromosome. Scales bars: 5 μm.

differentiated in length, the longer element also forms a lateral buckle. But in *L. agilis* the longer element starts to form a self-paired buckle when normal pairing is present only at one end, and is not yet constrained by the difference in length. In contrast, in the *S. graciosus* the buckle forms as the result of synaptic adjustment, when the axial elements are anchored at the ends and try to pair completely, compensating the difference in length (Reed et al. 1990). We examined a possibility that the additional region of the long variant is composed of repeated sequences. Our tests for NOR, $(TTAGGG)_n$, AT- and GC-rich repeats gave negative results. All major satellites which are characterized in the Lacertidae so far are AT-rich (Ciobanu et al. 2004). In *I. monticola*, the satellites are located in the centromeric and pericentromeric DAPI/C-positive bands (Giovannotti et al. 2014). These bands are similar to the bands seen in our samples (Fig. 5). Moreover, the *TaqI* satellite of *I. monticola* was found to have homologues in *Lacerta* species. Therefore, we suggest that the elongation of the chromosome 19 is not due to the accumulation of the known *L. agilis* satellites.

The polymorphic variant of the microchromosome seems not to affect the fitness of the carriers. Homozygous and heterozygous carriers of the variant were phenotypically normal compared with the specimens having the normal karyotype. The occurrence of the homozygote and the presence of the mature spermatids on the preparations from the heterozygotes (not shown) indicate that heterozygotes are fertile.

The five carriers of the long microchromosome were found in the same area of several thousand square meters of grassy river terrace slope, between a motorway and

a river. This variant is possibly local and shared by a group of related animals. If this is the case, a homozygote can be produced in the third generation after the origin of the variant chromosome.

Fusion of microchromosomes with each other and with macrochromosomes is considered as the main mechanism of the reduction of the number of the microchromosomes (Uno et al. 2012). However, our finding indicates that enlargement of a microchromosome by gaining additional DNA content can close the gap between micro- and macrochromosomes, and this can be an alternative route for microchromosome transformation. This may not be rare, since, in several reptile lineages, loss of the microchromosomes does not lead to a decrease in chromosome number (Olmo and Signorino 2005). Perhaps, this Siberian population of the sand lizard gives an insight of how the transition to all-macrochromosome karyotype might occur.

Conclusion

We found a polymorphic variant of the 19th chromosome in one population of the sand lizard, *Lacerta agilis*. It is presented in both heterozygous and homozygous states, and the carriers seem to be phenotypically normal and fertile. The polymorphic variant is two-fold larger than the normal one. Its exact content is unknown. We suggest that enlargement of an individual microchromosome by accumulating repetitive and other sequences may serve as alternative way in the process of the disappearance of the microchromosomes, along with the fusion events.

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Supplementary material 1

The colocalization of dense SYCP3 signal with the centromeres at meiotic metaphase I in the sand lizard

Authors: Artem P. Lisachov, Pavel M. Borodin

Data type: Tif file

Explanation note: The colocalization of dense SYCP3 signal with the centromeres at meiotic metaphase I in the sand lizard. Blue: DAPI, red: SYCP3, green: centromere. Scale bar: 5 μm.

Supplementary material 2

FISH with the telomeric probe on the SC spread of the sand lizard heterozygous for the long variant of chromosome 19

Authors: Artem P. Lisachov, Pavel M. Borodin

Data type: Tif file

Explanation note: FISH with the telomeric probe on the SC spread of the sand lizard heterozygous for the long variant of chromosome 19. Blue: DAPI, red: SYCP3, green: telomeric probe. Insert and arrowhead shows SC 19. Scale bar: 10 μm.

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RESEARCH ARTICLE

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Intense genomic reorganization in the genus *Oecomys* (Rodentia, Sigmodontinae): comparison between DNA barcoding and mapping of repetitive elements in three species of the Brazilian Amazon

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Abstract

Oecomys Thomas, 1906 is one of the most diverse and widely distributed genera within the tribe Oryzomyini. At least sixteen species in this genus have been described to date, but it is believed this genus contains undescribed species. Morphological, molecular and cytogenetic study has revealed an uncertain taxonomic status for several *Oecomys* species, suggesting the presence of a complex of species. The present work had the goal of contributing to the genetic characterization of the genus *Oecomys* in the Brazilian Amazon. Thirty specimens were collected from four locations in the Brazilian Amazon and three nominal species recognized: *Oecomys auyantepui* (Tate, 1939), *O. bicolor* (Tomes, 1860) and *O. rutilus* (Anthony, 1921). COI sequence analysis grouped *O. auyantepui*, *O. bicolor* and *O. rutilus* specimens into one, three and two clades, respectively, which is consistent with their geographic distribution. Cytogenetic data for *O. auyantepui* revealed the sympatric occurrence of two different diploid numbers, 2n=64/NFa=110 and

2n=66/NFa=114, suggesting polymorphism while *O. bicolor* exhibited 2n=80/NFa=142 and *O. rutilus* 2n=54/NFa=90. The distribution of constitutive heterochromatin followed a species-specific pattern. Interspecific variation was evident in the chromosomal location and number of 18S rDNA loci. However, not all loci showed signs of activity. All three species displayed a similar pattern for 5S rDNA, with only one pair carrying this locus. Interstitial telomeric sites were found only in *O. auyantepui*. The data presented in this work reinforce intra- and interspecific variations observed in the diploid number of *Oecomys* species and indicate that chromosomal rearrangements have led to the appearance of different diploid numbers and karyotypic formulas.

Keywords
Oryzomyini, FISH, telomere, rDNA, heterochromatin, COI

Introduction

The order Rodentia is divided into nine taxonomic families in Brazil. The family Cricetidae contains the most members, among which the subfamily Sigmodontinae includes 86 genera and 395 species (*sensu* Reig 1980) according to Prado and Percequillo (2013). Oryzomyini is the most diverse tribe of the Sigmondontinae, and the genus *Oecomys* Thomas, 1906 is one of the most diverse of the tribe Oryzomyini (Prado and Percequillo 2013). However, its morphological and karyological distinction and generic status were only recognized relatively recently (Andrades-Miranda et al. 2001, Carleton and Musser 1984, Gardner and Patton 1976, Reig 1984, 1986 as cited in Musser and Carleton 2005). Similarity among species and the limited understanding of morphological variations in *Oecomys* (including interspecific, intraspecific, geographic, and specimen age-inherent variations) have rendered species identification difficult.

Currently, 16 species are recognized within this genus (Musser and Carleton 2005, Carleton et al. 2009), but only nine species have been studied for karyotypes, showing 11 different diploid numbers, varying between 54 and 86 chromosomes (Table 1). In Brazil 12 species were registered and 9 of which can be found in Amazon biome; *O. auyantepui* Tate, 1939, *O. bicolor* (Tomes, 1860), *O. concolor* (Wagner, 1845), *O. paricola* (Thomas, 1904), *O. rex* Thomas, 1910, *O. roberti* (Thomas, 1904), *O. rutilus* Anthony, 1921, *O. superans* Thomas, 1911 and *O. trinitatis* (J. A. Allen & Chapman, 1893) (Bonvicino et al. 2008; Flores 2010). Variations in fundamental number have also been reported in species with the same diploid number, which is an indicator of chromosomal rearrangements within the group (Rosa et al. 2012). However, morphological and morphometric analysis in conjunction with molecular and cytogenetic approaches revealed uncertainty in the delimitation and distribution of *Oecomys* species, suggesting the presence of a complex of species (Patton and Sherwood 1983, Emmons and Feer 1997, Patton et al. 2000, Musser and Carleton 2005, Carleton et al. 2009, Flores 2010, Rosa et al. 2012).

Hence, in the present study, we used classic and molecular cytogenetics approaches in order to enable the genetic characterization of three species of the genus *Oecomys* from the Brazilian Amazon. Further, we used DNA barcoding to evaluate the intra- and interspecific distances, and infer the utility in species identification by combining this dataset with sequences deposited in GenBank.

Table 1. Karyotypes recorded for species of the genus *Oecomys*. Diploid Number (2n), fundamental number (FN) and location are listed.

Species	Location	2n	FN	Reference
O. auyantepui	Jari river – PA	72	80	Lira (2012)
O. auyantepui	Jatapu river – AM	64	110	Present paper
		66	114	Present paper
*O. bahiensis***	São Lourenço da Mata – PE	60	62	Langguth et al. (2005)
O. bicolor	Jari river – PA	54	82	Lira (2012)
O. bicolor	SUR	80	–	Baker et al. (1983)
O. bicolor	RR	80	124	Andrades-Miranda et al. (2000)
	Ipameri and Serra da mesa– GO			Andrades-Miranda et al. (2001)
O. bicolor	Curanja river – PER	80	134	Gardner and Patton (1976)
O. bicolor	Curanja river – PER	80	136	Gardner and Patton (1976)
O. bicolor	Juruá river – AM	80	140	Patton et al. (2000)
O. bicolor	Purus and Jatapu river – AM	80	142	Present paper
O. bicolor	?	82	110	Andrades-Miranda et al. (2000)
	Hydropower plant UEH Samuel – GO			Andrades-Miranda et al. (2001)
O. bicolor	Jari river – PA	82	116	Lira (2012)
O. bicolor	Jurua river – AM	86	98	Patton et al. (2000)
O. catherinae	GO, São Lourenço da Mata – PE Ubatuba – SP, Cruz do Espírito Santo – PB, Igarassú, Jaqueira and Paudalho – PE RJ	60	62	Andrades-Miranda et al. (2001) Andrade and Bonvicino (2003) Langguth et al. (2005) Pinheiro and Geise (2008) Asfora et al. (2011)
O. catherinae	Ubatuba – SP	60	64	Pinheiro e Geise (2008)
	RJ			Asfora et al. (2011)
O. catherinae	RJ, SP	86	98	Patton et al. (2000)
O. concolor	PAN	58	–	Baker et al. (1983)
O. concolor	SUR	60	–	Baker et al. (1983)
O. concolor	Villavicencio – COL	60	62	Gardner and Patton (1976)
O. concolor	MEX	60	–	Andrade and Bonvicino (2003)
O. concolor	MEX	61	–	Andrade and Bonvicino (2003)
O. concolor	Curanja River – PER	80	112	Gardner and Patton (1976)
O. concolor	DF, RJ, GO, SP, RO	60	62	Gardner and Patton (1976) Svartman (1989) Andrades-Miranda et al. (2000) Andrades-Miranda et al. (2001) Andrade and Bonvicino (2003)
O. paricola	Environment Park – PA	68	72	Rosa et al. (2012)
O. paricola	Marajó island – PA	70	72	Rosa et al. (2012)
O. paricola	Environment Park – PA	70	76	Rosa et al. (2012)
O. rex	Jari river – PA	62	80	Lira (2012)
O. roberti	AM	80	114	Patton et al. (2000)
O. roberti	Juruá river – AM	82	106	Langguth et al. (2005)
	Jamari river – RO			
O. rutilus	Negro river – AM	54	90	Present paper
O. superans	PER	80	108	Gardner and Patton (1976)
	Jurua river – AM			Andrade and Bonvicino (2003) Patton et al. (2000)
O. trinitatis	Jurua river – AM	58	96	Patton et al. (2000)
Oecomys sp.	Cuieiras river – AM	54	84	Lira (2012)
Oecomys sp.	Jatapu – AM	54	86	Lira (2012)
Oecomys sp.	MS	72	90	Andrade and Bonvicino (2003)

*The location indicates the sampled countries or Brazilian states. AM = Amazonas, GO = Goiás, MS = Mato Grosso do Sul, PA = Pará, PB = Paraíba, PE = Pernambuco, RJ = Rio de Janeiro, RO = Rondônia, RR = Roraima, SP = São Paulo, COL= Colombia, MEX = Mexico, PAN = Panama, PER = Peru, SUR = Suriname.
**Synonym of *O. catherinae*.

Materials and methods

Samples

Thirty specimens were collected from five locations in the Brazilian Amazon (Fig. 1, Table 2) and euthanized according to the recommendations of Resolution CFBIO N. 301 from December 8th, 2012. Voucher specimens were prepared or fixed, and stored in 70% ethanol; the specimens are currently stored in the mammal collection of the National Institute of Amazonian Research [Instituto Nacional de Pesquisas da Amazônia – INPA] (Table 2). The methods for the collection, maintenance and processing of the material complied with the guidelines of the Brazilian College of Animal Experimentation [Colégio Brasileiro de Experimentação Animal – COBEA] and were approved by the Ethics Committee On Animal Use of the Federal University of Amazonas [Comissão de Ética no Uso de Animais da Universidade Federal do Amazonas] (043/2013-CEUA/UFAM). Individuals were collected with the permission of the Chico Mendes Institute for Biodiversity Conservation [(Instituto Chico Mendes de Conservação da Biodiversidade – ICMBIO), License No. 10832-1 /35513-1). It must be noted that the collections took place outside of conservation units and that these species are not threatened with extinction. Samples were collected from the hematopoietic organ of each individual following euthanasia to obtain chromosome preparations and muscle tissue for DNA extraction.

Chromosome analysis

Mitotic chromosomal preparations were obtained using the protocol described by Ford and Harmerton (1956), with some modifications. Nucleolus organizing regions (NORs), heterochromatin and G-banding were identified through silver nitrate staining (Howell and Black 1980), the C-banding technique (Sumner 1972) and trypsin solution (Seabright 1971), respectively. 5S and 18S rDNA probes were obtained after PCR amplification using the following primers: 5Sf (5'-CAG GGT CGG GCC TGG TTA GTA-3') and 5Sr (5'-CTT CYG AGA TCA GAC GAG ATC-3'); 18Sf (5'-CCG CTT TGG TGA CTC TTG AT-3') and 18Sr (5'-CCG AGG ACC TCA CTA AAC CA-3') (Gross et al. 2010). For telomere sequences, DNA-free amplifications were performed using the primers $(TTAGGG)_5$ and $(CCCTAA)_5$ (Ijdo et al. 1991). Amplification reactions were conducted in a total volume of 25 µl (~100 ng of genomic DNA), containing 10x reaction buffer (final concentration: 10 mM Tris-HCl; 1.5 mM $MgCl_2$; 50 mM KCl; pH 8.3), 0.3 units of Taq DNA polymerase, 0.2 mM each dNTP, 0.2 µl of each primer and Milli-Q water to the final volume; the annealing temperature was 56 ºC for 18S rDNA and 59 °C for 5S rDNA, and the final volume was 25 µl. The 5S gene PCR product was labeled with Biotin (Biotin Nick translations mix, Roch) and the 18S gene and telomere sequences with digoxigenin (Dig-Nick Translation mix, Roche), following the manufacturer's instructions. Alexa Fluor 488-conjugated

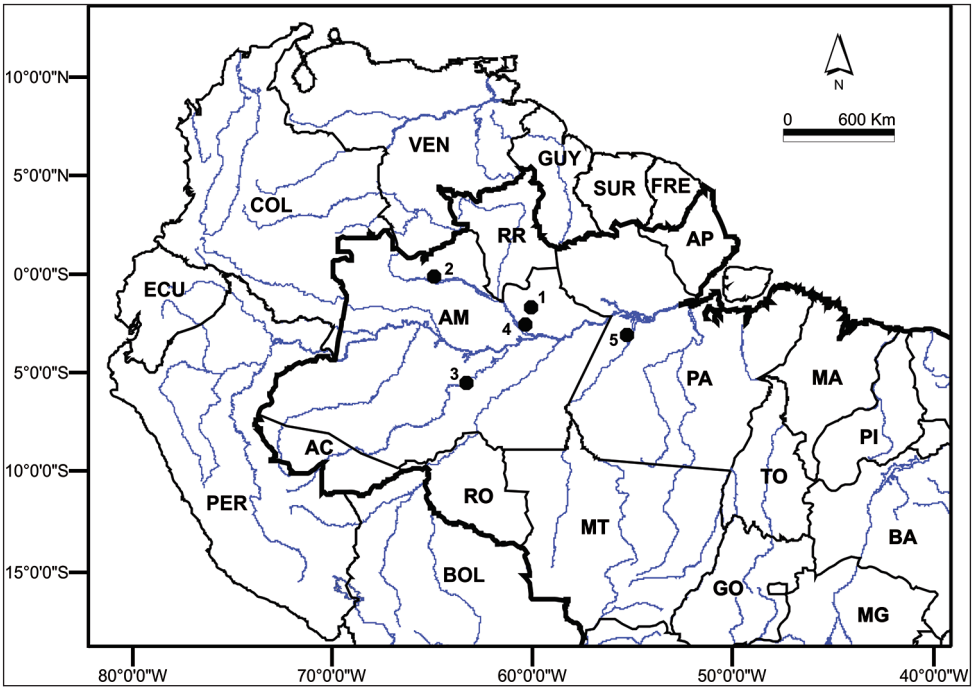

Figure 1. Map of the Brazilian Amazon, indicating the collection sites. The left and right banks of the following Amazonas state rivers were sampled: **1** Jatapú (near the city of São Sebastião do Uatumã - 0°50' to 1°55'S; 58°50' to 60°10'W) **2** Negro (near the city of Santa Isabel do Rio Negro - 0°24.4'N; 65°1.017'W) **3** Purus (near the city of Tapauá - 05°42.183'S, 63°13.967'W) **4** Cuieiras (02°47'S, 60°27'W) **5** Tapajós (03°21.283'S, 55°11.733'W). BOL = Bolivia, PER = Peru, ECU = Ecuador, COL = Colombia, VEN = Venezuela, GUY = Guyana, SUR = Suriname, FRE = French Guyana, RR = Roraima, AP = Amapa, AM = Amazonas, PA = Para, RO = Rondonia, AC = Acre, MA = Maranhão, PI = Piaui, TO = Tocantins, BA = Bahia, MT = Mato Grosso, GO = Goias, MG = Minas Gerais.

streptavidin (Life technologies) and anti-digoxigenin rhodamine (Roche) antibodies were used to detect the probe signal. Fluorescent *in situ* hybridization was carried out based on the protocols described by Pinkel et al. (1986).

