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New cytogenetic data for some Palaearctic species of scale insects (Homoptera, Coccinea) with karyosystematic notes

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Abstract

New cytogenetic data are reported for 17 species from 15 genera of the families Pseudococcidae, Eriococcidae, Kermesidae, and Coccidae. Twelve species and 6 genera (*Peliococcopsis* Borchsenius, 1948, *Heterococcopsis* Borchsenius, 1948, *Heliococcus* Šulc, 1912, *Trabutina* Marchal, 1904, *Lecanopsis* Targioni Tozzetti, 1868, and *Anapulvinaria* Borchsenius, 1952) were studied cytogenetically for the first time. The taxonomic problems in the genera *Trionymus* Berg, 1899, *Acanthopulvinaria* Borchsenius, 1952 and *Rhizopulvinaria* Borchsenius, 1952 are discussed based on karyotype characters. Two chromosomal forms (cryptic species) of *Acanthopulvinaria orientalis* (Nasonov, 1908), 2n=18 and 2n=16 were discovered.

Keywords

scale insects, karyotypes, chromosome numbers, karyosystematics

Introduction

In June 2009 the author and Dr. Mehmet Bora Kaydan made several joint collecting trips in Eastern Anatolia (Turkey). Part of the material collected during these trips, plus some other material collected by M.B. Kaydan without me in 2009, proved to be suitable for cytogenetic studies. Turkey in general and especially Eastern Anatolia have an exceptionally rich scale insect fauna (Kaydan and Kozár 2010, 2011, Scalenet: http://www.sel.barc.usda.gov/scalenet/scalenet.htm, accessed on 14 September, 2011) not only in terms of species and genera diversity (more then 300 species, 117 genera

have been recorded for Turkey), but also in terms of populations density. The latter fact is especially important for species, living on roots of wild plants. Most of these species, usually rarely collected in other Palaearctic regions as single adult females from one or two collecting points can be found in Eastern Anatolia comparatively easily as numerous females (and often with males and larvae of both sexes) in numerous localities. The high density of populations, in turn, is especially important for cytogenetic studies which often demand a high number of prepared insects. Hereby material collected in Eastern Anatolia provides a good possibility to clarify cytogenetic characteristics not only for newly studied species but also for some species that were insufficiently studied earlier.

Until now, Palaearctic scale insects were studied cytogenetically rather fragmentarily and significantly more poorly than tropical and subtropical species (Gavrilov 2007a). However, the available data (mainly from the author's previous papers) and comparison of these data with the new information reported here allow generation of some karyotaxonomic conclusions (see below).

The unique genetic systems of scale insects (*XX-X0, n-2n (Haplo-diploidy), Hermaphroditism, 2n-2n, Lecanoid, Comstockioid, Diaspidoid, obligate Thelytoky*) have been reviewed many times in special papers (Schrader 1923a, 1929a, Brown 1958-1969, Hughes-Schrader 1948, Nur 1962-1990, Haig 1993, Normark 2003, Gavrilov 2007a, Gavrilov and Kuznetsova 2007) and so will not be discussed here, except only for the following detail. Nur (1980), based on his own studies and literature data, noted that the Comstockioid genetic system differs from the Lecanoid system "…in the destruction or loss of from one to all the H chromosomes just prior to prophase I of spermatogenesis". This approach assumes that it is impossible to distinguish the Lecanoid and the Comstockioid systems without analysis of spermatogenesis. In practice the collecting of third-instar larvae of males (stage of spermatogenetic divisions) or even males themselves is a very rare event for most scale insect species. Even if these larvae are collected it is often rather difficult to prepare good slides of male meiotic chromosomes because of difficulties with the methods of squashing testis tissue. On the other hand, based on my long term work with scale insect chromosomes, it seems that the Comstockioid genetic system is visually different in easily studied male embryonic cells: heterochomatic elements usually do not form compact singular heterochomatic body in interphase nuclei (Fig. 21) (in contrast to the Lecanoid system) and at least some cells have fewer heterochomatic chromosomes than the haploid number (as, for example, on Fig. 19). According to this indirect evidence it may be possible to note Lecanoid or Comstockioid heterochromatinization for newly studied species and genera of the higher taxa for which Lecanoid or Comstockioid systems were previously detected by studies of spermatogenesis. In the present paper this admission was made for species of the families Pseudococcidae, Eriococcidae and Kermesidae.

Some scale insects (in particular, some of those listed below) are characterized by a unique individual development that is similar to a double fertilization in angiosperms. In this case each embryo develops from two different cells. One of those is a normal diploid zygote that gives rise to the majority of tissues. The other cell is a polyploid secondary

zygote that results from the fusion of a cleavage nucleus with the first or second polar bodies. The secondary zygote gives rise to the polyploid bacteriome (or mycetome). Each cell of the bacteriome (or mycetome) thus includes one haploid set of paternal chromosomes and several maternal sets (Schrader 1923b, Hughes-Schrader 1948, Brown 1965, Normark 2001, Gavrilov 2007a). This phenomenon has been studied mainly in Diaspididae and Pseudococcidae, which can display 5-ploid, 7-ploid or even 14-ploid bacteriomes (Brown 1965, Normark 2004). It is not known whether other coccid families also have "dizigotic soma" or other mechanisms of bacteriome-formation similar to some soft scales (Tremblay 1961) or to the genus *Puto* (Pseudococcidae s.l.) (Brown and Cleveland 1968).

Material and methods

All material for this study was collected in 2009 in Eastern Anatolia (Turkey). The detailed collecting data are listed below, separately for each species in order to avoid the double citations of taxonomic names and for more comfortable using of the paper.

The chromosomal plates were made as previously described (Gavrilov and Trapeznikova 2007, 2008).

All material is deposited at the Zoological Institute, Russian Academy of Sciences, St. Petersburg.

Results and discussion

Family Pseudococcidae

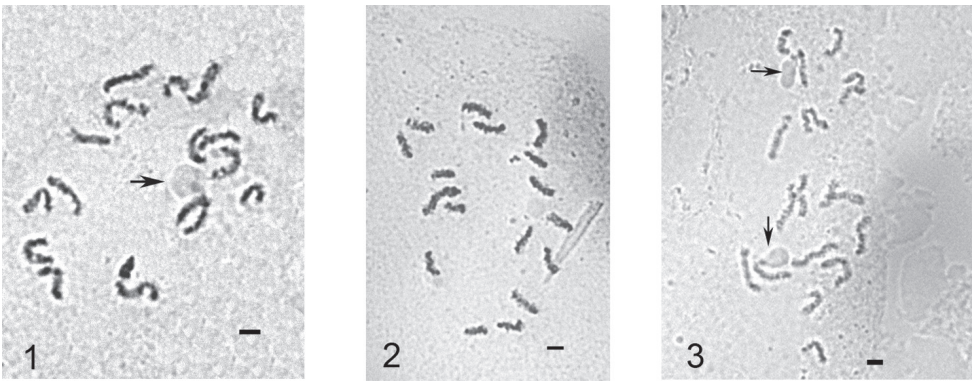
Puto superbus (Leonardi, 1907)
Figs 1–3

Material. K 607, Igdir-Digor road, 40°10'451"N, 43°40'389"E, on steams of grass, 04.06.2009, M.B. Kaydan & I. Gavrilov.

Embryos from female body. 2n=16 +XX (♀), 2n= 16 +X0 (♂).

Hitherto, only American species of the genus *Puto* Signoret, 1876 were studied cytogenetically (Hughes-Schrader 1944, Brown and Clevelend 1966). *P. superbus* is the first studied species of the genus from the Palaearctic fauna; it also has an ancient XX/X0 genetic system (as 5 other studied species of the genus), but demonstrates a different chromosome number (2n=18/17) in contrast to 2n=14/13, 16/15, 20/19 in American species.

Figures 1 & 3 illustrate nucleoli localized at the ends of the middle-sized chromosomes. The localizations of NORs in scale insects were discussed earlier by Gavrilov 2005, Gavrilov and Trapeznikova, 2007 based their own and literature data. *P. superbus* shows the new pattern of this localization in contrast to the position of NORs on longest or shortest chromosomes in other studied coccid species.



Figures 1–3. Mitotic chromosomes of *Puto superbus*. **1** cell of female embryo, 2n=18 **2, 3** cells of male embryo, 2n=17. The chromosomes with NORs are arrowed. Bar = 10 μm.

Phenacoccus Cockerell, 1893

Hitherto, 16 species of the large and widely distributed genus *Phenacoccus* have been studied by different authors (see the review of Gavrilov 2007a and Gavrilov and Trapeznikova 2007, 2010). Most of studied species demonstrate the modal chromosomal number 2n=10. Here I am adding the data on 3 species, which have not been studied before.

Sharing the same chromosomal number *Phenacoccus* spp. demonstrate, however, significant variation of chromosomal lengths in their karyotypes. This variation in combination with the data on differential staining of *Phenacoccus* spp. chromosomes will probably provide the basis for further karyotaxonomic studies of the genus.

Phenacoccus specificus Matesova, 1960

Fig. 4

Material. K 603 (4472), Kars - Kagizman road, 40°16'351" N/42°52'275" E, 1761 m alt., on roots of *Thymus* sp., 04.06.2009, M.B. Kaydan & I. Gavrilov.

Embryos from female body. 2n=10.

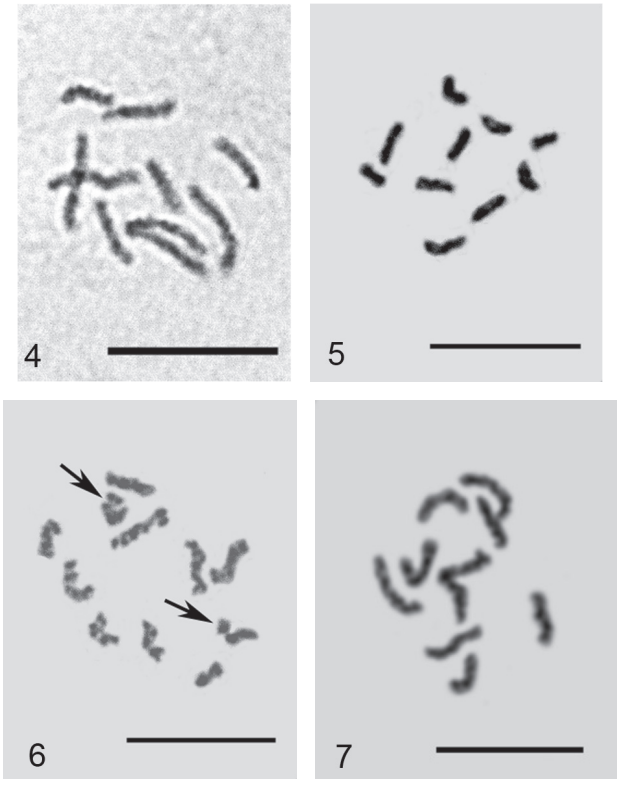
Phenacoccus phenacoccoides (Kiritshenko, 1932)

Figs 5–6

Material. K 612 (4483), Kars-Kagizman road, 40°12'011"N, 43°02'827"E, 1273 m alt., under the leaf sheaths of grass, 04.06.2009, M.B. Kaydan & I. Gavrilov.

Embryos from female body. 2n=10, 2n=10 + Bs, Lecanoid heterochromatinization.

The studied population of *Ph. phenacoccoides* demonstrates variation from 0 to 2 additional chromosomal elements (B-chromosomes) between embryonic cells like as seen in a population from the Voronezh region (central part of European Russia) studied earlier by me (Gavrilov 2004).



Figures 4–7. Embryonic cells of *Phenacoccus* spp. **4** *Ph. specificus*, 2n=10 **5, 6** *Ph. phenacoccoides*: **5** 2n=10, **6** 2n=10+Bs, additional chromosomal elements arrowed **7** *Ph. tergrigorianae*, 2n=10. Bar = 10 μm.

***Phenacoccus tergrigorianae* Borchsenius, 1956**
Fig. 7

Material. K 693, Van-Hakkari road, N37°32'340", E43°43'173", on roots of undetermined Asteraceae, 02.09.2009, M.B. Kaydan. K 689, the same data, but on *Sorghum halepense*.

2n=10, Lecanoid heterochromatinization.

***Peliococcopsis priesneri* (Laing, 1936)**
Figs 8–9

Material. K 601, Agri-Dogubeyazid-Ishakpasa, 39°31'905"N, 44°07'100"E, 2059 m alt., under the leaf sheaths of *Cynodon dactylon*, 03.06.2009, M.B. Kaydan & I. Gavrilov.

Embryos from female body. 2n=10, Lecanoid heterochromatinization.

It is the first species of the genus *Peliococcopsis* Borchsenius, 1948 studied cytogenetically.

Heterococcopsis opertus **Borchsenius, 1949**
Fig. 10

Material. 4530, Eastern Anatolia without concrete location, 2009, M.B. Kaydan.
Embryos from female body. 2n=10.
It is the first species of the genus *Heterococcopsis* Borchsenius, 1948 studied cytogenetically.

Heliococcus sulci **Goux, 1934**
Fig. 11

Material. K 677, Hatay-Erzin, 08.09.2009, M.B. Kaydan.
Only one female was available for cytogenetic studies and the specimen did not provide cells with chromosome plates suitable for karyotype study. However, some polyploid cells of the mycetome with about 140 chromosomes and numerous agglutinations were observed. In view of the absence till now of any cytogenetic data on the large and very important for phylogenetic reconstructions genus *Heliococcus* Šulc, 1912 I am presenting here the first photograph of *Heliococcus* chromosomes (Fig. 11). It appears that there is no significant size difference between chromosomes.

Trabutina crassispinosa **Borchsenius, 1941**
Fig. 12

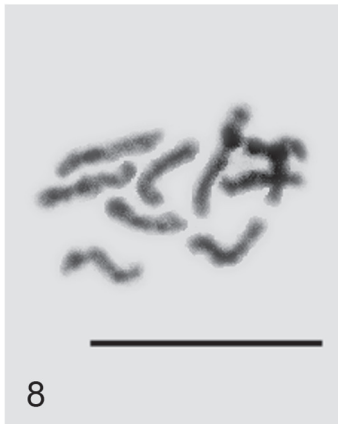
Material. K 605, Igdir-Digor road, Kars border, 40°07'278"N, 43°37'708"E, on branch of *Tamarix* sp., 04.06.2009, M.B. Kaydan & I. Gavrilov.
Embryos from the ovisacs. 2n=16.
It is the first species of the genus *Trabutina* Marchal, 1904 studied cytogenetically.

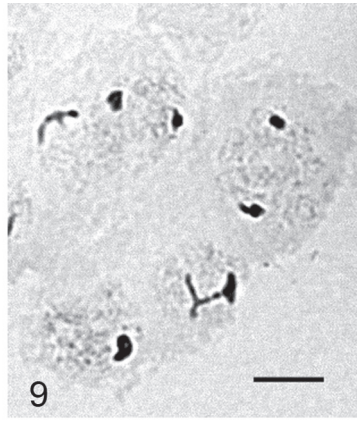
Planococcus vovae **(Nasonov, 1908)**
Figs 13–14

Material. K 686, Şanliurfa, on *Cupressus* sp., 09.09.2009, M.B. Kaydan.
2n=10, Lecanoid heterochromatinization. The studied population deviates morphologically from the usual *P. vovae* having 2 circuli in contrast to 1 (or, exceptionally, none) in huge material from different regions of the Palaearctic (Danzig and Gavrilov 2010). However, the karyotype characters seem to be the same as in a previously studied population from the Mediterranean coast of Turkey (Adana) (Gavrilov 2007 and unpublished) that included females with only 1 circulus.

Dysmicoccus multivorus **(Kiritshenko, 1936)**
Figs 15–16

Material. K 685, Van-Akdamar, on undetermined Apiaceae, 09.06.2009, M.B. Kaydan & I. Gavrilov.





Figures 8–9. *Peliococcopsis priesneri*. **8** mitotic chromosomes, 2n=10 **9** male embryonic cells at interphase stage with one haploid set heterochomatinized. Bar = 10 μm.