Slides were screened for metaphases, at least 30 for each technique were analyzed and the best metaphases were photo-documented using an Olympus BX-51 epifluorescence microscope. Chromosomes were organized by decreasing size, and their morphology was determined based on the centromere position, being classified as metacentric (m), submetacentric (sm), subtelocentric (st) or acrocentric (a) (Levan et al. 1964).

Mitochondrial DNA analysis

DNA was extracted according to the protocol described by Sambrook and Russel (2001). The cytochrome oxidase subunit I (COI) gene sequence was obtained through

Table 2. Species of *Oecomys* collected in present work: The voucher, collection sites, sex, diploid number (2n), fundamental number (FN), karyotype formula, Nucleolus organizer region (NOR), rDNA 18S (18S), rDNA 5S (5s) are listed; M = male; F = female; m = metacentric; sm = submetacentric; st = subtelocentric; a = acrocentric; X = Sexual chromosome X; Y = Sexual chromosome Y. Bold voucher were karyotyped in the present work.

Species	Voucher	Sex	Collection sites	2n	FN	Karyotype formula	NOR	18S	5S
O. auyantepui	**INPA 6754**	M	Brazil, AM – Jatapú River	64	110	12m+10sm+26st +16a+XY	10p and 14p	10p and 14p	5p
	INPA 6751	M		66	112	16m+6sm+26st+ 14a+XY			
	INPA 6753	M		–	–	–	–	–	–
	INPA 6747	M		–	–	–	–	–	–
O. bicolor	**INPA 6772**	M	Brazil, AM – Purus River	80	142	18m+10sm+36st+ 14a+XY	15p, 18p, 21p, 22p and 26p	2p, 3p, 13p, 15p, 16p, 18p, 19p, 21p, 22p, 25p 26p and 30p	7p
	INPA 6749	M	Brazil, AM – Jatapú River						
	INPA 6756	M							
	INPA 6758	M							
	INPA 6757	F							
	INPA 6752	M	Brazil, AM – Jatapú River	–	–	–	–	–	–
	INPA 6773	F	Brazil, AM – Purus River	–	–	–	–	–	–
	INPA 6770	M	Brazil, AM – Negro River	–	–	–	–	–	–
	INPA 6775	M	Brazil, PA – Tapajós River	–	–	–	–	–	–
O. rutilus	**INPA 6760**	F	Brazil, AM – Negro River	54	90	24m+6sm+8st+ 14a+XX	4p and 23p	4p and 23p	1p
	INPA 6761	F							
	INPA 6762	F							
	INPA 6768	M							
	INPA 6767	F		–	–	–	–	–	–
	INPA 6769	M		–	–	–	–	–	–
	INPA 6766	F		–	–	–	–	–	–
	INPA 6763	F		–	–	–	–	–	–
	INPA 6764	F		–	–	–	–	–	–
	INPA 6765	F		–	–	–	–	–	–
	INPA 6774	F		–	–	–	–	–	–
	INPA 6759	F		–	–	–	–	–	–
	INPA 6745	F	Brazil, AM – Cuieiras River	–	–	–	–	–	–
	INPA 6746	M		–	–	–	–	–	–
	INPA 6744	F		–	–	–	–	–	–
	INPA 6750	M	Brazil, AM – Jatapú River	–	–	–	–	–	–
	INPA 6755	M		–	–	–	–	–	–
	INPA 6748	F		–	–	–	–	–	–

polymerase chain reaction (PCR) using the universal primers described by Ivanova et al. (2007). The PCR products were purified with the ExoSap® kit (GE Healthcare) and sequenced using the method described by Sanger et al. (1977) on an ABI 3130XL automatic sequencer. The resulting sequences were submitted to the NCBI database under the following accession numbers: KT258600–KT258632.

Sequences were manually aligned using BioEdit v7.2.2 software (Hall 2001) and compared with sequences deposited in GenBank using BLASTn (Basic Local Alignment Search Tool). A Bayesian phylogenetic analysis was conducted with MrBayes 3.2 (Ronquist and Huelsenbeck 2003). For this analysis, Markov Chain Monte-Carlo sampling was conducted every 20,000th generation until the standard deviation of split frequencies was <0.01. A burn-in period equal to 25% of the total generations was required to summarize the parameter values and trees. Parameter values were assessed based on 95% credibility levels to ensure that the analysis had run for a sufficient number of generations. A genetic distance matrix was constructed using the MEGA 6 program (Tamura et al. 2013) and was obtained according to the Kimura 2 parameter (K2p) model. For Bayesian analysis, 53 *Oecomys* COI sequences available in GenBank were included (Appendix 1). One specimen of *Euryoryzomys macconnelli* was used as an outgroup.

Results

Chromosome analysis

Oecomys auyantepui – Jatapú River

Two different diploid numbers were observed along the same bank of the Jatapú River: Karyomorph "a" exhibited 2n=64 chromosomes, a fundamental number = 110, and a karyotypic formula of 16m+6sm+26st+14a+XY (Fig. 2), in which pairs 1, 4, 15, 22, 26, 28, 30 and 31 were metacentric; 2, 3 and 19 were submetacentric; 5-13, 23, 24, 25 and 27 were subtelocentric; and 14, 16, 17, 18, 20, 21 and 29 were acrocentric (Fig. 2a). Karyomorph "b" exhibited 2n=66 chromosomes, a fundamental number = 112 and a karyotypic formula of 12m+10sm+26st+16a+XY (Fig. 3), in which pairs 1, 4, 17, 21, 24 and 29 were metacentric; 2, 3, 15, 16 and 27 were submetacentric; 5, 6, 7, 8, 9, 12, 18, 19, 20, 23, 25, 26 and 28 were subtelocentric; and 10, 11, 13, 14, 22, 30, 31 and 32 were acrocentric (Fig. 3a). Chromosomes X and Y were submetacentric for 2n=64 (Fig. 2a), whereas for 2n=66, chromosome X was metacentric, and chromosome Y was submetacentric and half the size of chromosome X (Fig. 3a). The heterochromatin was predominantly centromeric for both 2n=64 and 2n=66 chromosomes, ranging between subtle and conspicuous (Figs 2b, 3b). The Y chromosome exhibited a heterochromatic long arm in both karyotypes, while the X chromosomes presented a centromeric block and bitelomeric labeling. G-banding patterns enabled the identification of homologous pairs for each karyomorph (Figures 2c, 3c) and homology detection among the largest pairs of the complement. Pairs

1, 2, 3, 4, 5, 6, 12 and 13 from the 2n=66 chromosome karyomorph were homologous to pairs 1, 3, 2, 4, 5, 6, 12 and 13 from the 2n=64 chromosome karyomorph, respectively. Silver nitrate staining of the 2n=64 karyomorph resulted in labeling of three terminal sites, on one of the chromosomes of pair 10 and on both of the pair 14 homologs (Fig. 2d). The 2n=66 karyomorph also exhibited labeling of three terminal sites, two on pair 10 and one on one of the pair 14 chromosomes (Fig. 3d).

18S rDNA loci were visualized on chromosome pairs 10 and 14 of both karyomorphs, while the single 5S rDNA loci was located on pair 5 of karyomorph "a" and pair 7 of karyomorph "b" (Figs 2e, 3e). Both karyomorphs presented interstitial telomeric sequences (ITSs) in the centromeric region of the X chromosome (Figs 2f and 3f).

Oecomys bicolor – Jatapú, Negro and Purus rivers

Oecomys bicolor was found to exhibit a diploid number 2n=80 chromosomes, a fundamental number = 142, and a karyotypic formula of 18m+10sm+36st+14a+XX or XY, wherein pairs 12, 32, 33, 34, 35, 36, 37, 38 and 39 were metacentric; pairs 7, 20, 25, 26 and 27 were submetacentric; 1, 2, 5, 6, 10, 11, 13, 14, 15, 16, 17, 19, 21, 22, 23, 24, 29 and 30 were subtelocentric; and 3, 4, 8, 9, 18, 28 and 31 were acrocentric (Figs 4a, 4b), with no differences being observed among individuals from the three collection sites. Sex chromosome X is the largest submetacentric chromosome of the complement, while sex chromosome Y is an average subtelocentric chromosome (Fig. 4b). Heterochromatin can be found in conspicuous blocks in the centromere region of all chromosomes, and in the case of the majority of metacentric, submetacentric (Fig. 4c), and the sex X chromosome, it also extends into the short arm (Fig. 4d). G-banding patterns enabled the correct identification of homologous pairs (Fig. 4e). Silver nitrate staining showed multiple terminal type-NORs on both homologous chromosomes of pairs 18 and 26 and on one of the homologous chromosome of pairs 15, 21 and 22 (Fig. 4f). 18S rDNA loci were identified on both homologous chromosomes of pairs 2, 3, 13, 15, 16, 18, 19, 21, 22, 25, 26 and 30, whereas 5S rDNA locus was located only on pair 7 (Fig. 4g). No ITSs could be observed (Fig. 4h).

Oecomys rutilus – Cuieiras, Jatapú and Negro rivers

Oecomys rutilus was characterized as showing a diploid number 2n=54 chromosomes and a fundamental number = 90, with a karyotypic formula 24m+6sm+8st+14a+XX or XY, in which pairs 3, 5, 11, 12, 13, 14, 15, 19, 21, 22, 25 and 26 were metacentric; 7, 8 and 20 were submetacentric; 1, 2, 4 and 6 were subtelocentric; and 9, 10, 16, 17, 18, 23 and 24 were acrocentric (Figs 5a, 5b), with no differences being detected between the specimens collected at three different sites. The X chromosome was large and submetacentric, while the Y chromosome was subtelocentric and approximately 3/4 of the size of chromosome X (Fig. 5b). Heterochromatic regions were characterized

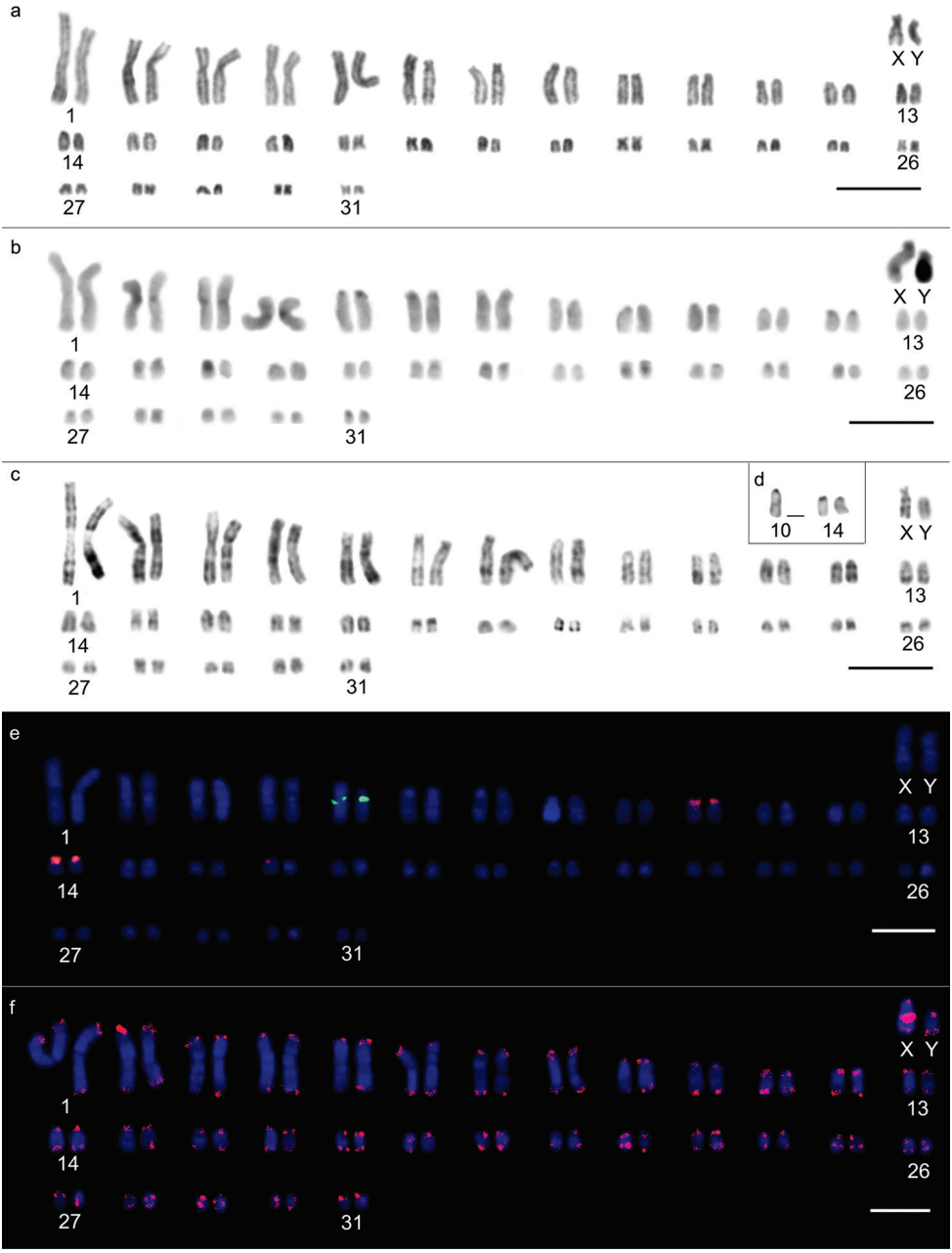

Figure 2. Karyotypic characteristics of male *Oecomys auyantepui*, karyomorph "a" (INPA 6754) with 2n=64: **a** conventional Giemsa staining **b** heterochromatic regions highlighted by C-banding **c** G-banding **d** nucleolus organizing region-carrying pairs evidenced by silver nitrate staining **e** fluorescent *in situ* hybridization of 5S rDNA (green) and 18S rDNA (red) probes **f** karyotype indicating the presence of telomeric sites as well as interstitial telomeric sequence in the sex X chromosome. Bars: 10 μm.

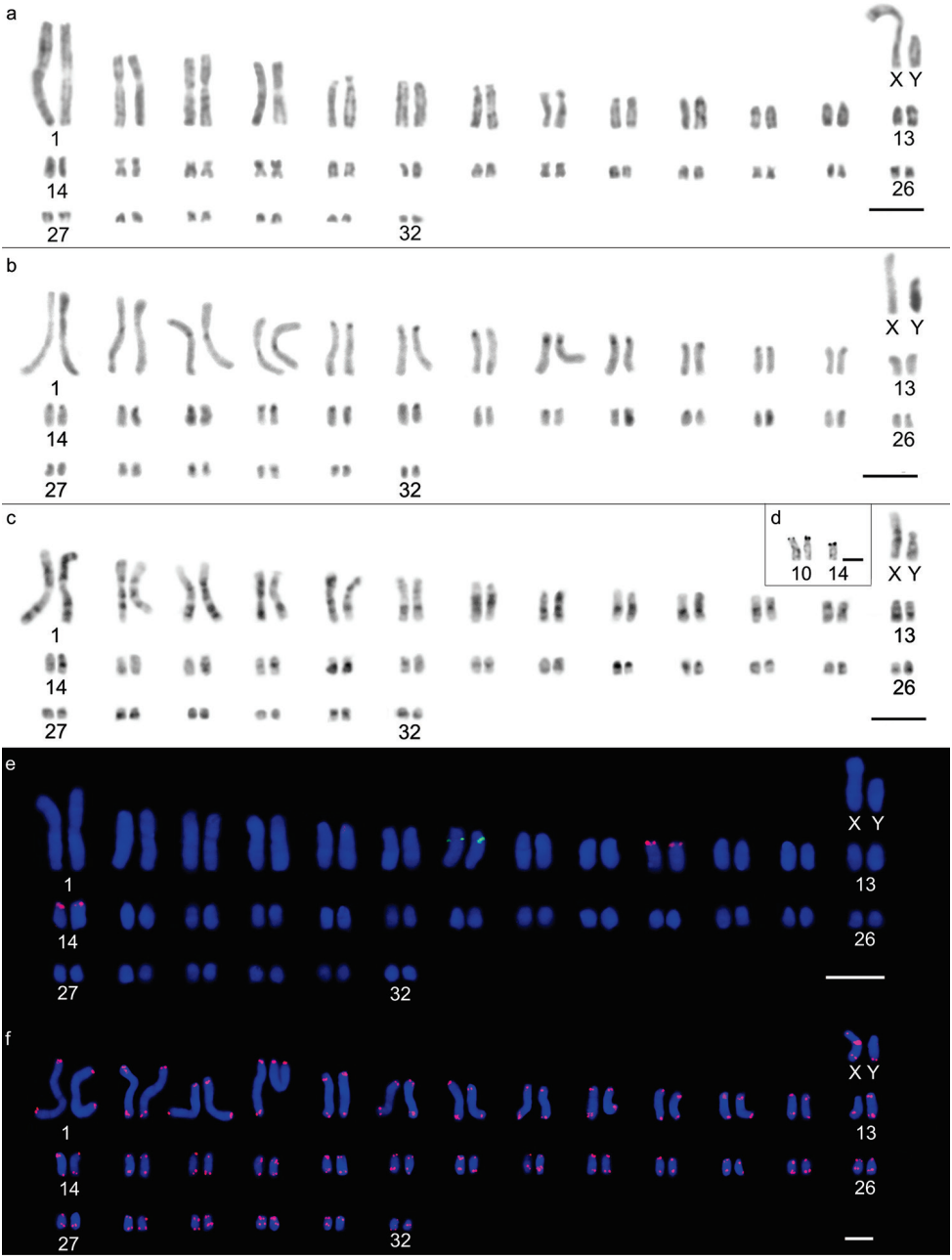

Figure 3. Karyotypic characteristics of male *Oecomys auyantepui* karyomorph "b", with 2n=66: **a** conventional Giemsa staining (INPA 6751) **b** heterochromatic regions highlighted by C-banding (INPA 6751) **c** G-banding (INPA 6751) **d** nucleolus organizing region-carrying pairs revealed by silver nitrate staining (INPA 6751) **e** fluorescent *in situ* hybridization of 5S rDNA (green) and 18S rDNA (red) probes (INPA 6751) **f** karyotype indicating the presence of telomeric sites as well as an interstitial telomeric sequence on the X sex chromosome (INPA 6754). Bars: 10 μm.