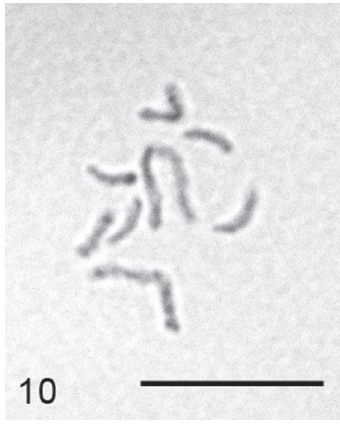


Figure 10. *Heterococcopsis opertus*, embryonic cell, 2n=10. Bar = 10 μm.

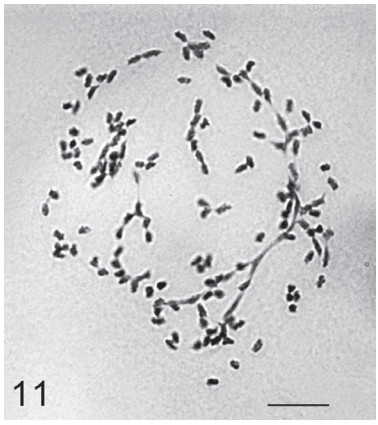
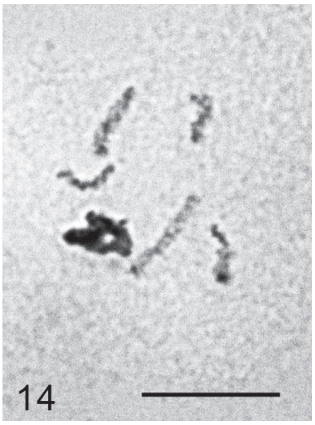
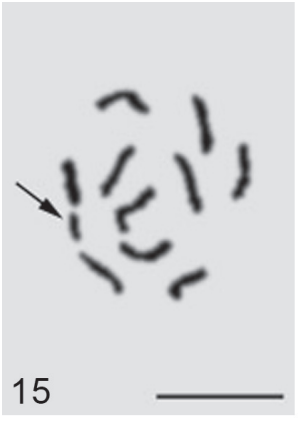


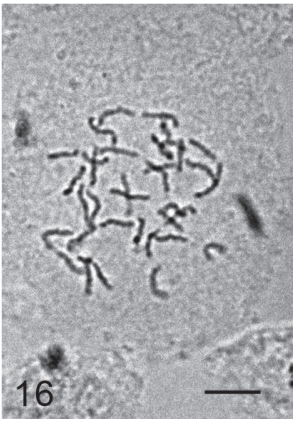
Figure 11. *Heliococcus sulci*, the cell of mycetome, about 140 chromosomes with numerous agglutinations. Bar = 10 μm

Figure 12. *Trabutina crassispinosa*, cell of embryo, 2n=16. Bar = 10 μm.



Figures 13–14. *Planococcus vovae* **13** mitotic chromosomes, 2n=10 **14** male embryonic cell with one haploid set heterochomatinized. Bar = 10 μm.





Figures 15–16. *Dysmicoccus multivorus*. **15** embryonic cell with 2n=10 + B, additional chromosomal element arrowed **16** cell of mycetome, 7x=35. Bar = 10 μm

2n=10+1-2 B. All previously studied populations of this species (Gavrilov and Trapeznikova, 2007) from the central part of European Russia and Crimea (Ukraine) showed a stable chromosomal number 2n=10. Turkish material shows 1 or 2 additional (B) chromosomes. Mycetocytes with 35 (7x) chromosomes.

***Trionymus artemisiarum* (Borchsenius, 1949)**
Fig. 17

Material. K 680 (4536), Ağri-Patnos-Adilcevaz road-Aktepe, on *Achillea* sp., 10.06.2009, M.B. Kaydan.

Embryos from female body. However, oviposition takes place during earlier stages of embryonic development, before gastrulation.

2n=10, Lecanoid heterochromatinization.

The species demonstrates another new example of karyotaxonomic rule discovered by the author (Gavrilov 2005, 2007a, Gavrilov and Trapeznikova 2007, 2010) in the genus *Trionymus* Berg, 1899. All species that significantly deviate from morpho-ecological diagnosis of the genus, have chromosome numbers different from the type species, *T. perrisii* (Signoret, 1875); the last one as well as morphologically similar with it *T. aberrans* Goux, 1938 and *T. haancheni* (McKenzie, 1960) have 2n=16. Morphologically deviating *T. multivorus* (Kiritshenko, 1936) and *T. radicum* (Newstead, 1895), both with 2n=10, are considered by me now in the genera *Dysmicoccus* Ferris, 1950 and *Balanococcus* Williams, 1962 correspondingly (Gavrilov 2005, 2007a, Gavrilov and Trapeznikova 2007, 2010). *T. artemisiarum* studied here for the first time also have deviating chromosomes number (2n=10) and deviating classic taxonomic characters. In contrast to other *Trionymus* spp., *T. artemisiarum* has a broadly oval body (not elongate body with parallel margins) and lives on roots of dicotyledonous herbs (not under the leaf sheaths of Poaceae).

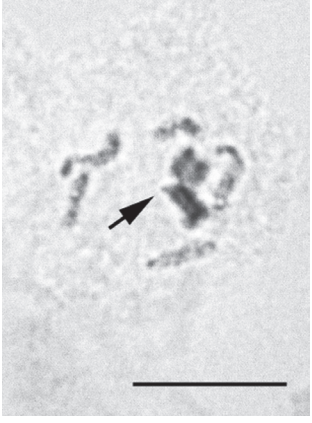


Figure 17. *Trionymus artemisiarum*, 2n=10, cell of male embryo, one haploid set (arrowed) is heterochromatinized. Bar = 10 μm.

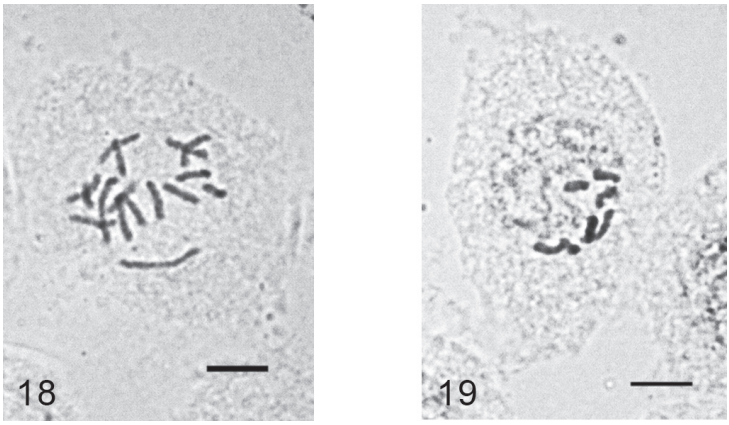
Family Eriococcidae

***Acanthococcus lactucae* Borchsenius, 1949**
Figs 18–19

Material. K 675 (4555), Hakkari-Çukurca road, on *Cichorium* sp., 01.09.2009, M.B. Kaydan.

Embryos from female body.

2n=16, heterochromatinization, presumably Comstockioid. The same characteristics have been previously detected in two other species of this genus (Gavrilov 2004, 2007a).



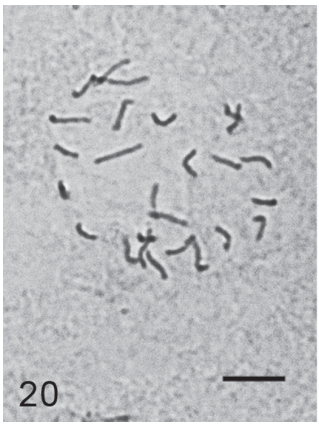
Figures 18–19. *Acanthococcus lactucae*. **18** mitotic chromosomes in female embryo, 2n=10. **19** - male embryonic cell in interphase with one haploid set heterochomatinized. Bar = 10 μm.

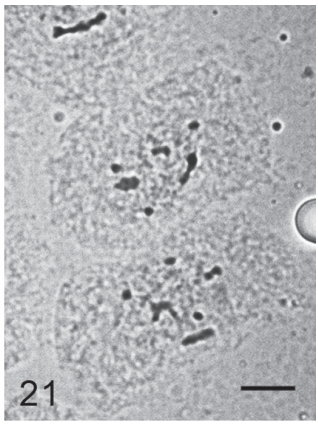
Family Kermesidae

***Kermes roboris* (Fourcroy, 1785)**
Figs 20–21

Material. K 636, Tatvan-Güroymak road, 38°33'187"N, 42°05'851"E, 1570 m alt., on twigs of *Quercus* sp., 10.06. 2009, I. Gavrilov.