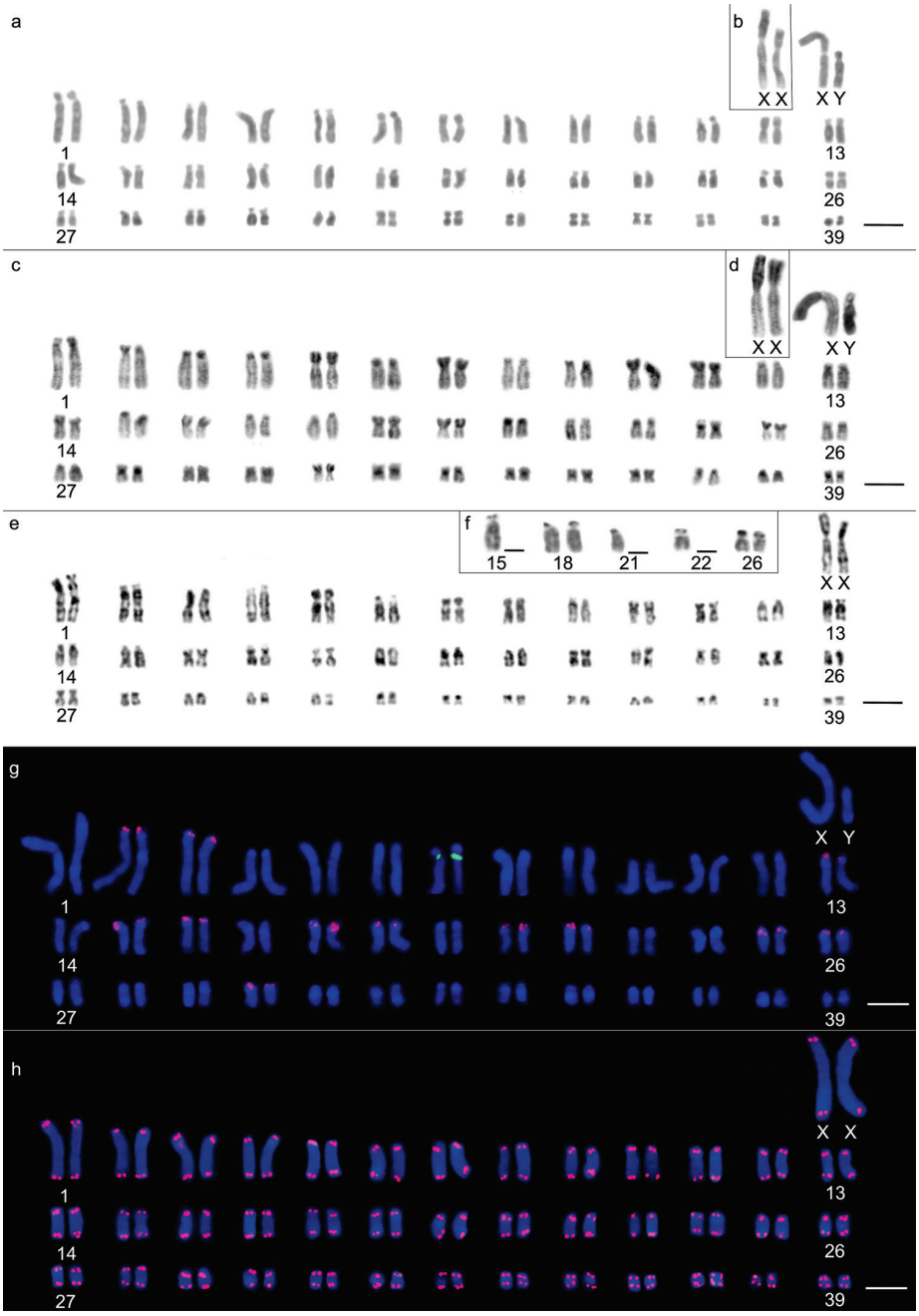

Figure 4. Karyotypic characteristics of *Oecomys bicolor*: **a** conventional Giemsa staining of a male (INPA 6749) **b** highlighted sex chromosomes of a female (INPA 6749) **c** heterochromatic regions revealed by C-banding in a female (INPA 6772) **d** highlighted C-banding on a male's sex chromosomes (INPA 6772) **e** G-banding of a female (INPA 6772) **f** nucleolus organizing region-carrying pairs revealed by silver nitrate staining (INPA 6749) **g** fluorescent *in situ* hybridization of 5S rDNA (green) and 18S rDNA (red) probes (INPA 6758) **h** karyotype indicating the presence of telomeric sites (INPA 6772). Bars: 10 μm.

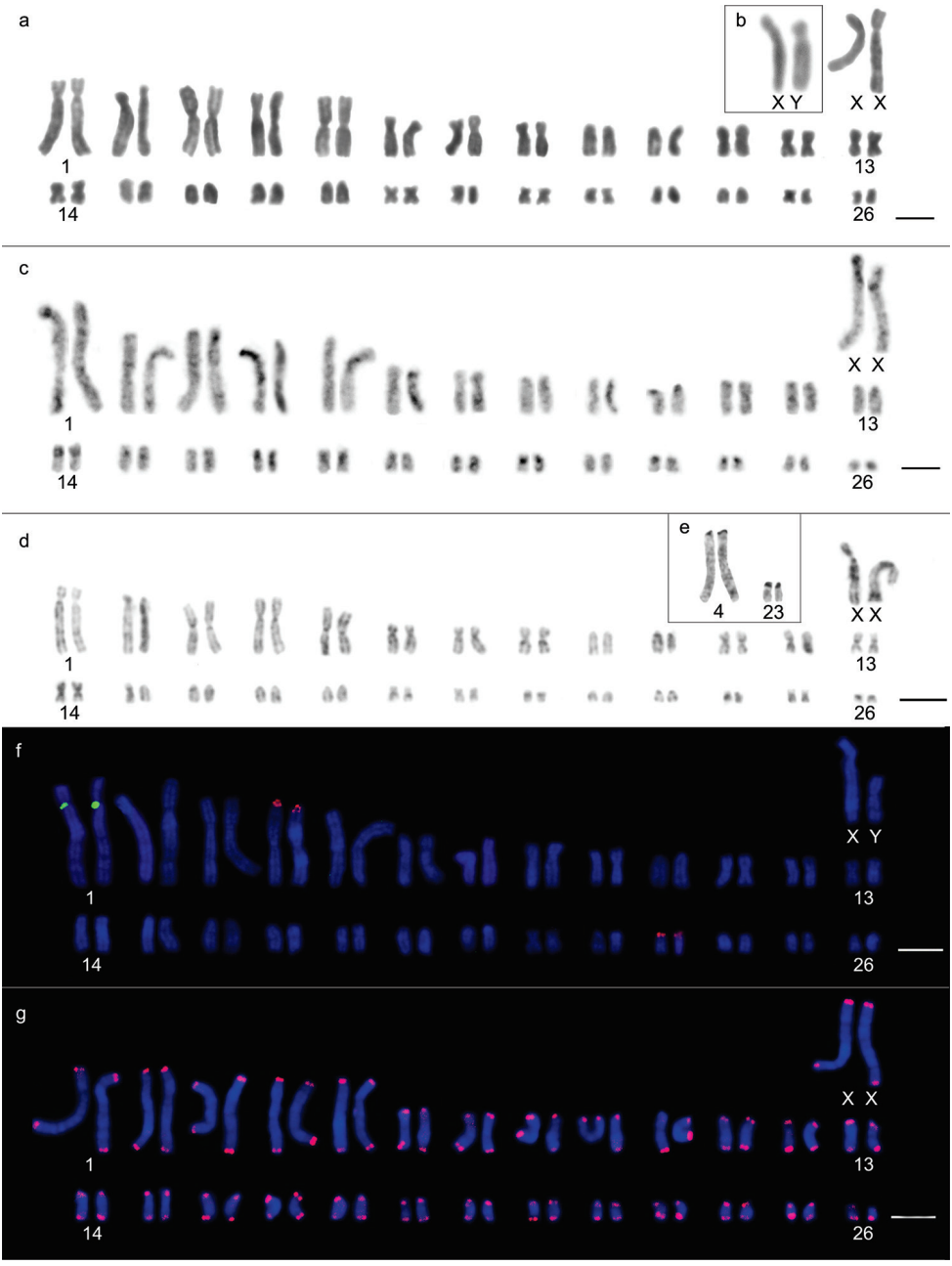

Figure 5. Karyotypic characteristics of *Oecomys bicolor*: **a** conventional Giemsa staining of a male (INPA 6761) **b** highlighted sex chromosomes of a male (INPA 6768) **c** heterochromatic regions revealed by C-banding of a male individual (INPA 6754) **d** G-banding of a female (INPA 6761) **e** nucleolus organizing region-carrying pairs revealed by silver nitrate staining (INPA 6762) **f** fluorescent *in situ* hybridization of 5S rDNA (green) and 18S rDNA (red) probes (INPA 6761) **g** karyotype indicating the presence of telomeric sites (INPA 6761). Bars: 10 μm.

by subtle or conspicuous centromeric labeling on some chromosome pairs (Fig. 5c). G-banding patterns enabled correct homologous pairing (Fig. 5d). Multiple NORs were revealed by silver nitrate staining in the terminal regions of both homologous chromosomes of pairs 4 and 23 (Fig. 5e), coinciding with 18S rDNA loci (Fig. 5f), which were also observed on both homologous chromosomes of pair 1, in a proximal position on the long arms (Fig. 5f). No ITSs were observed (Fig. 5g).

Mitochondrial DNA identification

A total of 86 *Oecomys* mitochondrial COI gene sequences were compared: 33 originating from the present work and 53 deposited in GenBank (Appendix 1). NJ, Bayesian and ML tree retrieved the same topology and showed differences mainly in relation to branch support values. The similarity index was greater than 98%, which allowed molecular identification of the species. The phylogenetic trees (Figure 6) grouped *O. rutilus* into two clades, one comprising individuals from Brazil, Suriname and Guyana (I), while the other consisted of one individual from Ecuador (J). The genetic distance between clades I and J was 7.33%, whereas the genetic distance within clade I was 1.62%. The individuals of *O. auyantepui* were grouped into a single clade (H), comprising individuals from Brazil, Guyana and Suriname, with an intraspecific genetic distance of 1.41%. One individual from Ecuador, whose species was not defined in GenBank, belonged to a distinct lineage (clade E). Two other specimens without species level-definition were grouped with *Oecomys concolor* (branch F), with a genetic distance of 0.79%. One other individual (clade G), also identified as *O. concolor* in GenBank, exhibited a distinct lineage, showing a large genetic distance (12.88%) from branch F. All *O. roberti* specimens were grouped together (clade D), with a genetic distance of 0.39%. *Oecomys bicolor* formed three clades (A, B and C) with large genetic distances: individuals from the Guyanas and Suriname were grouped together with high support, forming a moderately supported clade (C) with an individual from the Central Amazon (INPA 6775). *Oecomys bicolor* and *Oecomys* sp. from Ecuador and the Negro river (INPA 6770) formed a group with moderate-to-high support (clade B). One individual from the Purus River (clade C) showed a highly supported association with clade B, with a genetic distance of 7.12%. The genetic distance between clades A and B was 8.4%, and that between A and C was 9.89%. *Oecomys rex* also formed two clades (K and L), with a large genetic distance between them (11.92%).

Discussion

The identification of Rodentia species is often difficult using morphological criteria alone (Granjon et al. 2002, Lecompte et al. 2005, Ben Faleh et al. 2010). Such difficulties are evident in this order mainly because of the existence of cryptic species (Granjon et al.

2002, Musser and Carleton 2005, Lecompte et al. 2005) and new species are continually described (Helgen 2005, Musser et al. 2005). Species identification via molecular methods, such as molecular barcoding using a short genetic marker (Hebert et al. 2003), has been proposed to overcome some of the weaknesses of the traditional approach, which will aid non-taxonomists by fulfilling the urgent requirement for rapid and accurate species identification tools (Teletchea 2010). This approach is potentially useful in the study of rodents (Borisenko et al. 2008, Tamrin and Abdullah 2011, Barbosa 2013). In the present work, employing COI sequences as a tool for species identification was shown to be satisfactory, as the obtained distance patterns provided sufficient information for the identification of specimens whose taxonomic identification at the species level is not straightforward. Most of the species were recovered as monophyletic groups.

The available chromosomal data for *Oecomys* species consist mostly of descriptions of diploid and fundamental numbers, which restricts comparisons with the data obtained in the present work (Table 1). However, high karyotypic diversity can be observed, with countless chromosomal rearrangements between *Oecomys* species being responsible for this diversity. Neither of the two *O. auyantepui* karyomorphs reported in this work had been previously described in the literature. Both individuals (INPA 6751, INPA 6754) showing these two karyomorphs were captured on the same bank (right) of the Jatapú river, approximately 1 km from each other. The karyomorphs only exhibited one ITS, located on X chromosome. ITSs have been observed in other rodents as well (Castiglia et al. 2007, Rovatsos et al. 2011, Suárez-Villota et al. 2013). Short telomeric sequences $(TTAGGG)_n$ have been primarily classified as components of satellite DNA (Adegoke et al. 1993). These sequences may be located in subtelomeric and interstitial chromosome positions (Garrido-Ramos et al. 1998) and are subjected to amplification (Arnason et al. 1998, Castiglia et al. 2006). They may also appear during the double-stranded DNA nick repair process (Nergadze et al. 2004, 2007). However, the most commonly accepted scenario is that ITSs signal recent chromosomal rearrangements, such as the transposition of functional telomeric sequences to an interstitial position (Dobigny et al. 2003, Zhdanova et al. 2005), or chromosome fusion events, with the latter being the main source of ITSs in many organisms (Lee et al. 1993, Slijepcevic 1998). Nevertheless, it was not possible to determine the occurrence of either an increase in the diploid number from 2n=64 as a result of a fission event or a decrease from 2n=66 due to a fusion event.

Establishing the evolutionary direction of chromosomal rearrangements is not always possible because most of the available painting data for the Sigmodontinae group are incomplete, and it is not possible to draw definitive conclusions regarding the composition of a putative Sigmodontinae ancestral karyotype (Romanenko et al. 2012). The same is true for *Oecomys*, where it cannot be determined whether the diploid number has increased or decreased because the *in situ* hybridization method used in this study likely does not detect very short (< 1 kb) stretches of (TTAGGG)n sequences. Thus, even if chromosome fusions that would result in a decrease in diploid number have occurred, the fused chromosomes will not always possess an ITS, which

may have been lost prior to the fusion or been subjected to molecular erosion (Mandrioli et al. 1999).

Both *Oecomys auyantepui* karyomorphs exhibit similar, predominantly centromeric, subtle heterochromatic blocks. Their NORs are also similar, with three different labeled sites being observed on the same chromosome pairs. The largest chromosomes of both karyomorphs are homologous - those carrying 5S rDNA loci in particular - sharing the same chromosomal region (subtelocentric chromosomes), position (long arm, proximal) and number of labeled sites, as inferred based on the increased resolution provided by G-banding. Thus, much like the NOR-carrying pairs, these chromosomes were not involved in chromosomal alteration processes leading to the occurrence of two different diploid numbers in *O. auyantepui*. Mitochondrial DNA analysis grouped all *O. auyantepui* specimens onto a single branch (Fig. 6) with a high support value and low intraspecific genetic distance (1.41%), indicating that the occurrence of these two karyomorphs may be due to chromosomal polymorphism and not to the existence of two differentiated evolutionary units, as the intraspecific genetic distance is consistent with available data for other Sigmodontinae and the family Cricetidae in general (Smith and Patton 1993, Patton 1999,Ventura 2009).

Current phylogenetic and karyotypic data suggest the existence of a complex of *O. bicolor* species (Smith and Patton 1999, Flores 2010, Andrade and Bonvincino 2003). Four different diploid numbers have previously been characterized in the Brazilian Amazon, varying from 54 to 86 chromosomes, with 2n=80 being the most common (Gardner and Patton 1976, Patton et al. 2000, Andrades-Miranda et al. 2000, Andrades-Miranda et al. 2001, Lira 2012). Comparison of the karyotypic patterns of *O. bicolor* captured along the Jatapú and Purus rivers revealed a similar chromosomal organizational pattern for individuals with 2n=80 chromosomes. However, the karyotypic pattern of individuals collected on the banks of the Jari river diverges, with a diploid number 2n=82 and FN=116 (Lira 2012). The NORs described in the present work (7 labeled sites) occurred in larger numbers than what had been previously described for the species (1 to 4 labeled sited) (Andrades-Miranda 2001, Lira 2012). These NORs do not refer to the labeling of acidic heterochromatic regions, as fluorescent *in situ* hybridization using 18S rDNA probes revealed the existence of twelve chromosome pairs carrying these sequences. A larger number of sites compared with the number identified through silver nitrate staining, which is a common occurrence and is observed in other groups (Lira 2012). This disparity stems from the fact that the latter technique labels proteins associated with the nucleolar structure and not ribosomal DNA regions, thus identifying only NORs that had been active in the preceding interphase (Miller et al. 1976). Thus, the difference in silver-stained sites between different populations may stem from the activity of ribosomal RNA genes. Because rDNA sequence hybridization had not been performed in individuals from the analyzed populations in previous studies, this hypothesis cannot be verified. In contrast, the heterochromatin distribution pattern is similar, with centromeric blocks extending to the short arms of the majority of metacentric and submetacentric chromosomes and both sex chromosomes.

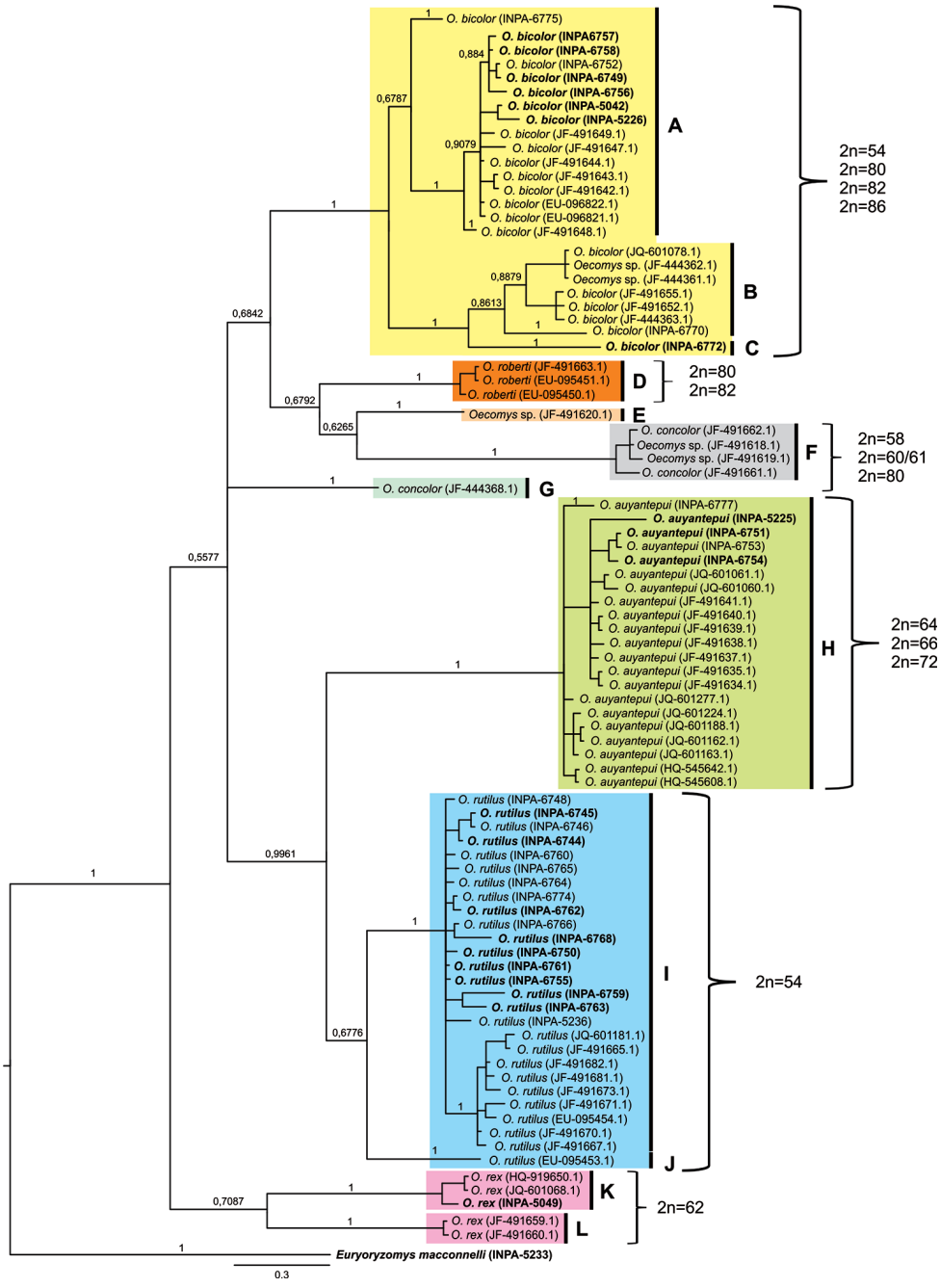

Figure 6. Bayesian tree of the cytochrome oxidase I gene. The probabilistic support is presented above the branches. Letters (**A–L**) represent the groups formed based on the analysis of the genetic distances between them. Sequences in bold were analyzed in the present work.

The diploid number determined for *O. rutilus* (2n=54, first described in Lira (2012) did not vary, regardless of the collection site, and no variations in karyotypic structure were observed in the present work. However, the three specimens described previously (Lira, 2012) exhibited differences in their autosomal fundamental number (82, 84 and 86). Such variation may be related to karyotype interpretation, given that it depends on the quality of chromosome preparations, DNA compaction patterns, size and number of chromosomes and errors in the measurement of chromosomal arms. The C-banding pattern observed in *O. rutilus* consisted of very subtle labeling on the majority of chromosomes but was consistent with the expected locations previously described for other *Oecomys* species and the tribe Oryzomyini (Yonenaga-Yassuda et al. 1987, Svartman and Almeida 1992, Silva and Yonenaga-Yassuda 1998, Aniskin and Volobouev 1999, Volobouev and Aniskin 2000, Andrades-Miranda et al. 2002, Bonvicino et al. 2005, Lira 2012).

Based on the amplitude of the genus distribution, Langguth et al. (2005) suggested *O. catherinae* (2n=60) as the ancestral taxon; the same finding was reported by Weksler (2006), based on phylogenetic analysis of the IRBP gene and morphological data for *O. bicolor*, *O. catherinae*, *O. concolor*, *O. mamorae* and *O. trinitatis*. Although the current phylogenetic analysis based on COI sequences was limited to a single marker and did not consider several of the taxa analyzed by Flores (2010), it showed similar results with high support values, such as monophyly of the genus *Oecomys*, which was also observed in molecular studies using other markers (Smith and Patton 1999, Weksler 2006). However, considering *O. rex* as a sister group of *O. catherinae*, which would classify both species as ancestral taxa (Flores 2010), the basal diploid number would be approximately 60/62 chromosomes. Therefore, it must be noted that molecular analyses did not detect an increasing or decreasing tendency in the diploid number between the branches, suggesting a complex karyotypic structure, as shown by the different diploid numbers obtained for the same morphological species. Moreover, the phylogenetic analysis placed all individuals in a single group.

In the present work, NORs were found to be preferentially located in the terminal regions of chromosomes, and their number increased with the diploid number; this pattern is also present in other members of the family Cricetidae (Lira 2012, Romanova et al. 2006, Ventura 2009, Fagundes et al. 1997). These data agree with FISH results obtained using the 18S ribosomal DNA probe, confirming the presence of two labeled pairs for *O. auyantepui* and *O. rutilus*. Labeling of four NORs was observed in *Oecomys rutilus*, whereas three were detected in *O. auyantepui*. Lira (2012) described four labeled sites in *O. rex*, again suggesting that it may constitute a basal taxon. In *Oecomys bicolor*, five chromosome pairs exhibited labeling, though not all displayed labeling on both homologous chromosomes. The multiple 18S rDNA sites observed in *O. bicolor* likely derive from duplication and dispersion. Di Meo et al. (1993) reported that the difference in the NOR distribution in correlated species is ascribed to rearrangements that have accumulated since the divergence of the common ancestor, mainly via inversions and Robertsonian translocations. Grozdanov et al. (2003) and Britton-Davidian et al. (2012) stated that NOR diversity among rodents is an indica-

tor of high intrachromosomal transposition rates in the absence of visible rearrangements, suggesting, once again, that this character represents a derived state for this taxon. Despite this fact, the interstitial position of 5S rDNA is related to sequence protection, thereby avoiding possible crossing-over or transposition events, which are more frequent in terminal regions (Martins and Galetti Jr., 1999). This scenario is made evident by comparing the degree of conservation in the position and location of this sequence compared with 45S rRNA. Ventura et al. (2012) described a similar situation in Akodontini, which shows conservation of 5S rDNA chromosomal sites, despite large chromosomal variability within the group.

Oecomys species have undergone intense chromosomal alteration processes, as confirmed by the observed karyotypic patterns, indicating high local diversity and an ample distribution for the taxa under study. However, the limited taxonomic sample available, in terms of both *Oecomys* individuals and molecular data renders the determination of which evolutionary processes have led to the variability in karyotype morphology more difficult. Furthermore, the current data reinforce the necessity for integrative taxonomy, where genetic tools should be used in conjunction with morphological analysis to delimit *Oecomys* taxa.

Conclusions

The intra- and interspecific variations observed in the diploid number of *Oecomys* species indicate that chromosomal rearrangements such as fusions/fissions, translocations and duplications have led to the appearance of different diploid numbers and karyotypic formulas. However, telomere sequence hybridization was not found to be a good indicator of autosomal chromosome rearrangements in the *Oecomys* species under study, as no autosomal ITSs could be observed. *O. bicolor*, which is considered to be a derived taxon of the genus (Flores 2010), exhibits the highest diploid number, possibly arising from chromosomal fission events that occurred during its evolutionary history.

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Appendix

COI sequences of *Oecomys* deposited in GenBank. The voucher, species and collection sites are listed.

Species	Genbank n°	Collection sites
O. auyantepui	JQ601277.1	Suriname – Sipaliwini river
	JQ601224.1	Suriname – Sipaliwini river
	JQ601188.1	Suriname – Kutari River
	JQ601163.1	Suriname – Kutari River
	JQ601162.1	Suriname – Kutari River
	JQ601061.1	Suriname: Brownsberg Nature Park
	JQ601060.1	Suriname: Brownsberg Nature Park
	JQ601049.1	Suriname: Brownsberg Nature Park
	HQ545642.1	Suriname: Sipaliwini River
	HQ545608.1	Suriname
	HQ545608.1	Suriname
	JF491641.1	Guiana: Upper Demerara-Berbice, West Pibiri, Mabura
	JF491640.1	Guiana: Upper Takutu-Upper Essequibo
	JF491639.1	Guiana: Upper Takutu-Upper Essequibo
	JF491638.1	Guiana: Potaro-Siparuni
	JF491637.1	Guiana: Potaro-Siparuni
	JF491635.1	Guiana: Cuyuni-Mazaruni
	JF491634.1	Guiana: Cuyuni-Mazaruni
O. bicolor	JQ601078.1	Equador: Parque Nacional Yasuni
	JF491655.1	Equador: Napo, Parque Nacional Yasuni
	JF491652.1	Equador: Orellana, Onkone Gare
	JF491649.1	Guiana: Demerara-Mahaic
	JF491648.1	Guiana: Barima-Waini
	JF491647.1	Guiana: Barima-Waini
	JF491644.1	Guiana: Upper Takutu-Upper Essequibo
	JF491643.1	Guiana: Potaro-Siparuni
	JF491642.1	Guiana: Potaro-Siparuni
	JF444363.1	Equador: Orellana
	EU096822.1	Suriname: Sipaliwini
	EU096821.1	Suriname: Sipaliwini
O. concolor	JF491662.1	Equador: Napo, Parque Nacional Yasuni
	JF491661.1	Equador: Orellana, Onkone Gare
	JF444368.1	Equador: Orellana
O. rex	JF491660.1	Guiana: Potaro-Siparuni
	JF491659.1	Guiana: Potaro-Siparuni
	JQ601068.1	Guiana: 40 Km NE of Surama
	HQ919650.1	Suriname
O. roberti	JF491663.1	Guiana: Potaro-Siparuni

Species	Genbank n°	Collection sites
O. rutilus	JQ601181.1	Suriname: Kutari River Camp
	JF491682.1	Guiana: Potaro-Siparuni
	JF491681.1	Guiana: Potaro-Siparuni
	JF491673.1	Guiana: Potaro-Siparuni Kabukalli Landing, Iwokrama Forest
	JF491671.1	Guiana: Siparuni river
	JF491670.1	Guiana: Barima-Waini, Baramita, Old World
	JF491667.1	Guiana: Barima-Waini, Baramita, Old World
	JF491665.1	Guiana: Upper Takutu-Upper Essequibo
	EU095454.1	Guiana: Upper Demerara-Berbice
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The presence of the ancestral insect telomeric motif in kissing bugs (Triatominae) rules out the hypothesis of its loss in evolutionarily advanced Heteroptera (Cimicomorpha)

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Abstract

Next-generation sequencing data analysis on *Triatoma infestans* Klug, 1834 (Heteroptera, Cimicomorpha, Reduviidae) revealed the presence of the ancestral insect $(TTAGG)_n$ telomeric motif in its genome. Fluorescence *in situ* hybridization confirms that chromosomes bear this telomeric sequence in their chromosomal ends. Furthermore, motif amount estimation was about 0.03% of the total genome, so that the average telomere length in each chromosomal end is almost 18 kb long. We also detected the presence of $(TTAGG)_n$ telomeric repeat in mitotic and meiotic chromosomes in other three species of Triatominae: *Triatoma dimidiata* Latreille, 1811, *Dipetalogaster maxima* Uhler, 1894, and *Rhodnius prolixus* Ståhl, 1859. This is the first report of the $(TTAGG)_n$ telomeric repeat in the infraorder Cimicomorpha, contradicting the currently accepted hypothesis that evolutionarily recent heteropterans lack this ancestral insect telomeric sequence.

Keywords

Cimicomorpha, kissing bugs, holocentric chromosomes, telomeres, NGS, $(TTAGG)_n$

Introduction

Telomeres, the physical ends of eukaryote chromosomes, are defined as specialized DNA-protein structures essential for chromosome replication, meiotic pairing and chromosome stability. In most organisms, telomeric DNA is composed by simple G-rich sequences repeats that extend for tens of base pairs (bp) as much as 150 kb, depending on the organism. Although telomeric repeats are diverse in their DNA sequence composition among different organisms (Zakian 1995), several taxonomic groups possess highly conserved motifs. Vertebrates, including bony fishes, reptiles, amphibians, and mammals exhibit the $(TTAGGG)_n$ repeat (Meyne et al. 1989) while the $(TTTAGGG)_n$ sequence appears highly conserved in the plant kingdom (Watson and Riha 2010). Extensive studies in arthropods have revealed that the predominant telomeric sequence is a pentanucleotide sequence repeat $(TTAGG)_n$, which has been considered as the ancestral telomeric motif in phylum Arthropoda, including insects (Sahara et al. 1999, Frydrychová et al. 2004, Vítková et al. 2005). However, numerous studies contradict this claim. For example several insect groups do not exhibit this telomeric repeat, such as Diptera, Ephemeroptera, Odonata, Dermaptera, Siphonaptera, Mecoptera, Raphidioptera and parasitic Hymenoptera. In addition, Coleoptera, Neuroptera and Hemiptera orders include species with and without the ancestral $(TTAGG)_n$ telomeric motif (Frydrychová et al. 2004, Gokhman et al. 2014, Korandová et al. 2014). In these insect groups, the ancestral telomeric motif is replaced by other alternative telomeric sequences such as $(TCAGG)_n$ in some coleopteran species (Mravinac et al. 2011), non-long terminal repeat (LTR) retrotransposons in *Drosophila* Fallén, 1823 (Mason et al. 2008), arrays of long satellite repeats in Culicomorpha dipteran (Walter et al. 2001), or by unknown sequences as in damselflies, mayflies and some aphid species (Frydrychová et al. 2004, Vítková et al. 2005). The most illustrative example of the variability of the telomeric sequences was observed in Coleoptera where ancestral $(TTAGG)_n$ has been lost at least eight times during the evolution of this insect group (Frydrychová and Marec 2002, Mravinac et al. 2011).

Among Hemiptera, the ancestral motif is present in the suborder Sternorrhyncha (coccids and aphids with some exceptions) (Mohan et al. 2011, Monti et al. 2011, Novotná et al. 2011), in several genera of Auchenorrhyncha (Frydrychová et al. 2004, Maryańska-Nadachowska et al. 2013, Golub et al. 2014, Kuznetsova et al. 2015a) and Coleorrhyncha (Kuznetsova et al. 2015b) suborders. In the suborder Heteroptera, only two species of the basal infraorders Nepomorpha and Gerromorpha show the ancestral telomeric motif (Kuznetsova et al. 2012, Mason et al. 2016). On the contrary, the most derived and specious heteropteran infraorders (Cimicomorpha and Pentatomomorpha) do not show the classic insect motif (for review see Grozeva et al. 2015, Mason et al. 2016). A recent survey of several sequenced genomes of these groups, including the triatomine *Rhodnius prolixus*, confirms the lack of the ancestral telomeric repeat and these groups are regarded as having a defective version of telomerase gene (Mason et al. 2016). Mason et al. (2016) have suggested the occurrence of a single loss event of the telomeric repeat, sometime before the Cimicomorpha and Pentatomomorpha divergence, and after their separation from Nepomorpha.

Kissing bugs (Triatominae, Reduviidae) are included within the infraorder Cimicomorpha (Heteroptera), constituting a group of medical relevance because they act as vectors of Chagas disease, also known as American trypanosomiasis. This subfamily includes 150 species, of which more than 80 have been cytogenetically studied (Panzera et al. 2010), having holocentric chromosomes. The current data, as above mentioned, suggest a high heterogeneity in insect telomere composition. One should also take into consideration that loss of the insect ancestral repeat in Cimicomorpha has been reported (Mason et al. 2016). For all these reasons it is important to explore for the first time in Triatominae the presence of $(TTAGG)_n$ motif, using next-generation sequencing (NGS) analysis tools and fluorescence *in situ* hybridization (FISH) in four triatomine species from three different genera. The results presented in this paper are in clear contradiction to the loss of ancestral telomeric repeats hypothesis in evolutionarily advanced Heteroptera.