Embryos from female body. 2n=26, heterochromatinization, presumably of the Comstockioid type. It is the second studied species of the genus *Kermes* Linnaeus, 1758 and of the whole family Kermesidae. The first studied species, *K. quercus* (Linnaeus, 1758), also has 2n=26 and presumable Comstockioid heterochromatinization (Gavrilov 2004, 2007a)





Figures 20–21. *Kermes roboris*. **20** mitotic chromosomes in female embryo, 2n=26 **21** male embryonic cell in interphase with one haploid set heterochomatinized. Bar = 10 μm.

Family Coccidae

***Lecanopsis turcica* (Bodenheimer, 1951)**
Figs 22

Material. K 592, Dogubeyazit – Igdir road, 39°46'51"N, 44°08'584"E, 1552 m alt., on roots of undetermined Poaceae, 03.06.09, I. Gavrilov.

Embryos from female body. 2n=18, heterochomatinization of an unidentified type. It is the first species of comparatively large Palaearctic genus *Lecanopsis* Targioni Tozzetti, 1868 studied cytogenetically.

***Acanthopulvinaria orientalis* (Nasonov, 1908)**
Figs 23–24

Material. K 631 (4502), Van Gurpinar road, on steams of *Artemisia* sp., M.B. Kaydan & I. Gavrilov.

Embryos from ovisacs. 2n=16, heterochromatinization of an unidentified type.

These new data revealed that *A. orientalis*, earlier studied from Astrakhan only (Russian coast of the Caspian Sea) (Gavrilov 2007b, Gavrilov and Trapeznikova 2008), hides a minimum 2 chromosomal forms (cryptic species) with 2n=16 and 2n=18 (Figs 23-24). Moreover, 16-chromosome form (present study) demonstrates a pair of extra-large chromosomes that probably originated (in phylogenetic meaning) from a fusion between two chromosome pairs in 18-chromosomal karyotype. It seems that the new chromosomal number does not concern to *A. discoidalis* (Hall, 1923), recently placed under synonymy of *A. orientalis* (Gavrilov 2007b). *A. discoidalis* has never been noted anywhere outside of Egypt and has not clear morphological differences from *A. orientalis*. The two populations studied by me cytogenetically (Russian and Turkish) also have

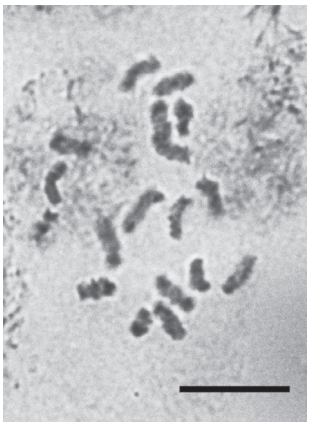
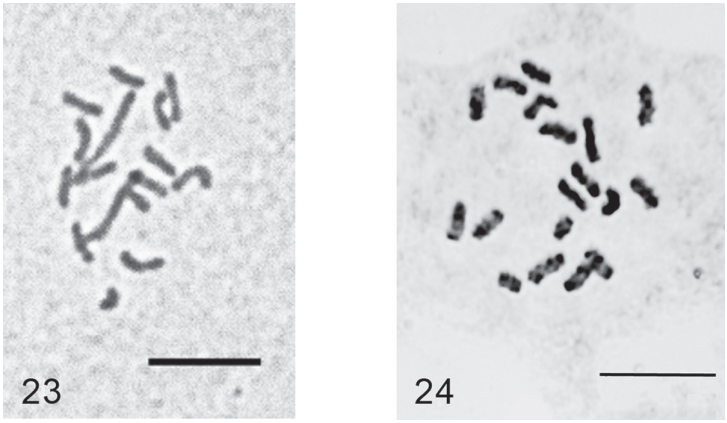


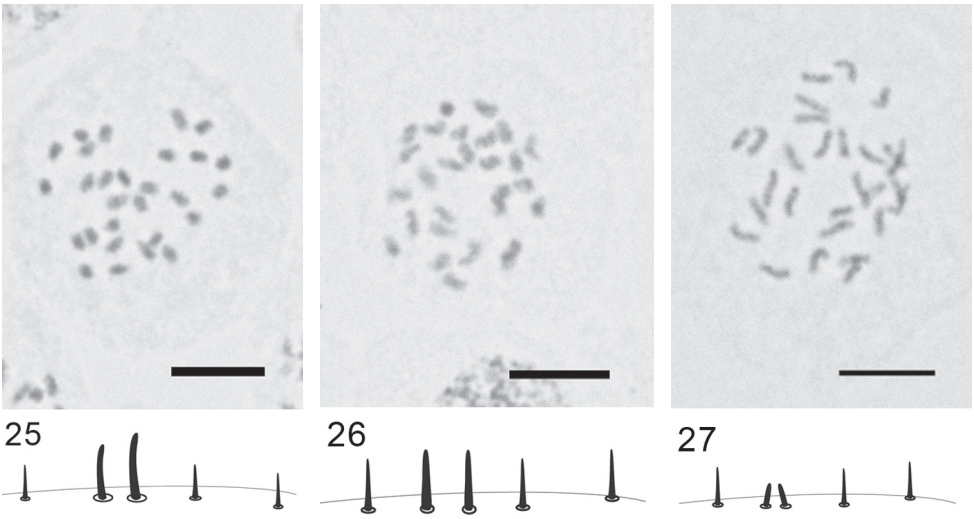
Figure 22. *Lecanopsis turcica*, cell of female embryo, 2n=18. Bar = 10 μm.



Figures 23–24. *Acanthopulvinaria orientalis*, cells of female embryo **23** Turkey, 2n=16 **24** Astrakhan (Russia), 2n=18, after Gavrilov 2007a. Bar = 10 μm.

not structural morphological differences lying outside the usual variability of *A. orientalis*. However, Astrakhan females (2n=18) are smaller (about 3 mm long) than the Turkish specimens (2n=16 and about 4 mm long) and both are significantly larger than noted in the original description of Hall (1923) – 1.25–1.5 mm long. It is interesting that in a similar situation with two cryptic species, *Pulvinaria ribesiae* Signoret, 1873 (2n=18) and *P. vitis* Linnaeus, 1758 (2n=16), the first one, having higher chromosomal number, is also smaller-sized (Drozdovskiy 1966, Gavrilov and Trapeznikova 2008).

Since 2n=16 and 2n=18 chromosomal sets obviously cannot produce fertile hybrid progeny due to meiotic abnormalities they should be treated as two separate species. However, for a final taxonomic decision it is necessary to study more populations from different parts of *A. orientalis* geographic area.



Figures 25–27. *Rhizopulvinaria artemisiae,* 2n=28, embryonic cells and marginal setae **25** population K 595 **26** population K 598 **27** population K 610. Bar = 10 μm

***Rhizopulvinaria artemisiae* (Signoret, 1873)**
Figs 25–27

Material. K 595 (4462), Çaldiran-Dogubeyazit road, 39°11'71"N, 44°00'784"E, on *Acantholimon* sp., 03.06.09, M.B. Kaydan & I. Gavrilov. K 598 (4467), Agri-Dogubeyazit-Ishakpaşa Palace, 39°31'905"N, 44°07'100"E, 2059 m alt., on *Artemisia* sp., 03.06.09, M.B. Kaydan & I. Gavrilov. K 610 (4481), Kars-Kagizman road, 40°12'011"N, 43°02'827"E, 1273 m alt., on *Artemisia* sp., 04.06.09, M.B. Kaydan & I. Gavrilov.

Embryos from female body. 2n=28, male embryos with heterochromatinization are absent.

Three Turkish populations studied here show the same karyotype with 28 approximately equal in size chromosomes as in a previously studied population from Astrakhan (Gavrilov 2007a, 2009, Gavrilov and Trapeznikova 2008). These new data confirm the author's conception of polytypic variable species *R. arthemisiae* sensu lato (Gavrilov 2009) and the synonymization of numerous nominal species (=forms), described by different authors without any clear differential characters (see the references in the revision of Gavrilov 2009). The studied Turkish populations fortunately show most usual and representative examples of morphological variation of marginal and stigmatic conical setae in *R. arthemisiae* s. l. (Figs 25-27) and none the less the karyotype stability, that seems especially important as an additional taxonomic character in view of significant variability of chromosomal number in the Pulvinariini in general.