Materials and methods

Material

Four species where analyzed, involving three different genera from the two principal tribes of the subfamily: Triatomini (*Dipetalogaster maxima*, *Triatoma infestans*, and *T. dimidiata*) and Rhodniini (*Rhodnius prolixus*). The last three species are the main vectors of Chagas disease. Origin and cytogenetic traits of each species are detailed in Table 1.

Telomere detection by genome sequencing

A *Triatoma infestans* (non-Andean lineage) specimen collected in Tacuarembó (Uruguay) was used for sequencing. Approximately 3 μg of genomic DNA were employed in a low coverage Illumina® Hiseq™ 2000 paired-end sequencing. Graph-based clustering analysis was carried out using RepeatExplorer (Novák et al. 2013), implemented within the Galaxy environment (http://repeatexplorer.umbr.cas.cz/) (Novák et al. 2010). RepeatExplorer also allow quantifying the abundance of the repeated sequences in the genome in base to the number of reads in each cluster.

Telomere detection by FISH

Chromosome preparations for FISH analyses were obtained from male gonads. Testes were removed from live adult insects, fixed in an ethanol–glacial acetic acid mixture (3:1) and stored at -20°C. Squashes were made in a 50% acetic acid drop, coverslips were removed after freezing in liquid nitrogen and the slides were air dried and then stored a 4°C.

Table 1. Geographical origin and male diploid chromosome number in the four species here analyzed. A = autosomes.

Species	Geographical origin	Male diploid chromosome number (2n)
Tribe *Rhodniini*		
Rhodnius prolixus	Guatemala, Quezaltenango, Insectary CDC (USA)	22= 20A + XY
Tribe *Triatomini*		
Dipetalogaster maxima	Baja California, Mexico	22= 20A + XY
Triatoma dimidiata	Jutiapa, Guatemala	23= 20A + X_1X_2Y
Triatoma infestans	Tacuarembó, Uruguay	22= 20A + XY

Telomeric TTAGG probe generation and FISH assays were carried out following Lorite et al. (2002) and Mora et al. (2015). Telomeric probes were generated by PCR using the primers $(TTAGG)_6$ and $(TAACC)_6$, following a similar procedure as described by IJdo et al. (1991). PCR was performed in 100 µl using 100 pmol of each primer and 2.5 units of Taq polymerase, in the absence of a template. PCRs were carried out using the following cycling profile: 30 cycles at 95°C (60 sec), 50°C (1 min), 72°C (3 min), with a final elongation step of 72°C for 10 min. PCR generated fragments (between 200 bp and 1 kb) were purified and labeled with biotin-16-dUTP (Roche) out using the Nick Translation Kit (Roche), following manufacturer's instructions. The labelled probe was precipitated and dissolved in 50% formamide.

Previously to hybridization, slides were treated with RNase A, pepsin and formaldehyde and dehydrated in 70%, 90% and 100% ethanol for 5 min each. Hybridization was performed applying 25 µl of DNA labelled solution to each slide, which was heated for 3 min at 80°C to denature the DNA, and immediately chilled on ice for 3 min. The hybridization mix consisted of (final concentrations) 50% formamide, 2xSSC, 50 mM sodium phosphate, 0.1 mg/ml sonicated salmon sperm DNA, 0.1 mg/ml yeast RNA, and 5 ng/ml labeled telomere probe. The slides were transferred to a moist chamber humidified with formamide (50%) and incubated overnight at 37°C. After incubation, the slides were washed in 50% formamide at 37°C, three times, 3 min each; followed by 2xSSC, 0.05% Tween-20, pH 7.5, three times, 5 min each. Fluorescence immunological detection was performed using the avidin-FICT/ anti-avidin-biotin system with four rounds of amplification. Slides were mounted with Vectashield (Vector). DAPI in the antifade solution was used to counterstain chromosomes.

Results and discussion

The data obtained from the *T. infestans* genome sequencing were analyzed with RepeatExplorer (Novák et al. 2013). One of the obtained clusters was formed by a telomeric sequence TTAGG array. In order to test if this repeat represents the putative telomere, FISH was carried out using the TTAGG repeat as probe. Hybridization signals were clearly seen at the ends of the mitotic chromosomes (Fig. 1A), revealing that telomeres in this species are really composed by this ancestral insect motif. The cluster of

the $(TTAGG)_n$ sequences was estimated for about 0.0266% of the total genome size, i.e. 395.5 kb. Considering that the haploid genome content in *T. infestans* is 1.52 pg (1.487 Mb) (Panzera et al. 2007, 2010) and that the chromosome number is 2n=22, the average telomeres length motifs in each chromosome end would be almost 18 kb long. This value is in the range of the telomere length observed in other insects with the ancestral motif or a variant of this repeat such as *Tenebrio molitor* Linnaeus, 1758 (15 kb) (Richards et al. 2008) but higher than the observed in other species with holocentric chromosomes as lepidopteran species (6-9 kb) (Okazaki et al. 1993, Mandrioli 2002), or in the homopteran coccid *Planococcus lilacinus* Cockerell, 1905 (6.4 kb) (Mohan et al. 2011).

Furthermore, we tested the telomeric motif presence by FISH in other three triatomine species with $(TTAGG)_n$ probe. Hybridization signals were clearly seen on the chromosomal ends of mitotic and meiotic chromosomes (Fig. 1B–D), revealing that Triatominae telomeres are composed by the ancestral insect motif. FISH technique in triatomines is highly sensitive to material fixation conditions. Cytoplasmic remnants in the slides represent the greatest challenge because it hinders the access of the telomeric probes to the chromosomes. This can be partially avoided using recently extracted gonads. In addition, access of the telomeric probes to the chromosome and its visualization are very sensitive to the chromosomes being on the same plane. As a result, differences in hybridization signals can be observed in the same slide or even within chromosomes of the same cell (Fig. 1).

Given our positive FISH hybridization results on *R. prolixus* chromosomes, we additionally conducted a BLAST search of telomeric sequences in the published genome of this species, available at https://www.vectorbase.org/. Similar as reported by Mason et al. (2016), we did not find $(TTAGG)_n$ repeats, so that these tandem sequences and probably others repeated sequences are not included in the published genome of *R. prolixus* (Mesquita et al. 2015). This reveals the difficulty of the repetitive DNA fraction assembly, as has been reported in different organisms including the well-studied human genome, making that many repetitive sequences have been omitted from the reference assembly and from most genome-wide analyses (Altemose et al. 2014).

Heteroptera or true bugs are a hemipteran suborder comprising seven infraorders and 40,000 species. All phylogenetic studies agreed that the infraorders Cimicomorpha and Pentatomomorpha are the most evolutionarily derived groups, with a common ancestor and involving about 80% of heteropteran species (Weirauch and Schuh 2011). Until now, the detection by FISH, Southern and/or dot-blot hybridization of telomeric repeat motif $(TTAGG)_n$ in Heteroptera has been unsuccessful in nine genera from five families of the infraorders Cimicomorpha and Pentatomomorpha (Sahara et al. 1999, Kuznetsova et al. 2011, Frydrychová et al. 2004, Grozeva et al. 2011, Golub et al. 2015). Only two heteropteran species from the basal infraorders Nepomorpha and Gerromorpha exhibit the ancestral telomeric motif (Kuznetsova et al. 2012, Mason et al. 2016). The $(TTAGG)_n$ motif was suggested to be lost in the early evolution being and secondarily replaced by another motif or an alternative telomerase-independent mechanism of telomere maintenance (Frydrychová et al. 2004, Lukhtanov and Kuznetsova 2010). Although several authors have suggested the loss of TTAGG repeat in all

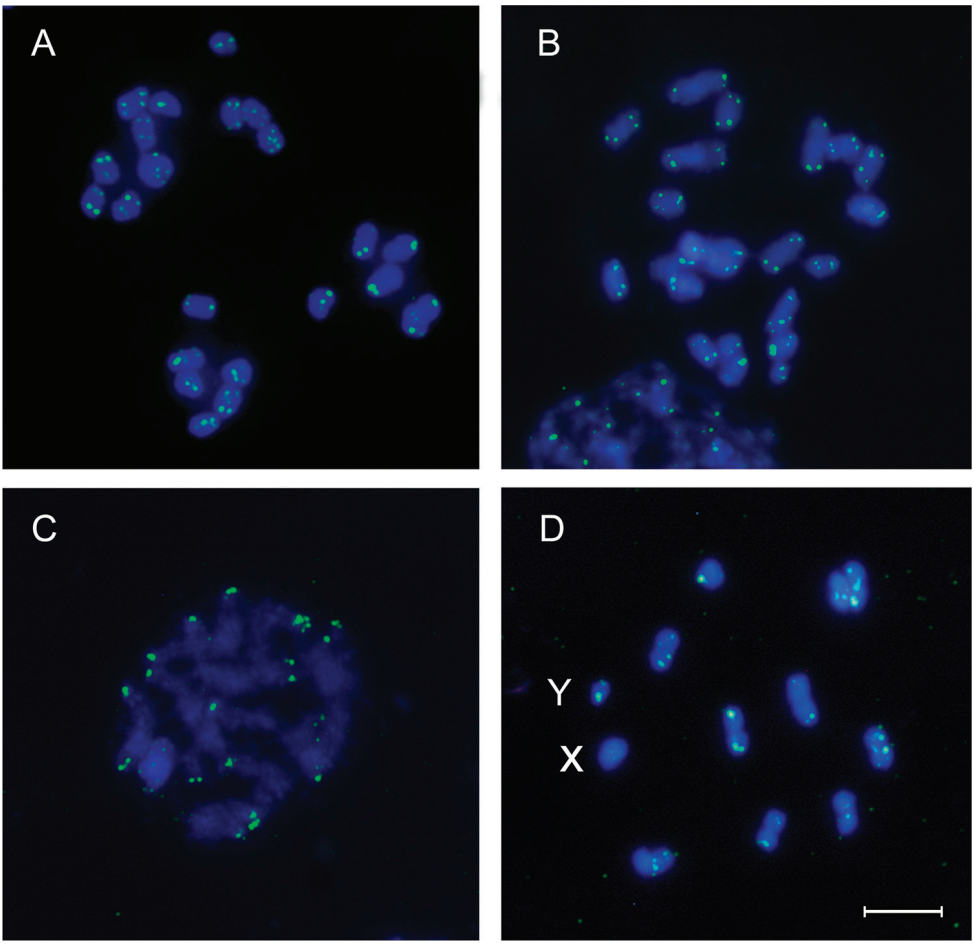

Figure 1. Fluorescence *in situ* hybridization with $(TTAGG)_n$ telomeric probe (green signals) on mitotic and meiotic chromosomes (counterstained with DAPI in blue) of four Triatominae species. **A** *Triatoma infestans* (2n=22), spermatogonial prometaphase **B** *Triatoma dimidiata* (2n=23), spermatogonial prometaphase **C** *Dipetalogaster maxima* (2n=22), pachytene stage **D** *Rhodnius prolixus* (2n=22), first meiotic division showing 10 bivalents and two sex chromosomes (X and Y). Scale bar: 5 μm.

Cimicomorpha species (Grozeva et al. 2015, Mason et al. 2016), the results presented here clearly contradict this hypothesis. According to the most comprehensive phylogeny of assassin bugs, the subfamily Triatominae is the youngest within Reduviidae, having evolved in the Oligocene, approximately 32 million years ago (24–38 Ma) (Hwang and Weirauch 2012). Whereas, a new acquisition of telomeric repeat in this recent evolutionary group seems unlikely, probably this lack of detection in Cimicomorpha and Pentatomomorpha is due to a methodological problem of the telomeric probe rather than a loss process during their evolution. Detailed analyses of the genomes repetitive fraction as well as exhaustive bioinformatics search on genomic databases might clarify the existence of these repeat sequences in other heteropteran groups.

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SHORT COMMUNICATION

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Divergent karyotypes of the annual killifish genus *Nothobranchius* (Cyprinodontiformes, Nothobranchiidae)

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Abstract

Karyotypes of two species of the African annual killifish genus *Nothobranchius* Peters, 1868*, N. brieni* Poll, 1938 and *Nothobranchius* sp. from Kasenga (D.R. Congo) are described. Both species displayed diploid chromosome number 2n = 49/50 for males and females respectively with multiple-sex chromosome system type $X_1X_2Y/X_1X_1X_2X_2$. The karyotypes of studied species are considerably different from those previously reported for the genus *Nothobranchius* and similar to the Actinopterygii conservative karyotype.

Keywords

Africa, chromosome number, karyotype, killifish, *Nothobranchius*

Introduction

Annual killifishes belonging to the genus *Nothobranchius* Peters, 1868 are mainly distributed in eastern Africa but several species are found in central Africa (Wildekamp 2004). They inhabit temporary pools that dry out during the dry season and have specific adaptations for extreme environments. Annual fishes are characterised by specific life history traits of extremely short lifespan and diapause in embryonic development (Furness 2015, Nagy 2015). Their unique biology makes them a model taxon with

which to investigate aging, embryonic development, ecology, and natural selection (Cellerino et al. 2015).

Killifishes of the genus *Nothobranchius* comprise 71 valid species (FishBase 2015). In this genus karyologicaly were described only 23 species (Arai 2011). These species have variable karyotypes with diploid chromosome numbers (2n) ranging from 2n = 16 for *N. rachovii* Ahl, 1926 to 2n = 43 for *N. thierryi* (Ahl, 1924) (Scheel 1990). More than 60% of karyotypes in *Nothobranchius* are characterised by a modal diploid number of 2n = 36-38.

A multiple-sex chromosome system of $X_1X_1X_2X_2/X_1X_2Y$ type has been reported for only one species of *Nothobranchius*, *N. guentheri* (Pfeffer, 1893) with a female karyotype consisting of 36 chromosomes and the male karyotype consisting of 35 chromosomes (Ewulonu et al. 1985).

In this paper, the karyotypes of two species, *Nothobranchius brieni* Poll, 1938 and *Nothobranchius* sp. from Kasenga, were studied, bringing the number of species studied to 25.

Material and methods

Specimens of *N. brieni* were collected from a large ephemeral swamp in the Lualaba drainage, near the village of Bukama in Katanga province (Democratic Republic of Congo, 09°11.374'S 25°51.334'E) on 2 April 2013 by E. Abwe, B. Katemo Manda, and B. Nagy, whereas specimens of *Nothobranchius* sp. from Kasenga (*Nothobranchius* sp. 'Kasenga') were collected in an ephemeral swamp in the Luapula drainage, near Kasenga, a village in Katanga province (D.R. Congo, 10°31.360'S, 28°27.368'E) on 17 April 2015, by E. Abwe, A. Chocha Manda, B. Katemo Manda, and T. Popp (Fig. 1).

Cytogenetic analysis

Chromosomes were prepared according to the Kligerman and Bloom method (1974). The chromosome preparations were obtained from head kidney tissue. Before preparation fish were treated intraperitoneally with 0.1% colchicine for 3–4 hours. The hypotonisation lasted 20–30 min at room temperature in 0.075 M KCl. Then tissue samples were fixed in 3:1 methanol : acetic acid for 24 hours. Six specimens of *N. brieni* (three males and three females) and three specimens of *Nothobranchius* sp. 'Kasenga' (one male and three females) were karyotyped with this method. Meiotic chromosome preparations of *N. brieni* were acquired from testes by the same technique.

Slides were dried by air and stained with 2% Giemsa solution in phosphate buffer at pH 6.8 for 10 min. Karyotypes were analysed under microscope "AxioImager" Karl Zeiss (Germany) equipped with CCD camera and "KaryoImage" Metasystems Software (Germany). In each specimen the chromosome number and type was determined on metaphase plate. Chromosome morphology was determined according to Levan et

Figure 1. Localities of specimen collections in the Democratic Republic of Congo (**1** *Nothobranchius brieni* **2** *Nothobranchius* sp. 'Kasenga').

al. (1964). The chromosomes were classified as metacentric (M), submetacentric (SM), and acrocentric (A). To determine the fundamental number (NF), chromosomes of the M and SM groups were considered bi-armed and those of group A as uni-armed.

Results

The diploid chromosome numbers of *N. brieni* were 2n = 49 for males and 2n = 50 for females with NF = 50/50 respectively. The female karyotype consisted of 25 pairs of acrocentric chromosomes gradually decreasing in size (Fig. 2a). The male karyotype consisted of 23 pairs of acrocentric chromosome and one bi-armed pair and two unpaired acrocentric chromosomes (Fig. 2b). In the first meiotic chromosomes during spermatogenesis 23 bivalents and a trivalent were observed at diakinesis (Fig. 2c).

The karyotype *Nothobranchius* sp. 'Kasenga' had diploid number 2n = 49 for males and 2n = 50 for females with NF = 68/68 respectively. The female karyotype had two pairs of metacentric, seven pairs of sub-metacentric, and 16 pair of acrocentric chromosomes varying in size from large to small (Fig. 3a). The male karyotype had 23 pair of chromosomes similar to the female with one bi-armed and two unpaired acrocentric chromosomes (Fig. 3b).