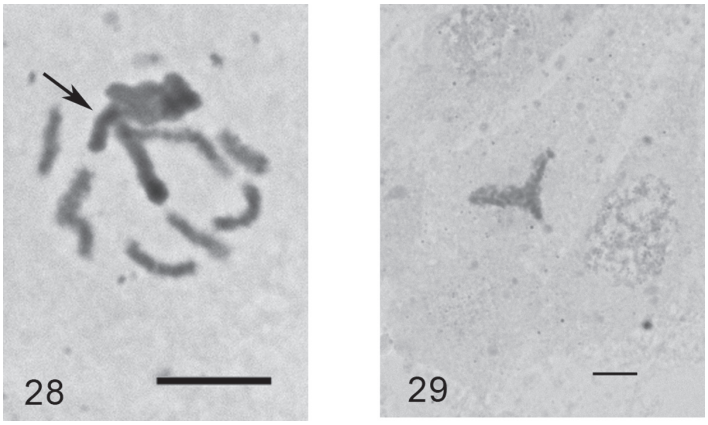
***Anapulvinaria pistaciae* (Bodenheimer, 1926)**
Figs 28–29

Material. K 647, Bitlis, ruins of ancient fortress, on twig of *Pistacia* sp., 10.06.2009, M.B. Kaydan & I. Gavrilov.

Eggs from female body. The laid eggs are of two colors: white and brown.

2n=16?, heterochromatinization.

The monotypic genus *Anapulvinaria* Borchsenius, 1952 was studied here cytogenetically for the first time. Unfortunately, the only female with ovisac was collected and analyzed; the embryos (more than 100 were squashed) demonstrated numerous tripolar mitotic divisions. According to this abnormality and also due to a small number of chromosomal plates suitable for karyotype analysis (2 cells of female embryo and 3 cells of male embryo in total) I am giving the chromosomal number with small doubt. Some embryos contained polyploid cells with about 50 chromosomes.



Figures 28–29. *Anapulvinaria pistaciae,* embryonic cells **28** cell of male embryo with one haploid set heterochromatinized (arrowed) and 8 euchomatic chromosomes **29** tripolar mitosis. Bar = 10 μm.

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SHORT COMMUNICATION

COMPARATIVE Cytogenetics
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International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

A cytogenetical study on *Economidichthys pygmaeus* Holly, 1929 (Pisces, Gobiidae), an endemic freshwater goby from Western Greece

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In memory of Angelo Libertini, 1957–2010

Abstract

A cytogenetic study was carried out on the chromosomes and the nuclear DNA content of the freshwater goby *Economidichthys pygmaeus* (Pisces, Gobiidae). The species is characterized by a 2n=46 karyotype consisting of 12 submetacentric and 11 subtelocentric chromosome pairs (NF=70). Major (45S) rDNA genes are terminal-centromeric located on the short arm of a single medium-small sized submetacentric pair as assessed by *in situ* hybridization, CMA_3 staining, and Ag-NOR banding. The haploid (C-value) nuclear DNA content is 0.93±0.003 picograms. The cytogenetical data of *E. pygmaeus* were compared with those ones already available for other related gobies.

Keywords

Goby fish, karyotype, fluorescent *in situ* hybridization, genome size

Introduction

Economidichthys pygmaeus is an endemic small-bodied, short-lived species restricted to the north and western part of Greece (Miller 1990). Specifically, it is distributed in the Thiamis, Louros, Arahcthos and Achelloos Rivers and in Lakes Trichonis, Lyssimachia, Ziros and Ozeros. Recently it has been recorded in Lake Pamvotis where it was introduced probably from River Louros and/or Kalamas (Thyamis) (Leonardos et al. 2008). The species appears to be extinct on Lefkas Island (Economidis 1991) and is protected under Greek law No. 67/1981. Greece's updated edition of the Red Book of Endangered Species (2009) has evaluated its conservation status and it is considered now a 'least concerned species', without facing any critical dangers (Crivelli 2006, Economidis 2009). Despite the importance of this species in terms of conservation, information on many aspects of its biology, ecology and behaviour are lacking in the current literature, and no data on karyotype are available at present (Miller 1990). In the aim of promoting the conservation of this species a set of investigations was carried out in order to better understand its biology and life-history. In such research frame the present cytogenetical study is included.

Materials and methods

Ten adult males and five females of *E. pygmaeus* were collected from Lake Pamvotis (Ioannina, NW Greece) and used for this study. Animals were injected with colchicine and were killed with an overdose of MS222. After that, the fish were sacrificed and chromosome preparations were obtained from spleen and testis by using the conventional air-drying technique. Chromosome plates were conventionally stained with Giemsa and eventually re-stained with silver nitrate (Howell and Black 1980) to get an Ag-NOR banding. Chromosome classification follows Levan et al. (1964). Mapping of rDNA major complex genes were performed by fluorescent *in situ* hybridization (FISH) with the pDm238 probe (Roiha et al. 1981) containing the 18S-5.8S-28S gene cluster and intergenic spacers of the fruit fly *Drosophila melanogaster* Meigen, 1830, according to the procedure in Libertini et al. (2008). Plates previously analysed by FISH were sequentially stained with chromomycin A_3 (CMA_3) (Schweizer 1976). Genome size (GS) was assessed by flow cytometry on peripheral erythrocytes, according to the method in Libertini et al. (2003).

Results

The haploid n=23 and the diploid 2n=46 chromosome numbers were determined for *E. pygmaeus* (Fig. 1 and Fig. 2) from the counts of 50 and 94 plates, respectively. All the analysed specimens, regardless of sex, shared the same 2n=46 karyotype (Fig. 2), composed of 24 submetacentric (Fig. 2, pairs 1-12) and 22 subtelocentric (Fig.

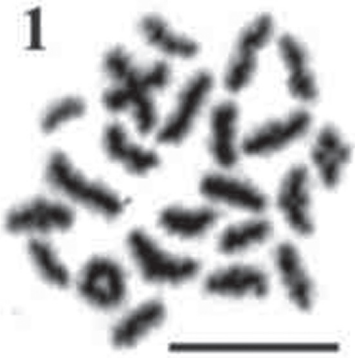

Figure 1. *E. pygmaeus* first spermatocyte metaphase. Bar = 10μm

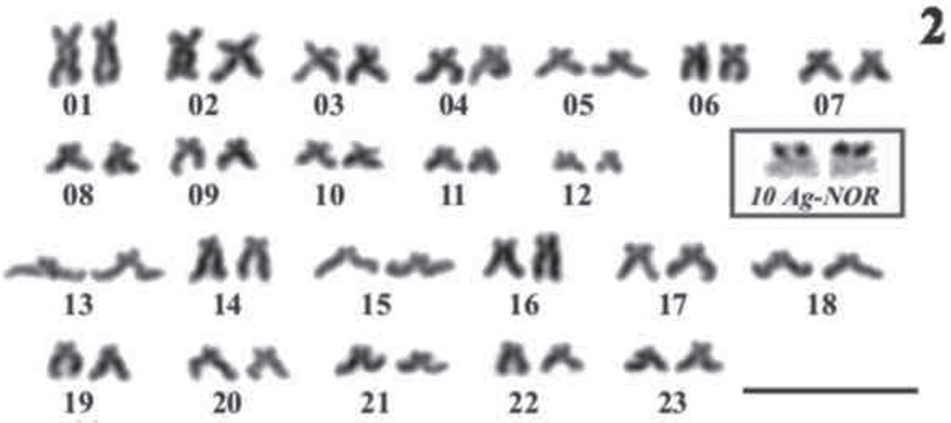

Figure 2. *E. pygmaeus* karyotype. Pairs 1–12 submetacentrics; pairs 13-23 subtelocentrcs. Inset Ag-NOR staining of pair 10. Bar = 10μm.

2, pairs 13-23) chromosomes. Therefore, the fundamental number of chromosome arms (NF) is 70. Ag-NOR banding (Howell and Black 1980) showed a terminal-centromeric location (following the scheme in Caputo 1998) of the active nucleolar organiser regions (Fig. 2, blue inset) on the short arm in a medium-small sized submetacentric pair (Fig. 1, pair 10). FISH results from first spermatocyte metaphase bivalents (Fig. 3A) and mitotic chromosomes (Fig. 4A) confirmed the previous observations with Ag-NOR banding. In fact, a single bivalent showed hybridization signals with the NORs probe on both ends (Fig. 3A, arrows) in the spermatocyte plates, while in mitotic plates a couple of chromosomes showed rDNA major complex FISH signals on the short arm (Fig. 4A, arrows). CMA_3 produced overlapping bright signals in the same location of hybridization signals (Figs 3B and 4B, see arrows), indicating that rDNA major complex gene sequences contain GC-rich DNA. Through flow cytometric essay the GS (haploid C-value) of *E. pygmaeus* was evaluated as 0.93±0.003 picograms.

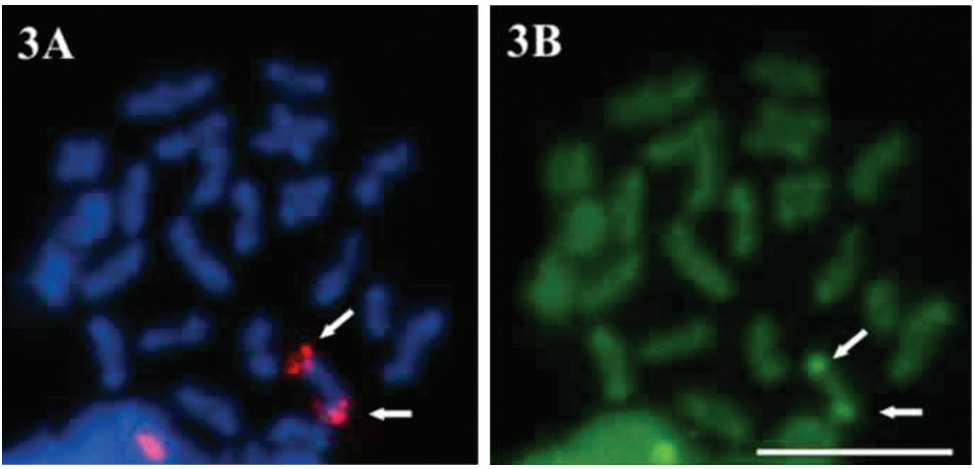

Figure 3. A–B *E. pygmaeus* first spermatocyte metaphase **A** FISH with a rDNA major complex probe **B** Sequential staining with CMA_3.

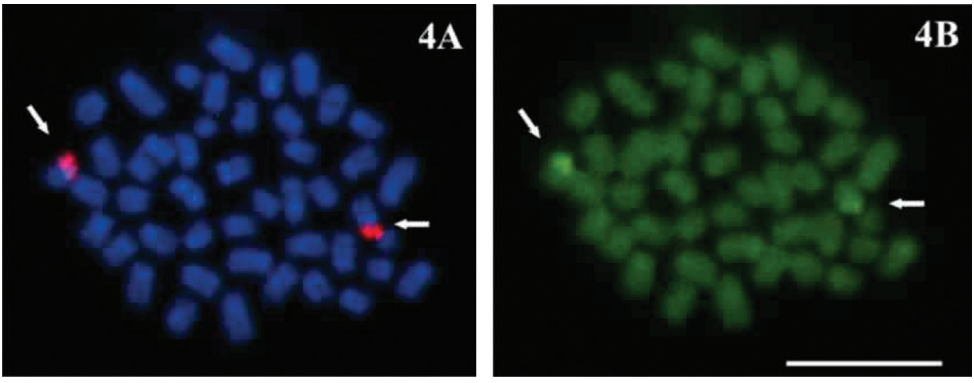

Figure 4. A–B *E. pygmaeus* mitotic metaphase **A** FISH with rDNA major complex probe **B** Sequential staining with CMA_3. Bar = 10μm.

Discussion

About one hundred species of Gobiidae have been analysed cytogenetically and show great karyotypic diversity. Diploid number varies from 30 to 62 and variation in chromosome morphology is also wide (NF=40-98). High intraspecific chromosomal variability has been detected in some species (Galetti et al. 2000, and reference therein). In the comparison with the other gobies *E. pygmaeus* is characterized by the most common chromosome number (2n=46) and by an unusual karyotype composed exclusively by submetacentric and subtelocentric elements. The resulting NF=70 is intermediate among NF values of Gobiidae (Klinkhardt et al. 1995, and reference therein). The GS of *E. pygmaeus* (0.93 pg) is also intermediate among the GS values of the Gobiidae (range 0.42-1.68 pg) (Animal Genome Size Database 2009). A single pair of medium-small

chromosomes bearing a GC-rich NOR on a terminal-centromeric zone in the short arm is the most common location of major rDNA genes in Gobiidae, being shared by the genera *Gobius* Linnaeus, 1758, *Pomatoschistus* Gill, 1863, *Knipowitschia* Iljin, 1927 and *Economidichthys* Bianco, Bullock, Miller et Roubal, 1987 (Caputo 1998; present paper; A. Libertini unpublished data), and this location represents probably the plesiomorphic character state (Caputo 1998). The genus *Economidichthys* along with the genera *Pomatoschistus*, *Gobiusculus* Duncker, 1928, and *Knipowitschia* were gathered in the so-called sand goby group (McKay and Miller 1997, Huyse et al. 2004). The sand gobies were clustered as a monophyletic group on morphological (McKay and Miller 1997), molecular (Huyse et al. 2004) and behavioural (Malavasi et al. 2008) grounds. Monophyly of sand gobies is also suggested by the sharing of common, probably plesiomorphic, cytogenetical characters: the chromosome number 2n=46 is present in all the four genera (Klinkhardt et al. 1995, Animal Genome Size Database 2009, present paper), the common NOR location in a terminal-centromeric zone in the short arm in a single submetacentric pair for the genera *Pomatoschistus*, *Knipowitschia* and *Economidichthys* so far studied (Caputo 1998, present paper, Libertini unpublished data), and similar GS values in a narrow range around 1 pg (0.91–1.04 pg) (Animal Genome Size Database 2009, present paper). The wide variability of karyotype formula and NF vs a general constancy of chromosome numbers indicate that non-Robertsonian mechanisms of chromosome rearrangements were more frequently involved in karyotype evolution of the sand gobies.

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RESEARCH ARTICLE

COMPARATIVE Cytogenetics
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International Journal of Plant & Animal Cytogenetic, Karyosystematics, and Molecular Systematics

Cytogenetic characterization of four species of the genus *Hypostomus* Lacépède, 1803 (Siluriformes, Loricariidae) with comments on its chromosomal diversity

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Abstract

Cytogenetic analyses were performed on fishes of the genus *Hypostomus* (*H. ancistroides* (Ihering, 1911), *H. strigaticeps* (Regan, 1908), *H. regani* (Ihering, 1905), and *H. paulinus* (Ihering, 1905)) from the seven tributaries of the Paranapanema River Basin (Brazil) by means of different staining techniques (C-, Ag-, CMA_3- and DAPI-banding) and fluorescence in situ hybridization (FISH) to detect 18S rDNA sites. All species showed different diploid numbers: 2n=68 (10m+26sm+32st-a) in *H. ancistroides*, 2n=72 (10m+16sm+46st-a) in *H. strigaticeps*, 2n=72 (10m+18sm+44st-a) in *H. regani* and 2n=76 (6m+16sm+54st-a) in *H. paulinus*. Ag-staining and FISH revealed various numbers and locations of NORs in the group. NORs were usually located terminally on the subtelocentric/acrocentric chromosomes: on the long arm in *H. strigaticeps* (2 to 4) and *H. paulinus* (2); and on the short arm in *H. ancistroides* (2 to 8) and *H. regani* (2 to 4). Conspicuous differences in heterochromatin distribution and composition were found among the species, terminally located in some st-a chromosomes in *H. ancistroides*, *H. strigaticeps*, and *H. paulinus*, and interstitially dispersed in most st-a chromosomes, in *H. regani*. The fluorochrome staining indicated that different classes of GC and/or AT-rich repetitive DNA evolved in this group. Our results indicate that chromosomal rearrangements and heterochromatin base-pair composition were significant events during the course of differentiation of this group. These features emerge as an excellent cytotaxonomic marker, providing a better understanding of the evolutionary mechanisms underlying the chromosomal diversity in *Hypostomus* species.