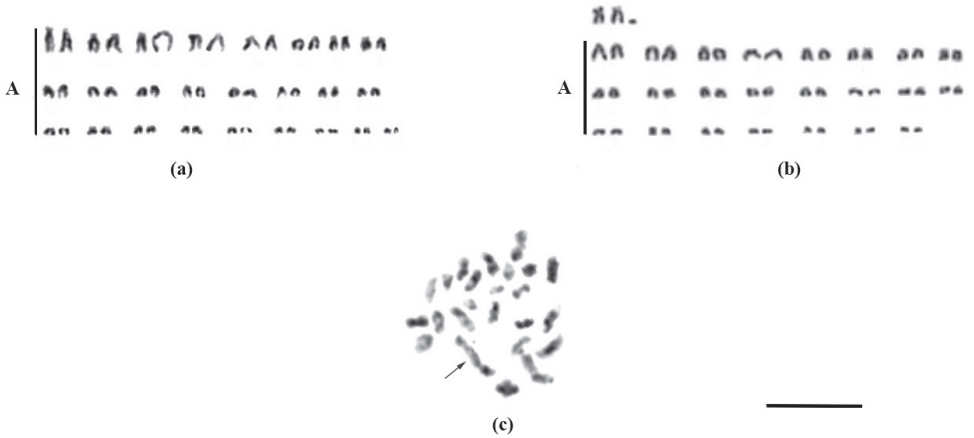

Figure 2. Karyotypes of *Nothobranchius brieni* **a** somatic chromosomes of female **b** somatic chromosomes of male **c** meiotic metaphase I (testicular). (A – acrocentric chromosomes). Note trivalent chromosome (arrowed). Scale bar: 10 μm.

Figure 3. Karyotypes of *Nothobranchius* sp. 'Kasenga' **a** somatic chromosomes of female **b** somatic chromosomes of male (M – metacentric chromosomes, SM – submetacentric chromosomes, A – acrocentric chromosomes). Scale bar: 10 μm.

Discussion

Cytogenetic characteristics

The described karyotypes stand apart from those already reported for species of genus *Nothobranchius*. The karyotype of *N. brieni* has the chromosomal number 2n = 49/50 and 25 pairs of uni-armed chromosomes in female (50A) and 23 pairs of uni-armed homomorphic and three heteromorphic chromosomes in male (1M + 48A). The karyotype of *Nothobranchius* sp. 'Kasenga' has the same diploid number 2n = 49/50 but a different karyotype structure possessing metacentric, sub-metacentric, and uni-armed chromosomes with 4M + 14SM + 32A for females and 5M + 14SM + 30A for males, while other species of the genus have a considerably lower modal diploid number of only 36 chromosomes (Table 1).

Table 1. The diploid number (2n) of *Nothobranchius* species (from Arai 2011 with modifications). *sex chromosome system of the type $X_1X_2Y/X_1X_1X_2X_2$.

Species	2n	References
N. brieni Poll, 1938*	49♂/50♀	Current study
N. eggersi Seegers, 1982	36	Scheel 1990
N. elongatus Wildekamp, 1982	38	Scheel 1990
N. foerschi Wildekamp & Berkenkamp, 1979	34	Ewulonu et al. 1985, Scheel 1990
N. furzeri Jubb, 1971	38	Reichwald et al. 2009
N. guentheri (Pfeffer, 1893)*	35♂/36♀	Ewulonu et al. 1985, Scheel 1990
N. hengstleri Valdesalici, 2007	38	Wildekamp et al. 2009
N. janpapi Wildekamp, 1977	38	Scheel 1990
N. jubbi Wildekamp & Berkenkamp, 1979	34	Scheel 1990
N. kirki Jubb, 1969	36	Scheel 1990
N. korthausae Meinken, 1973	36	Scheel 1990
N. krysanovi Shidlovskiy, Watters & Wildekamp, 2010	18	Shidlovskiy et al. 2010
N. kuhntae (Ahl, 1926)	38	Scheel 1990
N. lucius Shidlovskiy, Watters & Wildekamp, 2010	36	Wildekamp et al. 2009
N. makondorum Shidlovskiy, Watters & Wildekamp, 2010	38	Wildekamp et al. 2009
N. melanospilus (Pfeffer, 1896)	38	Ewulonu et al. 1985
N. microlepis (Vinciguerra, 1897)	24	Scheel 1990
N. palmqvisti (Lönnberg, 1907)	36	Ewulonu et al. 1985, Scheel 1990
N. polli Wildekamp, 1978	36	Ewulonu et al. 1985
N. patrizii (Vinciguerra, 1897)	36	Ewulonu et al. 1985
N. pienaari Shidlovskiy, Watters & Wildekamp, 2010	34	Shidlovskiy et al. 2010
N. rachovii Ahl, 1926	16	Ewulonu et al. 1985, Krysanov 1992
N. steinforti Wildekamp, 1977	36	Scheel 1990
N. thierryi (Ahl, 1924)	43	Scheel 1990
Nothobranchius sp. 'Kasenga'*	49♂/50♀	Current study

Sex chromosomes

The reduced diploid numbers and heteromorphic chromosomes in males suggest the occurrence of a multiple-sex chromosome system. A trivalent observation in the first meiotic chromosomes in *N. brieni* and the presence of a bi-armed chromosome exclusively in the male karyotype indicate a multiple-sex chromosome system of the type $X_1X_2Y/X_1X_1X_2X_2$. One bi-armed neo-Y chromosome has most likely resulted from the Robertsonian fusion between the Y chromosome and an autosome, as has been described for other fish species (e.g., Kitano and Peichel 2012). In *N. brieni* and *Nothobranchius* sp. 'Kasenga' the Y chromosomes is a large metacentric one, and X_1 and X_2 chromosomes are acrocentric of different sizes. The same-sex chromosome system has been reported only for *N. guentheri* (Ewulonu et al. 1985) among the 23 previously karyotyped species.

Karyotype evolution

In the genus *Nothobranchius* and the related *Aphyosemyon* Mayers, 1924 the evolutionary trend to reduce the total number of chromosomes via acrocentric chromosome fusion was specified (Scheel 1990, Völker et al. 2005). This assumption has been confirmed by the data presented in Table 1. According to this hypothesis, basal taxa have higher chromosome numbers and more acrocentric chromosomes while derived taxa have lower numbers of chromosomes with metacentric chromosomes (Agnese et al. 2006). It is widely accepted that the hypothetical ancestral karyotype of teleostean fishes consisted of 2n = 48-50 acrocentric chromosomes (Ohno et al. 1969, Nakatani et al. 2007). The two species presented in this study have numbers and a structure of karyotype conservative for Actinopterygii fishes (Mank and Avise 2006, Molina et al. 2014). It is supposed that karyotype of *N. brieni* is similar to that of the hypothetical ancestor of the genus *Nothobranchius*. There is a lack of molecular genetic data on this species, therefore we are not able to consider its phylogenetic position within the clade.

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RESEARCH ARTICLE

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First detailed karyo-morphological analysis and molecular cytological study of leafy cardoon and globe artichoke, two multi-use Asteraceae crops

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Abstract

Traditionally globe artichoke and leafy cardoon have been cultivated for use as vegetables but these crops are now finding multiple new roles in applications ranging from paper production to cheese preparation and biofuel use, with interest in their functional food potential. So far, their chromosome complements have been poorly investigated and a well-defined karyotype was not available. In this paper, a detailed karyo-morphological analysis and molecular cytogenetic studies were conducted on globe artichoke (*Cynara cardunculus* Linnaeus, 1753 var. *scolymus* Fiori, 1904) and leafy cardoon (*C. cardunculus* Linneaus, 1753 var. *altilis* De Candolle, 1838). Fluorescent *In Situ* Hybridization In Suspension (FISHIS) was applied to nuclei suspensions as a fast method for screening of labelling probes, before metaphase spread hybridization. Classic Fluorescent *In Situ* Hybridization (FISH) on slide, using repetitive telomeric and ribosomal sequences and Simple Sequence Repeats (SSRs) oligonucleotide as probes, identified homologous chromosome relationships and allowed development of molecular karyotypes for both varieties. The close phylogenetic relationship between globe artichoke and cardoon was supported by the very similar karyotypes but clear chromosomal structural variation was detected. In the light of the recent release of the globe artichoke genome sequencing, these results are relevant for future anchoring of the pseudomolecule sequence assemblies to specific chromosomes. In addition, the DNA content of the two crops has been determined by flow cytometry and a fast method for standard FISH on slide and methodological improvements for nuclei isolation are described.

Keywords
Cynara, SSR simple sequence repeats, repetitive sequences, flow cytometry, FISHIS, FISH

Introduction

The globe artichoke (*Cynara cardunculus* Linnaeus, 1753 var. *scolymus* (L.) Fiori, 1904) and the cultivated leafy cardoon (*C. cardunculus* Linneaus, 1753 var. *altilis* De Candolle, 1838) are dicotyledonous angiosperms belonging to the family *Asteraceae* and originate from the Mediterranean area (Sonnante et al. 2007a, b). They contribute significantly to the agricultural economy of this area, mainly of Italy, Egypt, Spain, France, Algeria and Morocco, which yields more than 70% of the total world globe artichoke production of 1.70 Mtons (FAOSTAT 2013). Peru, Argentina, China and USA are emerging countries for artichoke production outside Mediterranean region.

In spite of the agronomic, nutritional and industrial importance of globe artichoke and leafy cardoon for the Mediterranean basin, their genetics and cytogenetics is relatively poorly characterized, as recently stated by Scaglione et al. (2016). The unambiguous identification of individual chromosomes in the karyotype of a species is a cornerstone in understanding the genome organization and in identifying useful genes for breeding, but the small size and the remarkable similarity in the chromosome morphology (Falistocco 2016) still represent a challenge in defining a detailed karyotype for both *Cynara* varieties.

In addition to standard chromosome morphological analysis, cytogenetics can take advantage of a molecular approach based on fluorescence *in situ* hybridization (FISH) of repetitive sequences on metaphase chromosomes. This approach is very informative in recognising individual chromosomes and in delineating the structure and composition of genomic regions (Jiang and Gill 2006; Chester et al. 2010). This methodology enables the physical localization of one or more DNA probes along chromosomes. Among the different classes of repetitive sequences, SSRs represent one of the most valuable cytological markers in chromosome discrimination (Sharma et al. 2007; Cuadrado et al. 2008) due to their abundance and wide distribution in plant genomes (Heslop-Harrison and Schwarzacher 2011). In addition, the repeat sequences coding for ribosomal DNA (rDNA) have been widely used to characterize plant chromosome complements (Jiang and Gill 1994; Sharma et al. 2012). In the present study, a detailed karyo-morphological analysis and FISH characterization using a number of probes, that is, SSR derived oligonucleotides, telomeric repeats and the 18S-5.8S-26S rDNA, were performed to produce the first measures of single chromosomes and the molecular cytogenetic characterization of the globe artichoke and cardoon complements. FISHIS (Giorgi et al. 2013a) was used on nuclei suspensions as a fast and effective way to screen and select probes producing strong and localized signals, particularly useful in those species, such as *C. cardunculus*, where mitotic index remained quite low, even after using mitosis blocking agents (Giorgi et al. 2013b). Finally, flow cytometry genome size estimation was performed for both crops.

Materials and methods

Plant materials

Globe artichoke cultivar (cv) Istar and cardoon cv Bianco Avorio seeds were kindly provided by the Seed Company Topseed (Sarno, Salerno, Italy) while *Pisum sativum* (Linnaeus, 1573) cv Citrad seeds were generously provided by Dr. J. Doležel (Centre of Plant Structural and Functional Genomics, Institute of Experimental Botany, Olomouc, Ceck Republic). For both DNA content determination and cytogenetic analysis, *Cynara* spp. seeds were germinated in the dark on moist filter paper at 24±1 °C for 5-10 days, after a hot treatment at 50 °C for 10 min (for *P. sativum*, no hot treatment was performed). Actively growing roots and young leaves were excised for further treatment.

Nuclei isolation and DNA staining

Nuclei were extracted from 50 mg of young leaves using two different protocols. The first was performed according to Doležel and Greilhuber (2010) using fresh tissue while the second was carried out on tissue fixed in 2% formaldehyde solution/Tris buffer (10 mM Tris, 10 mM $Na_2EDTA.2H_2O$, 100 mM NaCl and 0.1% Triton X-100) for 20 min at 4±0.5 °C. After rinsing in Tris buffer, three times for 5 min each at 4 ±0.5 °C, leaves were briefly chopped in 1 ml of lysis buffer LB (15 mM Tris, 2 mM Na_2EDTA, 0.5 mM sperminetetrahydrochloride, 80 mM KCl, 20 mM NaCl, 15 mM β-mercaptoethanol, 0.7% (v/v) Triton X-100; pH 7.5) and homogenized with Ultraturrax T10 and G5 generator (IKA, Germany) at 10.000 rpm for 5 sec. The resulting homogenate was filtered through a 36 μm nylon mesh to remove debris, and the nuclei stained with 75 μM propidium iodide (PI) and 100 μg ml^{-1} RNase (Doležel et al. 1989).

Genome size determination

Flow cytometric estimation of nuclear DNA content stained with PI (λ ext max: 293 nm and 514 nm; λ ems max 625 nm) was performed using a FACS Vantage SE flow cytometer (BD Bioscience, San Jose, CA) with a solid state laser (Genesis CX STM, Coherent, Santa Clara, CA), UV emission at 200 mW, and a 70 μm flow tip running at 27 psi with a solution of 50 mM NaCl as sheath fluid. *P. sativum* cv "Citrad" was used as internal standard (2C = 9.09 pg) (Doležel et al. 1998). Nuclei of globe artichoke, cardoon and pea were isolated, stained and simultaneously analysed. The histogram of DNA fluorescence intensity was obtained following flow cytometric analysis of PI-stained nuclei. The flow cytometer was set for measuring with a CV of 2.5% or better; the measurements of relative florescence intensity of stained nuclei were performed on a linear scale, and typically 5.000–10.000 nuclei were analysed for each sample (samples

were run in triplicates). The peak of fluorescence of G0/G_1 nuclei from *P. sativum* was tuned to mean channel 400. The genome size (pg DNA) of globe artichoke and cardoon was calculated using DNA fluorescence measurements and the following equation:

unknown 2C DNA content = [(unknown G_1 peak mean)/ (standard G_1 peak mean)] × standard 2C DNA content.

2C DNA content (pg) was converted to base pairs (bp) following the factor: 1 pg DNA = 0.978 × 10^9 bp (Doležel et al. 2003).

Chromosome slide preparation and DNA staining

Actively growing roots were excised and pre-treated in 2 mM 8-Hydroxyquinoline (8HQ) for 3-5 h at room temperature (r.t.) or in 30 μM Oryzalin for 20 h at 4 °C, and then fixed in Carnoy solution (ethanol : glacial acetic acid = 3:1) at -20 °C for at least 18 h. Chromosome spreads were prepared according to the "drop spreading method" developed by Andras et al. (1999). Chromosomes were stained for morphometric analysis and karyotype definition with 4,6-diamidino-2-phenylindole (DAPI) at a final concentration of 2 μg ml^{-1}, according to Schwarzacher and Heslop-Harrison (2000).

FISH

In order to better discriminate each chromosome and pairing homologous chromosomes, single and double-target fluorescence *in situ* hybridization (FISH) was performed using ribosomal DNA (rDNA) sequences, telomeric and synthetic SSR oligonucleotides.

The 18S-5.8S-26S rDNA clone pTa71 (Gerlach and Bedbrook 1979) was labelled by nick-translation with Cy3 using standard kits (Nick Translation Mix, Roche) according to the manufacturer's instructions. FISH with the pTa71 probe was performed according to Andras et al. (1999) with minor modifications: the hybridization mixture (40 μL/slide) was prepared by adding 50% (v/v) formamide, 10% (w/v) dextran sulphate, 10% (v/v) 20X SSC and 120-160 ng of probe per slide, denatured at 75 °C for 10 min, and then put on ice.

Metaphases were denatured at r.t for 5 min in 70% ethanol (pH13 using 4 N NaOH). Preparations were dehydrated at r.t. through an ice-cold ethanol series (70%, 85% and 100%) for 2 min each, and air dried. The denatured probe was applied after chromosomes alkaline denaturation and plastic cover slips were placed over the specimens and the slides were incubated in a humid chamber at 37 °C for 16 h. After hybridization, the coverslips were removed and the preparations were subjected to a single stringency wash in 50% (v/v) formamide in 1 x SSC, followed by 2 additional washes in 2 x SSC, for 5 min at 45 °C each. Finally, samples were counterstained with DAPI and mounted in a Vectashield antifade solution (Vector Laboratories, Burlingame, CA, USA).

Fluorescence *In Situ* Hybridization In Suspension (FISHIS)

Before FISH analysis on metaphase spreads, a selection of SSR oligonucleotides was carried out on nuclei using FISHIS. Nuclei were isolated from roots following the same procedure previously described for fixed leaves. Hybridization was performed according to Giorgi et al. (2013a) using these SSR-oligonucleotides as probes: $(AT)_{12}$, $(AAC)_5$, $(AGG)_5$, $(ATC)_5$, $(GAA)_7$, $(CAT)_5$, $(CA)_{10}$, $(GA)_{10}$, $(CAG)_5$, $(GACA)_4$, $(TTTAGGG)_5$ and only those with clear hybridization signals were selected for FISH on slide. The SSR probes were single stranded oligos labeled at one end with FITC (fluorescein-5-isothiocyanate) or Cy3 (Cyanine 3), synthesized by Eurofins MWG Operon (Ebersberg, Germany). After FISHIS, 3–5 µl of labelled nuclei suspension was placed on a glass slide and mounted in Vectashield antifade solution (Vector Laboratories, Burlingame, CA, USA) for microscope image analysis.

Fast FISH

A fast FISH method was developed and carried out on metaphase spreads of artichoke and cardoon using selected SSR oligonucleotides as probes. Chromosome DNA was denatured in an alkaline 70% ethanol solution, as previously described, and the preparations were dehydrated at r.t. through an ice-cold ethanol series (70%, 85% and 100%) for 2 min each, and air dried. A mix containing 1.5 - 3 ng μl^{-1} of labelled oligonucleotide in 2X SSC (300 mM sodium chloride, 0.3 mM trisodium citrate) was applied on the slide (final volume 60 µl per slide) and hybridization was carried on at r.t. for 1 h After washing in 4X SSC, 0.2% Tween20 for 10 min, samples were counterstained with DAPI and mounted in antifade solution.