Keywords
loricariid catfishes, chromosome banding, NORs, fluorochromes, fluorescence in situ hybridization (FISH)

Introduction

The suckermouth armored catfishes *Hypostomus* Lacépède, 1803 (Siluriformes, Loricariidae) represent one of the most specious genus of the family Loricariidae, with 127 nominal species (Zawadzki et al. 2008).

Most species of this family have a wide distribution in Central and South America. They usually dwell in the rapids, but may be present in different aquatic habitats and in sand banks or rocky rivers. The species of Hypostominae are restricted to freshwater habitats, with the exception of *Hypostomus watwata* Hancock, 1828, which is a benthic species that lives in estuarine waters. Most of these animals have twilight habits and during daylight hours remain under stones or trunks of dead trees (Weber 2003).

The taxonomy of the Loricariidae family has constantly been reviewed through morphological studies (Reis et al. 2006), molecular phylogenies (Montoya-Burgos et al. 1998), allozymes (Zawadzki et al. 2005), and cytogenetic studies (Artoni and Bertollo 2001, Alves et al. 2006). In the most recent taxonomic study (Reis et al. 2006), this family was subdivided into six subfamilies: Lithogeneinae, Neoplecostominae, Hypoptopomatinae, Loricariinae, Hypostominae, and a new subfamily, Delturinae.

Among Hypostominae, only eight of its 30 genera (Armbruster 2004), namely *Ancistrus* Kner, 1854, *Hemiancistrus* Bleeker, 1862, *Hypostomus*, *Baryancistrus* Rapp Py-Daniel, 1989, *Panaque* Eigenmann and Eigenmann, 1889, *Pogonopoma* Regan, 1904, *Pterygoplichthys* Gill, 1858, and *Rhinelepis* Agassiz, 1829, have been object of cytogenetic studies. However, most of these reports are limited to the diploid number, silver staining of the nucleolus organizer regions (Ag-NORs), and chromosome C-banding (Artoni and Bertollo 2001, Alves et al. 2006). Among these genera, *Hypostomus* has the largest number of karyotyped species; however, the number of the studied species versus the species ascribed to the genus is scarce, i.e. approximately 10% (Table 1).

Concerning the cytotaxonomy, this genus shows a wide variation in diploid number, ranging from 2n=52 in *H. emarginatus* Valenciennes, 1840 (Artoni and Bertollo 2001) to 2n=84 in *Hypostomus* sp. 2-Rio Perdido NUP 4249 (Cereali et al. 2008). The most frequent diploid number was 2n=72 (Table 1). The occurrence of multiple NORs located in terminal position on the chromosomes is most common in this genus (Artoni and Bertollo 2001). Regarding the repetitive DNA in *Hypostomus*, different classes of GC and/or AT-rich heterochromatin, usually with segments located in terminal and/or interstitial chromosome regions, were observed in this fish group (Artoni and Bertollo 1999, Kavalco et al. 2004, Cereali et al. 2008).

Table 1. A summary of cytogenetic data available for the genus *Hypostomus*.

Species	Locality	2n	FN	KF	NORs	CB	Ref.
Hypostomus affinis (Steindachner, 1877)	Jacuí stream (SP)	66	94	14m 14sm 12st 26a	5,t, la	t, la,pc	9,10
Hypostomus albopunctatus (Regan, 1908)	Mogi-Guaçu river (SP)	74	104	10m 20sm 44st-a	6,t,sa,la	n.d.	3
Hypostomus albopunctatus	Piracicaba river (SP)	74	104	10m 20sm 44st-a	3,t,sa,la	i,la,t,sa,pc	7
Hypostomus ancistroides	n.d.	68	106	10m 28sm 30st-a	n.d.	n.d.	2
Hypostomus ancistroides	Mogi-Guaçu river (SP)	68	102	16m 18sm 34st-a	6,t,sa	n.d.	3
Hypostomus ancistroides	Araquá river (SP)	68	96	18m 10sm 12st 28a	6,t,sa	n.d.	12
Hypostomus ancistroides	***	68	104	10m 26sm 32st-a	6,t,sa	t,la,pc	16
Hypostomus prope *auroguttatus* Kner, 1854	Mogi-Guaçu river (SP)	76	114	8m 30sm 38st-a	2,t,la	n.d.	3
Hypostomus cochliodon Kner, 1854	Salobra river and Salobrinha stream (MS)	64♂	100	16m 20sm 28st-a	n.d.	t,la	11
		64♀	97	16m 19sm 27st-a	n.d.	t,la	11
Hypostomus emarginatus	Araguaia river (MT)	52	98	16m 30sm 6st	2,t,la	n.d.	5
Hypostomus goyazensis (Regan, 1908)	Vermelho river (GO)	72	98	10m 16sm 10st 36a	2,t,sa	n.d.	12
Hypostomus macrops (Eigenmann et Eigenmann, 1888)	n.d.	68	92	10m 14sm 44st-a	n.d.	n.d.	2
Hypostomus nigromaculatus (Schubart, 1964)	Mogi-Guaçu river (SP)	76	104	8m 20sm 48st-a	3,t,la	t,la,pc	15
Hypostomus nigromaculatus	Três Bocas stream (PR)	76	102	6m 20sm 50st-a	3,t,sa,la	t,la,sa,pc	15
Hypostomus paulinus	n.d.	74	104	10m 20sm 44st-a	n.d.	n.d.	2
Hypostomus paulinus	Três Bocas and Apertados streams (PR)	76	98	6m 16sm 54st-a	2,t,la	t,la,pc	16
Hypostomus plecostomus (Linnaeus, 1758)		54	90	24m 12sm 18st-a	n.d.	n.d.	1
Hypostomus regani	Mogi-Guaçu river (SP)	72	102	10m 20sm 42st-a	n.d.	n.d.	3
Hypostomus regani	Araquá river (SP)	72	102	12m 18sm 26st 16a	4,t,la	n.d.	12
Hypostomus regani	Piumhi river (MG)	72	116	8m 16sm 48st-a	4,t,la	i	13
Hypostomus regani	Jacutinga river	72	100	10m 18sm 44st-a	4,t,sa	i,pc	16
Hypostomus strigaticeps	n.d.	74	86	8m 4sm 62st-a	n.d.	n.d.	2

Species	Locality	2n	FN	KF	NORs	CB	Ref.
Hypostomus strigaticeps	***	72	98	10m 16sm 46st-a	4,t,la	t,la,pc	16
Hypostomus sp. A	Córrego Rincão (SP)	70	102	18m 14sm 38st-a	4,t,sa,la	n.d.	3
Hypostomus sp. B	Mogi-Guaçu river (SP)	72	102	12m 18sm 42st-a	2,t,la	t,la,pc	3,4
Hypostomus sp. C	Mogi-Guaçu river (SP)	72	102	10m 18sm 44st-a	4,t,la	n.d.	3
Hypostomus sp. D1	Mogi-Guaçu river (SP)	72	108	10m 26sm 36st-a	4,t,la	n.d.	3
Hypostomus sp. D2	Mogi-Guaçu river (SP)	72	106	14m 20sm 38st-a	4,t,la	n.d.	3
Hypostomus sp. E	Mogi-Guaçu river (SP)	80	104	8m 16sm 56st-a	2,t,sa	t,la,sa,i,pc	3,4
Hypostomus sp. F	São Francisco river (MG)	76	102	10m 16sm 50st-a	n.d.	pc,t,i	4
Hypostomus sp. G	Araguaia river (MT)	64	102♂	14m 24sm 26st-a	2,sa	pc,t,i	6
		64	103♀	15m 24sm 25st-a	2,sa	pc,t,i	6
Hypostomus sp.1	Paranapanema river (SP)	64	n.d.	n.d.	n.d.	n.d.	8
Hypostomus sp.2	Alambari and Jacutinga streams (SP)	68	n.d.	n.d.	n.d.	n.d.	8
Hypostomus sp. 3	Quinta and Edgardia stream, Paranapanema river (SP)	72	n.d.	n.d.	n.d.	n.d.	8
Hypostomus sp. 4	Paranapanema river; Hortelã stream (SP)	76	n.d.	n.d.	n.d.	n.d.	8
Hypostomus sp. 2-rio Perdido NUP 4249	Perdido river (MS)	84	106	6m 16sm 62st-a	2,t,la	pc,t,la	14
Hypostomus sp. 3-córrego Salobrinha NUP 4247	Salobra river and Salobrinha stream (MS)	82	102	6m 14sm 62st-a	2,t,la	pc,t,la	14
Hypostomus sp.1a	Patos stream (MG)	76	106	6m 8sm 62st-a	3,t,sa,la	t,la	13
Hypostomus sp.1b	Araras stream (MG)	76	106	6m 8sm 62st-a	3,sa,la	t,la	13
Hypostomus sp.2	Araras stream (MG)	74	106	10m 6sm 58st-a	2,la	t,la	13