Microscope and image analysis

Microscope slides with chromosomes or nuclei were examined with a Nikon Eclipse TE2000-S inverted microscope equipped with an HB0100 W lamp and a CFI Plan Apo oil objective 100X and appropriate filter sets for DAPI, FITC and Cy3 fluorescence. Separate images from each filter set were digitalized and analysed using a DXM1200F Nikon camera and the NIS AR 3.1 software (Nikon Instruments S.p.A, Florence, Italy), respectively. Image analysis and measurements were performed using ImageJ v1.46 (Abramoff et al. 2004). Chromosomes were arranged in decreasing order according to their total length (µm) using the ImageJ plugin Chias IV. Manual adjustment and chromosome pairing were performed also according to FISH hybridization results. Chromosomes were classified on the basis of arm ratio (AR = length of the long arm/length of the short arm) as metacentric (M, AR = 1.00÷1.49), submetacentric (SM, AR = 1.50÷2.99) or acrocentric (A, AR > or = 3), following Guerra's nomenclature (Guerra 1986).

Results

Flow cytometric analysis of DNA content

After optimization of the isolation procedure, flow cytometry estimation of nuclear DNA content was performed analysing nuclei isolated from globe artichoke and cardoon using *Pisum sativum* cv. Citrad as internal standard, When using the most common isolation buffers and the classical method of chopping of fresh tissues (Doležel and Greilhuber 2010), a low yield for *Cynara* nuclei suspensions was obtained. An increased nuclei yield (of about 5 times) was achieved after fixation of fresh tissues in 2% formaldehyde, standard chopping of leaves in LB isolation buffer, containing a larger amount of Triton-X100, and by performing an additional homogenization step. These modifications resulted in an average amount of 600 nuclei per milligram of plant tissue, sufficient for an operational flow cytometric analysis, and were effective to get a good DNA fluorescence histogram, with a low noise background (Figure 1). The DNA content for cardoon and globe artichoke was estimated to be 2C = 2.20 pg ± 0.04 and 2C = 2.40 pg ± 0.04 (Figure 1) corresponding to a C genome size of 1.07 $\times 10^9$ bp and 1.17 $\times 10^9$ bp, respectively.

Cytogenetics: morphometric analysis

In order to perform a good morphological analysis, the quantity and quality of metaphase spreads, in terms of absence of cytoplasm and low percentages of overlapping chromosomes, is of critical relevance. Here a pre-treatment with oryzalin, as antimitotic agent, was tested for the first time in *Cynara* and compared to the 8-hydroxyquinoline used in previous studies; a further slight increase in methaphases number (about 5%) was observed.

The morphometric analysis of cardoon and globe artichoke chromosomes was carried out by measuring the total length (tl), the arm length and the arm ratio of all 34 individual chromosomes (Table 1, A and B, respectively). Based on their size, chromosome pairs of both varieties could be divided into three main groups: large (2.1μm<tl<3.2μm), medium (1.6μm<tl<2.0μm) and small (tl<1.6μm), with seven, six and four chromosome pairs, respectively. Within each group, chromosomes were similar in size and morphology and difficult to distinguish and arrange. Large differences in chromatin condensation made it difficult to compare measurements from different metaphases in the same preparation (Figure 2A and 2B). Figure 3 shows two typical mitotic metaphase spreads where chromosome sizes are close in the two crops, ranging from 3.19–3.16 μm for the biggest chromosome pairs, to 1.22 μm for the smallest ones in globe artichoke and cardoon, respectively. The calculated karyotypic formula is similar for the two crops, 2n = 16M+8SM+10A for *C. cardunculus* L. var. *scolymus* (L.) Fiori and 2n = 16M+6SM+12A for *C. cardunculus* L. var. *altilis* DC, the only exception being chromosome pair 14, which is acrocentric in the cultivated cardoon and metacentric in globe artichoke, as shown at the ideogram.

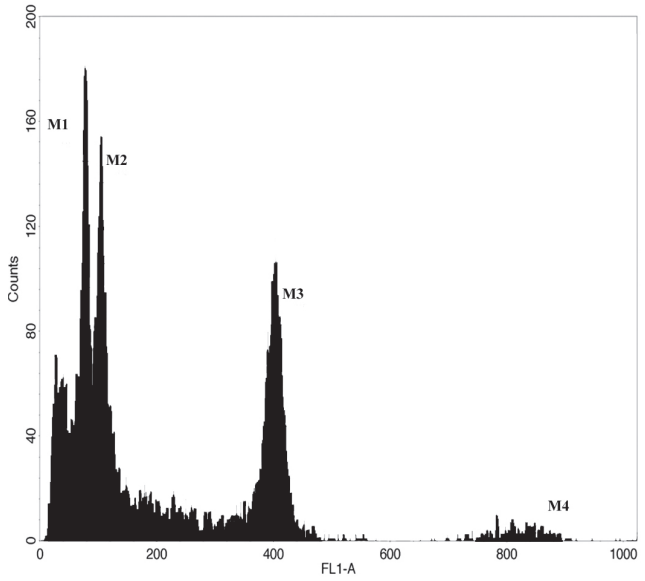

Figure 1. Flow Cytometry DNA content histogram. Flow cytometry analysis of DNA fluorescence peaks (FL1A) from G0/G_1 (2C) PI stained leaf nuclei from cultivated cardoon (M1), globe artichoke (M2) and *P. sativum* (pea) (M3). Pea was used as an internal standard and the peak was set at channel 400; M4 shows pea G_2 nuclei (4C).

Table 1. Morphometric analysis of *C. cardunculus* chromosomes. Morphological analysis of the 2n = 34 chromosomes of cultivated cardoon (**A**) and globe artichoke (**B**) based on Fig. 3A, B, respectively. Abbreviations: **C.p.** Chromosome pairs **T.l.** Total length **p** Average length of short arm **q** Average length of long arm; **AR** Arm Ratio (p/q) **Class** classification: **m** metacentric **sm** submetacentric **a** acrocentric.

C.p.	T.l. (µm)		p (µm)		q (µm)		AR		Class	
	A	B	A	B	A	B	A	B	A	B
1	3.16	3.19	1.23	1.27	1.93	1.92	1.57	1.51	s	s
2	2.97	3.16	1.07	1.24	1.78	1.93	1.78	1.55	sm	sm
3	2.58	2.81	1.05	1.21	1.55	1.60	1.46	1.32	m	m
4	2.35	2.65	0.97	1.16	1.38	1.49	1.42	1.28	m	m
5	2.35	2.45	0.78	0.95	1.57	1.50	2.01	1.57	sm	sm
6	2.12	2.27	0.85	0.95	1.27	1.32	1.49	1.39	m	m
7	2.10	2.25	0.87	0.93	1.23	1.32	1.41	1.42	m	m
8	2.03	2.00	0.79	0.78	1.24	1.22	1.56	1.56	sm	sm
9	2.00	1.98	0.92	0.92	1.08	1.06	1.17	1.15	m	m
10	2.00	1.98	–	–	2.00	1.98	>3	>3	a	a
11	1.95	1.95	–	–	1.95	1.95	>3	>3	a	a
12	1.79	1.95	0.81	0.71	0.98	1.24	1.20	1.74	m	sm
13	1.68	1.70	0.62	0.79	1.06	0.91	1.70	1.15	sm	m
14	1.40	1.41	–	0.65	1.40	0.76	>3	1.17	a	m
15	1.30	1.38	–	–	1.30	1.38	>3	>3	a	a
16	1.25	1.27	–	–	1.25	1.27	>3	>3	a	a
17	1.22	1.22	–	–	1.22	1.22	>3	>3	a	a

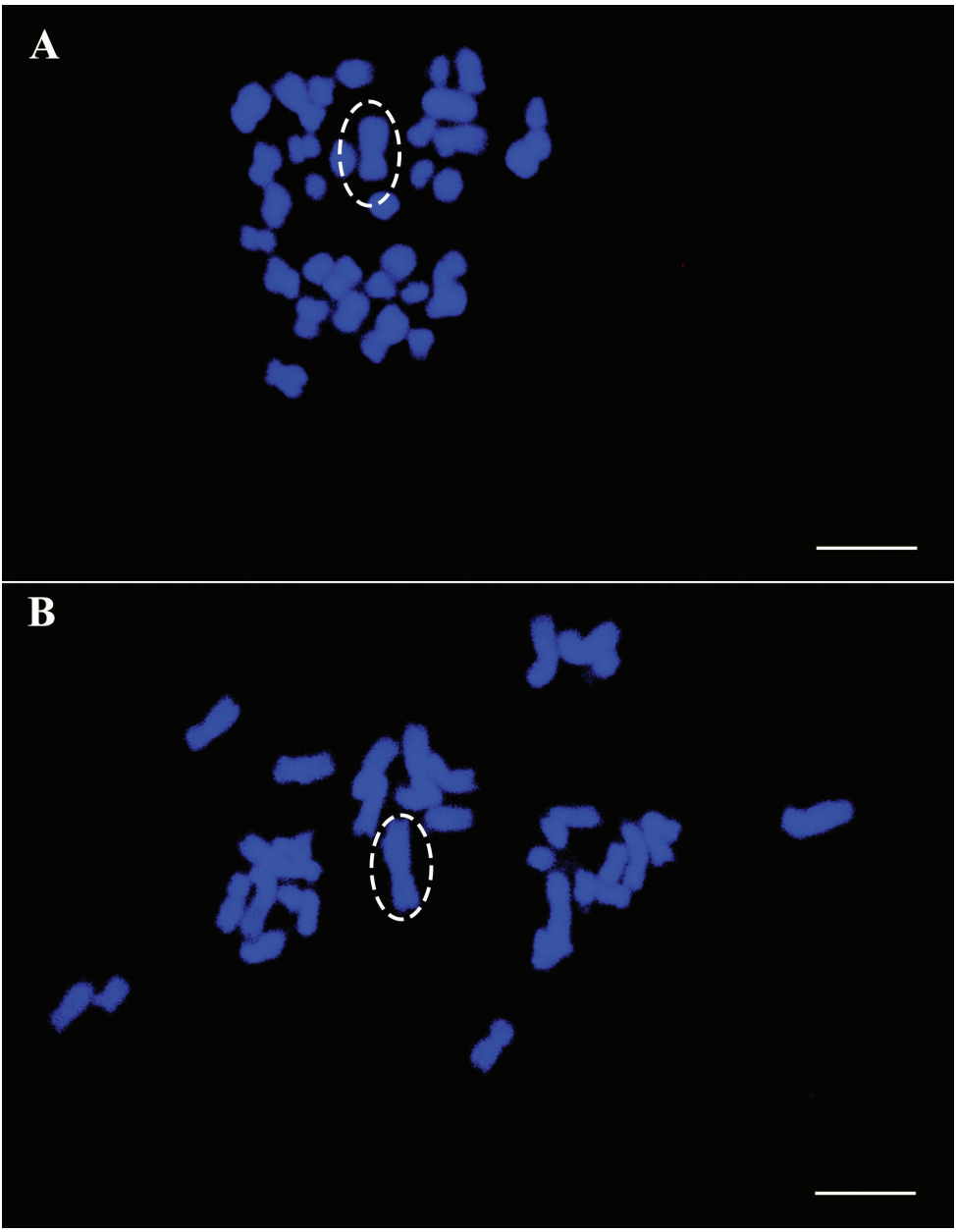

Figure 2. The smallness and variable sizing of *Cynara* chromosomes is shown; in both panel **A** and **B**, leafy cardoon chromosome 1 has been circled as an example of the different condensation level of the same metaphase chromosomes. In **A** chromosome 1 is 3.2 μm, in **B** it is 4.3 μm (a 36% size variation). Scale bars: 5 μm.

Molecular cytogenetics: rDNA localization FISHIS and FISH with SSR-oligonucleotides

FISH localization of rDNA was investigated using the pTa71 sequence as a probe. For both crops there were eight hybridization signals (Figures 4A and 4B), localized at the very distal part of chromosomes, and sometimes appearing almost detached from them (data not shown). Detailed morphological analysis identified very small satellite bodies on some acrocentric chromosomes, which were not consistently present, probably because they were damaged during slide preparation and FISH labelling. However, according to our observations, we identify two medium acrocentric and two small acrocentric chromosomes (most probably chromosomes 15 and 16) as the ones having satellites.

Screening SSR by FISHIS analysis revealed that only the telomeric sequence $(TTTAGGG)_5$ and the oligonucleotide $(GAA)_7$ showed clear and discrete hybridization signals on nuclei of both crops (Figure 5); while the two di-nucleotide $(CA)_{10}$ and $(GA)_{10}$ probes had a weak and diffuse signals (data not shown).

All four oligonucleotides $(TTTAGGG)_5$, $(GA)_{10}$, $(CA)_{10}$, and $(GAA)_7$ were used for fast standard FISH on chromosome spreads. As expected, $(TTTAGGG)_5$ hybridized at the telomeres, facilitating identification of the ends of the chromosome and more accurate measurements (Figures 6A and B).

The $(CA)_{10}$ and $(GA)_{10}$ di-nucleotides showed very similar hybridization patterns on the chromosomes of the two crops (Figures 6C-F). In most chromosomes, hybridization

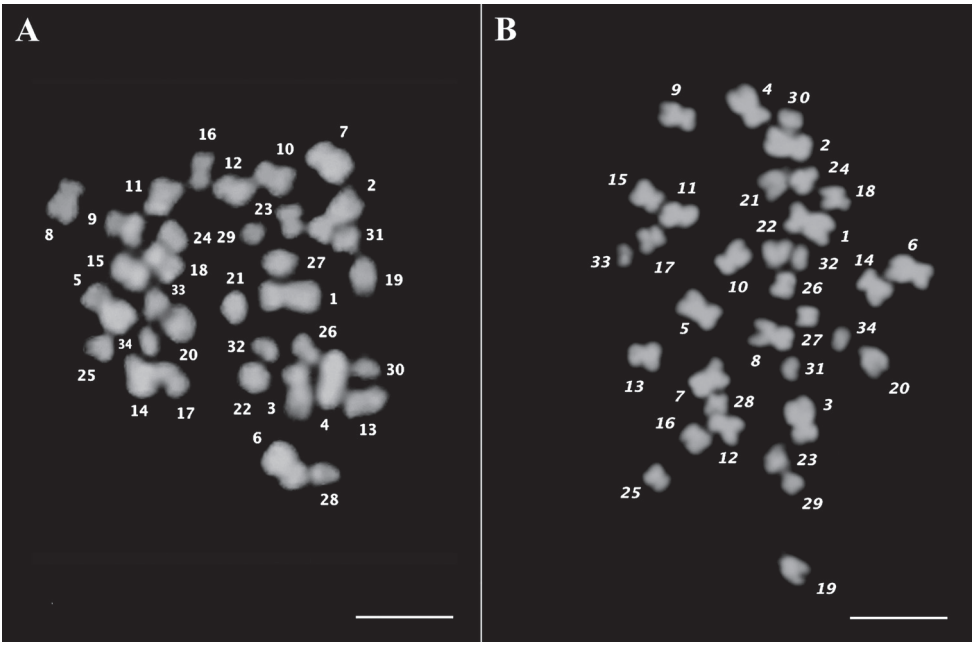

Figure 3. Chromosome numbering. Metaphase spreads showing numbered chromosomes of cardoon (**A**) and globe artichoke (**B**) counterstained with DAPI. Scales bars: 5 μm.

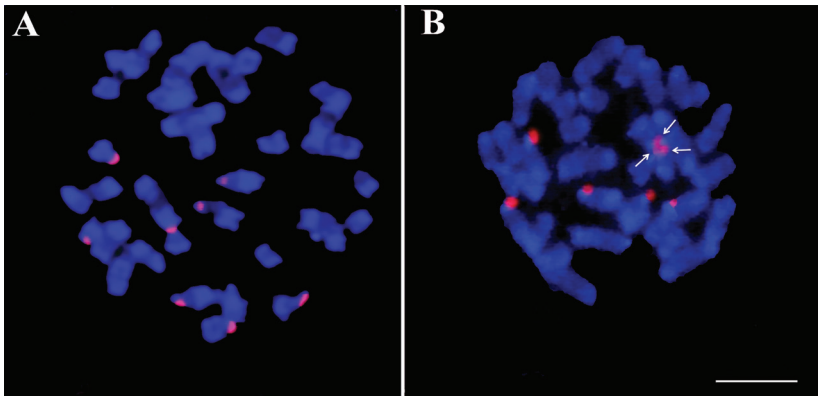

Figure 4. FISH molecular cytogenetic analysis with rDNA. FISH on metaphase spreads of cultivated cardoon (**A**) and globe artichoke (**B**) using the rDNA probe pTa71-Cy3 (red fluorescence). In Fig. 3B, arrows point to the hybridization spots localized on the three small acrocentric chromosomes, placed head to head. Scale bar: 5 μm.

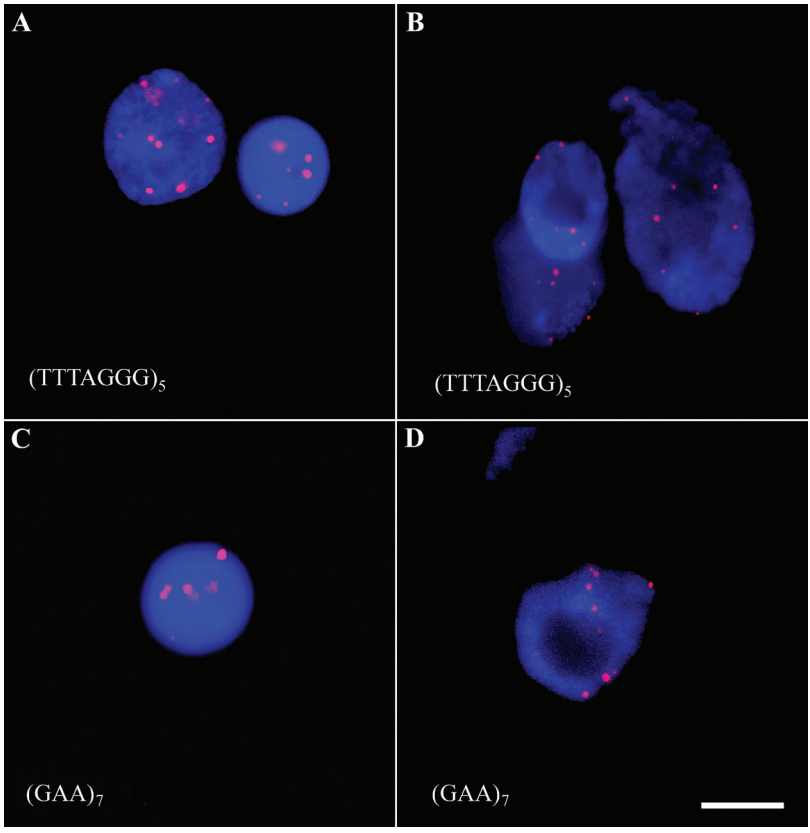

Figure 5. Fast screening of labelling probes was performed by FISHIS on cultivated cardoon (panel **A** and **C**) and globe artichoke (panel **B** and **D**) nuclei suspensions. The nuclei with the clearest and discrete telomeric $(TTTAGGG)_5$ and SSR $(GAA)_7$ signals are shown. All oligonucleotides were fluorescently labelled by Cy3 (red fluorescence). Nuclear DNA was counterstained with DAPI (blue fluorescence). Scale bar: 5 μm.

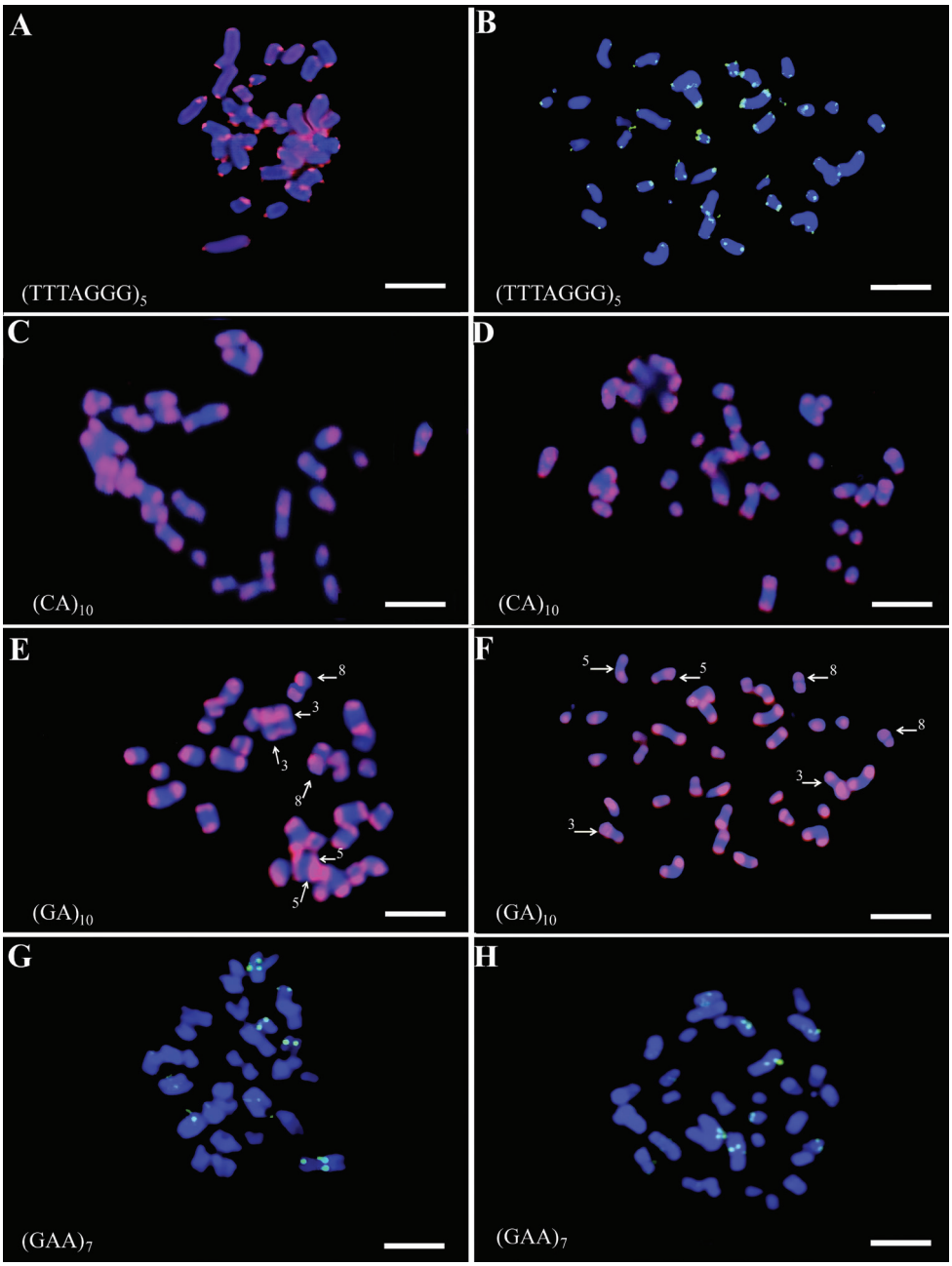

Figure 6. FISH molecular cytogenetic analysis with SSR probes. FISH on metaphase spreads of cardoon (left hand side) and of globe artichoke (right had side). The oligonucleotides sequence is indicated in each panel. Oligonucleotides were labelled with Cy3 (red fluorescence) or FITC (green fluorescence) fluorochromes. In (**E**) and (**F**) chromosomes 3, 5 and 8 of cardoon and globe artichoke, respectively, are indicated by arrows and can be discriminated by the widespread $(GA)_{10}$ hybridization pattern on the long arms. Scale bars: 5 μm.

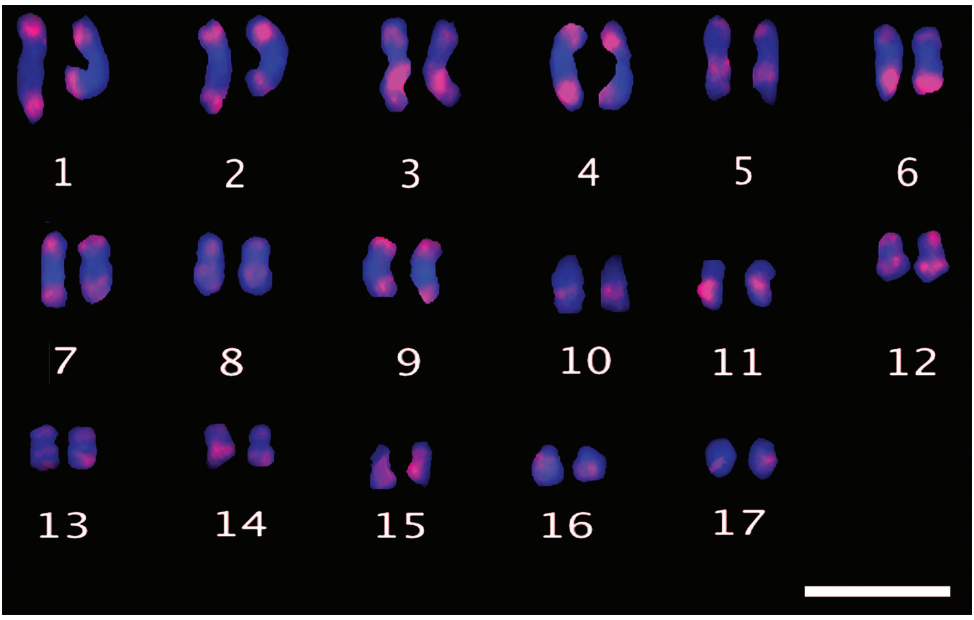

Figure 7. An example of globe artichoke pairing of homologous chromosomes based on $(GA)_{10}$ labelling pattern. Scale bar: 5 μm.

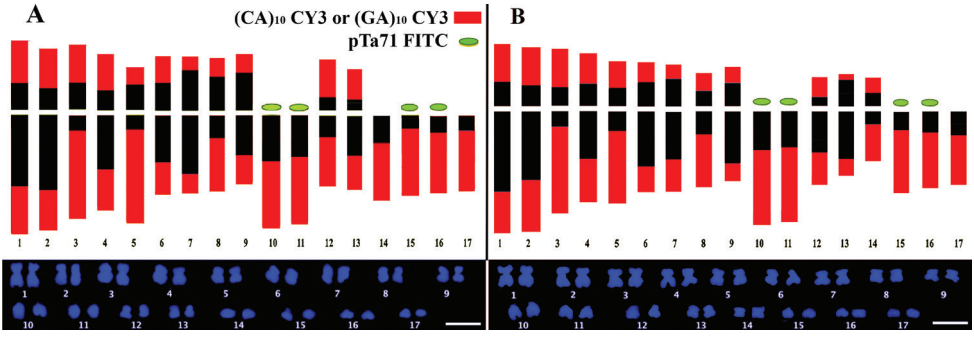

Figure 8. Ideogram with molecular characterization of cardoon and globe artichoke complement. Ideogram showing chromosome morphology (in black) and the $(CA)_{10}$, $(GA)_{10}$ di-nucleotides (red) and pTa71 sequence (green) distribution on cardoon (**A**) and globe artichoke (**B**) complement. The two di-nucleotides localize at similar chromosome regions. Scale bar: 5 μm.

signals were localized mainly at the telomeric and subtelomeric regions of both arms, with a slightly different band sizes, which helped in pairing homologous chromosomes (Figure 7). On chromosome pairs 3, 5 and 8, the extent of the hybridization signal was more widespread on the long arm (Figures 6E and 6F). Cardoon and globe artichoke acrocentric chromosomes can be discriminated from all other chromosomes by the distribution of the two SSR oligonucleotides along only on one side of the chromosome arms.

A less clear hybridization pattern was obtained using the $(GAA)_7$ probe which showed a variable and sometimes asymmetric distribution of the signals on sister chromatids, mainly on the large chromosome of globe artichoke and cardoon. For at least two large, one medium and one small chromosome pair a hybridization signal was visible in all the observed metaphases for both crops (Figures 6G and 6H), but further analyses are required.

Ideograms summarising chromosome morphology and molecular karyotyping with the $(CA)_{10}$/$(GA)_{10}$ and pTa71 DNA probes of the two crops are shown in Figure 8.

Discussion

In spite of the recent release of globe artichoke genome sequence (Scaglione et al 2016), the physical structure in which DNA is organized, that is the chromosomes, has been poorly investigated for cardoon and globe artichoke. The knowledge of chromosome organisation is important for studies of plant species evolution and is also relevant in plant breeding programmes. In the present paper, after the DNA content determination, we developed a detailed karyo-morphological analysis and a FISH-based molecular characterization of the chromosome complement of both crops

The genomes size measured in this study for leafy cardoon (2C = 2.20 pg) and globe artichoke (2C = 2.40 pg) are slightly different to those previously reported by Khaldi et al. (2014) and Marie and Brown (1993), respectively. Khaldi et al. (2014) reported a range of variability in DNA content, from 2C = 1.98 to 3.03 pg, in 10 populations of wild cardoon, and 2C = 2.05 and 2.10-2.11 pg for a single variety of globe artichoke and for two varieties of cultivated cardoon, respectively, while Marie and Brown (1993) stated a DNA content of 2C = 2.22 pg for globe artichoke. The differences between our estimates in DNA content and those of Marie and Brown (1993) and Khaldi et al. (2014) can be attributed to the DNA content estimation for the internal standard (*P. sativum*). Both works used 2C = 8.37 pg, while we employed 2C = 9.09 pg from Doležel et al. (1998). Possible intra-specific genome size variability was reported by Khaldi et al. (2014) which may also account for differences in DNA content estimates.

For cytogenetic studies in globe artichoke we have previously tested several microtubule assembling inhibitors, that is, 8-hydroxyquinoline, amiprophos-methyl, colchicine and a-bromonaphtalene, to increase the number of metaphases. 8-hydroxyquinoline was identified as the most effective inhibitor but even so, the mitotic index of *Cynara* remained as low as 10% (Giorgi et al. 2013b). Here a further slight increase in metaphase number was obtained using oryzalin.

To enhance metaphase spread quality, a drop spreading method recommended for plants with small chromosomes was used (Andras et al. 1999). This method produced good results in terms of cytoplasm removal and reduction of overlapping chromosomes, but it required a large number of root apexes to be processed. Considering that root tips are composed of cells at different cell cycle stages we observed high variation in the level of chromatin condensation among metaphases on the same slide, even

using antimitotic drugs. This heterogeneous chromosome condensation can make it difficult to clearly identify chromosome structures such as centromere position and secondary constrictions, and complicates chromosome length measurements. The morphological analysis and FISH signals of the chromosome complements of cultivated cardoon and globe artichoke revealed similar karyotypes, with analogous rDNA gene and SSR distribution in the two crops, supporting their close phylogenetic relationship. In fact, Fiori (1904) and Wiklund (1992) considered that both wild cardoon and the cultivated crops (cardoon and globe artichoke) belong to the same species (i.d. *C. cardunculus* L). Further studies indicated that wild cardoon is the common progenitor for both crops, which subsequently diverged in type of reproduction system and end use, probably following two distinct domestication events, separated in time and space (Sonnante et al. 2007b; Gatto et al. 2013). Our karyo-morphological analysis of globe artichoke and cardoon is largely different from the basic study reported by Falistocco (2016) in which all chromosomes of both crops were defined as metacentric and divided into three groups without showing single chromosome measurements. In the present study, a number of sub-metacentric and acrocentric chromosomes were identified and a clear difference was observed between the two crops in chromosome 14, which is acrocentric in cardoon and metacentric in artichoke. Such structural chromosome differences could indicate either the deletion of the small arm of the cardoon, or a translocation or insertion on chromosome 14 in artichoke, most likely during the process of domestication. The classification as acrocentric, of four chromosome pairs in cardoon and three chromosome pairs in globe artichoke was further supported by the distribution of the $(GA)_{10}$ and $(CA)_{10}$ SSR oligonucleotides along only one side of those chromosome arms, compared to the distribution on both arms of all the remaining chromosome pairs of the complement detected by FISH.

Chromosome characterization by FISH labelling was preceded by FISHIS on nuclei suspensions. This procedure was initially developed to label chromosomes in suspension for flow karyotyping and sorting, as an effective method to discriminate, purify and isolate specific plant chromosomes (Lucretti et al. 2014). Here we propose to use FISHIS on nuclei suspensions as a fast way for screening labelling probes. We found this method particularly effective for the selection of probes producing strong, discrete and localized hybridization signals, the kind usually most useful for identification of chromosomes; while it was less valuable for sequences more scattered and widespread.

The rDNA genes sites identified using traditional FISH analysis agrees, as number, with that reported in the recent work of Falistocco (2016), but appeared localized on acrocentric chromosomes in both crops. This ascertainment is consistent with Roa and Guerra (2012), who observed a very high frequency of rDNA sites on the short arms of acrocentric chromosomes in several genera.

The publication of the globe artichoke genome sequence (Scaglione et al. 2016) showed a high level of SSRs in artichoke DNA (41.73%) with di-nucleotides as the most frequent class (73%). Our FISH analysis with SSR oligonucleotides confirms the abundance of di-nucleotides, mainly $(AG)_{10}$ and $(AC)_{10}$ localized at telomeric and subtelomeric positions. These di-nucleotides co-localize in their FISH distribution and are probably organized in alternating tandem clusters, both in cultivated cardoon and globe artichoke. The $(GA)_{10}$

and $(CA)_{10}$ hybridization patterns enabled us to pair homologous chromosomes and to discriminate chromosomes 3, 5 and 8 which, compared to other chromosomes, had a wider distribution of SSRs on the long arms. The di-nucleotide localization at only one end of all acrocentric chromosomes enabled their identification in very condensed metaphases where the centromeric primary restriction is seldom visible. A *Cynara* specific centromeric probe would help in better defining chromosome arms in future studies.

Scaglione et al. (2016) also reported that AT di-nucleotides are quite abundant in the genome of globe artichoke, but it was not possible to detect any FISH signal with the $(AT)_{12}$ probe for us. This may be due to a low level of AT repeats in each cluster of tandem repeats and/or to a very scattered distribution of AT in the genome. The self-complementary nature of the di-nucleotide could also contribute to reducing the amount of available probe for hybridization to chromosome DNA.

Conclusion

Here we propose the karyo-morphological and molecular karyotype and the first ideogram of both cultivated cardoon and globe artichoke. Our results enable the identification of chromosomes pairs 3, 5 and 8 and the discrimination of acrocentric chromosomes in the complement of the two crop. Their karyotype revealed close affinity, but also chromosome structural variation among the two *C. cardunculus* varieties. Differences have been detected in the number of acrocentric chromosome, with cardoon showing an additional chromosome pairs, and also in the DNA content of the two varieties. The proposed karyotypes could help future anchoring of pseudomolecules from globe artichoke genome sequencing to chromosomes and contribute in locating important genes involved in the divergent evolution and domestication of *C. cardunculus*, for example, those associated with the development of different leaf structure and flower architecture.

Disclaimer

Andrea Gennaro is employed with the European Food Safety Authority (EFSA), the present paper is published under the sole responsibility of the authors. The positions and opinions presented in this paper are those of the author alone and are not intended to represent the views or scientific works of EFSA.

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