Diploid numbers (2n), number fundamental (NF), karyotype formula (KF), metacentric (m), submetacentric (sm), subtelocentric (st) and acrocentric (a); *** several collection sites of the Paranapanema river basin. Number of nucleolar organizing region (NORs), C-banding (CB). Interstitial (i), terminal (t), pericentromeric (pc), short arm (sa), long arm (la). No data (n.d.). References (Ref.): (1) Muramoto et al. (1968), (2) Michele et al. (1977), (3) Artoni and Bertollo (1996), (4) Artoni and Bertollo (1999), (5) Artoni and Bertollo (2001), (6) Artoni et al. (1998), (7) Camilo (2004), (8) Fenerich et al. (2004), (9) Kavalco et al. (2004), (10) Kavalco et al. (2005), (11) Cereali (2006), (12) Alves et al. (2006), (13) Mendes Neto (2008), (14) Cereali et al. (2008), (15) Rubert et al. (2008), (16) Present study.

The aim of this work was to analyze specimens of four species of the genus *Hypostomus* from different populations of the Paranapanema River Basin by means of conventional and molecular cytogenetic techniques and compare the obtained data with the cytogenetic records available for other species of the genus.

Material and methods

Cytogenetic analysis was performed on a total of 148 specimens of four *Hypostomus* species collected at different sites of the Paranapanema River Basin (southern Brazil) (Table 1). The specimens were deposited in the Museu de Zoologia of the Universidade Estadual de Londrina (MZUEL), Londrina, Paraná State, Brazil.

Conventional staining. Metaphase chromosomes were obtained through the air-drying technique (Bertollo et al. 1978) and stained with 5% Giemsa stain solution (diluted with phosphate buffer, pH 6.8). The karyotypes were organized in groups of metacentric (m), submetacentric (sm), and subtelocentric-acrocentric (st-a) chromosomes.

Chromosome banding. C-banding was performed according to Sumner (1972). The silver staining of the nucleolus organizer regions (Ag-NORs) was performed according to Howell and Black (1980). The GC- and AT-rich bands were detected by staining with Chromomycin A_3 (CMA_3) and 4'6-diamidin-2-phenylindole (DAPI), respectively, according to Schweizer (1980). The slides were stained with 0.5 mg/mL CMA_3 for 1 h, washed in distilled water and sequentially stained with 2 µg/mL DAPI for 15 min. Slides were mounted with a medium composed of glycerol/McIlvaine buffer (pH 7.0) 1:1 supplemented with 2.5 mM $MgCl_2$.

Fluorescence in situ hybridization (FISH). The fluorescence in situ hybridization procedure was performed according to Swarça et al. (2001). The 18S rDNA probe of *Prochilodus argenteus* Spix and Agassiz, 1829 (Hatanaka and Galetti Jr 2004) was labeled with biotin-14-dATP by nick translation. Slides were treated with 30 µL of the hybridization mixture containing 100 ng of labeled probe (4 µL), 50% formamide (15 µL), 50% polyethylene glycol (6 µL), 20xSSC (3 µL), 100 ng of calf thymus DNA (1 µL) and 10% SDS (1 µL). The slides and the hybridization mixture were denatured at 90°C for 30 min in a Termocycler, and hybridization was performed overnight at 37°C in a humidified chamber. Post-hybridization washes were carried out in 2x SSC, 20% formamide in 0.1x SSC and 4xSSC/0.2% Tween 20, all at 42°C. The hybridized probe was detected with FITC-conjugated avidin. The post-detection washes were performed in 4xSSC/0.2% Tween 20 at RT. The slides were mounted in 23 µL DABCO solution consisting of the following: 90% glycerol, 2% Tris HCl 20 mM, pH 8.0, and 2.3% (wt/vol) 1,4-diazabicyclo (2,2,2) octane, pH 8.6), 1 µL of propidium iodate (1 µg/mL) and 1 µL of $MgCl_2$ 50 mM.

Images were acquired with Leica DM 4500 B microscope equipped with a DFC 300FX camera and Leica IM50 4.0 software.

Results

Specimens of *Hypostomus ancistroides* showed a diploid number 2n=68 and a fundamental number (FN) of 104, with a karyotype formula of 10m+26sm+32st-a. One chromosome of pair 26 showed size heteromorphism (Fig. 1a). Silver nitrate staining (Fig. 1a left box) and FISH (Fig. 1a right box) revealed up to four pairs of subtelocentric/acrocentric NOR-bearing chromosomes. CMA_3 marked the terminal region of the long arms of pair 26, the pericentromeric region of the second pair of metacentric chromosomes, and probably the NOR-bearing chromosomes (Fig. 2a). No fluorescent staining was observed after DAPI staining (Fig. 2b). Heterochromatin was distributed in the pericentromeric region of the second pair (m) of the complement and in the terminal region of the long arm (pair 26) (Fig. 3a).

Hypostomus strigaticeps presented a diploid number 2n=72 and a FN of 98, with a karyotype formula of 10m+16sm+46st-a (Fig. 1b). The Ag-NOR site numbers ranged from two to four marked chromosomes (st-a) located in the terminal region of the long arm (pairs 18 and 28) (Fig. 1b left box), similar to the number observed in FISH (Fig. 1b right box). CMA_3 marked four chromosomes, possibly the Ag-NOR sites, and the pericentromeric regions of most subtelocentric/acrocentric chromosomes (Fig. 2c). Staining with DAPI revealed large blocks in the terminal regions of four-eight subtelocentric/acrocentric chromosomes (Fig. 2d). C-banding revealed the occurrence of heterochromatic blocks in the pericentromeric region of the third pair of metacentric chromosomes and of up to eight large blocks in the terminal regions of the long arms of subtelocentric/acrocentric chromosomes. In one of those chromosome pairs, the heterochromatic block was adjacent to the secondary constriction (Fig. 3b).

Hypostomus regani had 2n=72 with a karyotype formula of 10m+18sm+44st-a and FN of 100 (Fig. 1c). Ag-NORs were located in the terminal position on the short arms of four subtelocentric/acrocentric chromosomes (pairs 26 and 27) (Fig. 1c left box). The same number of NOR-bearing chromosomes was observed after FISH (Fig. 1c right box) and CMA_3-staining (Fig. 2e). Interstitial CMA_3-negative blocks were observed in most of the subtelocentric/acrocentric chromosomes, which, in contrast, were positive after DAPI staining (Fig. 2f). Heterochromatin was distributed in the interstitial region of most st-a chromosomes and in the pericentromeric region of one metacentric pair (Fig. 3c).

Hypostomus paulinus showed 2n=76, FN=98 and a karyotype formula of 6m+16sm+54st-a (Fig. 1d). NORs were located in the terminal position on the long arms of chromosome pair 16 (Fig. 1d left box), similar to the chromosomes observed in FISH (Fig. 1d right box). CMA_3-banding marked up to eight chromosomes (st-a) with large GC-rich blocks, and one st-a pair, probably corresponding to NOR-bearing chromosomes, and in the pericentromeric region of the first (m) pair (Fig. 2g); after DAPI staining, eight fluorescent bands were observed (Fig. 2h). Heterochromatin was distributed in the pericentromeric region of the first pair of metacentric chromosomes, in the terminal region of the long arms of eight pairs of

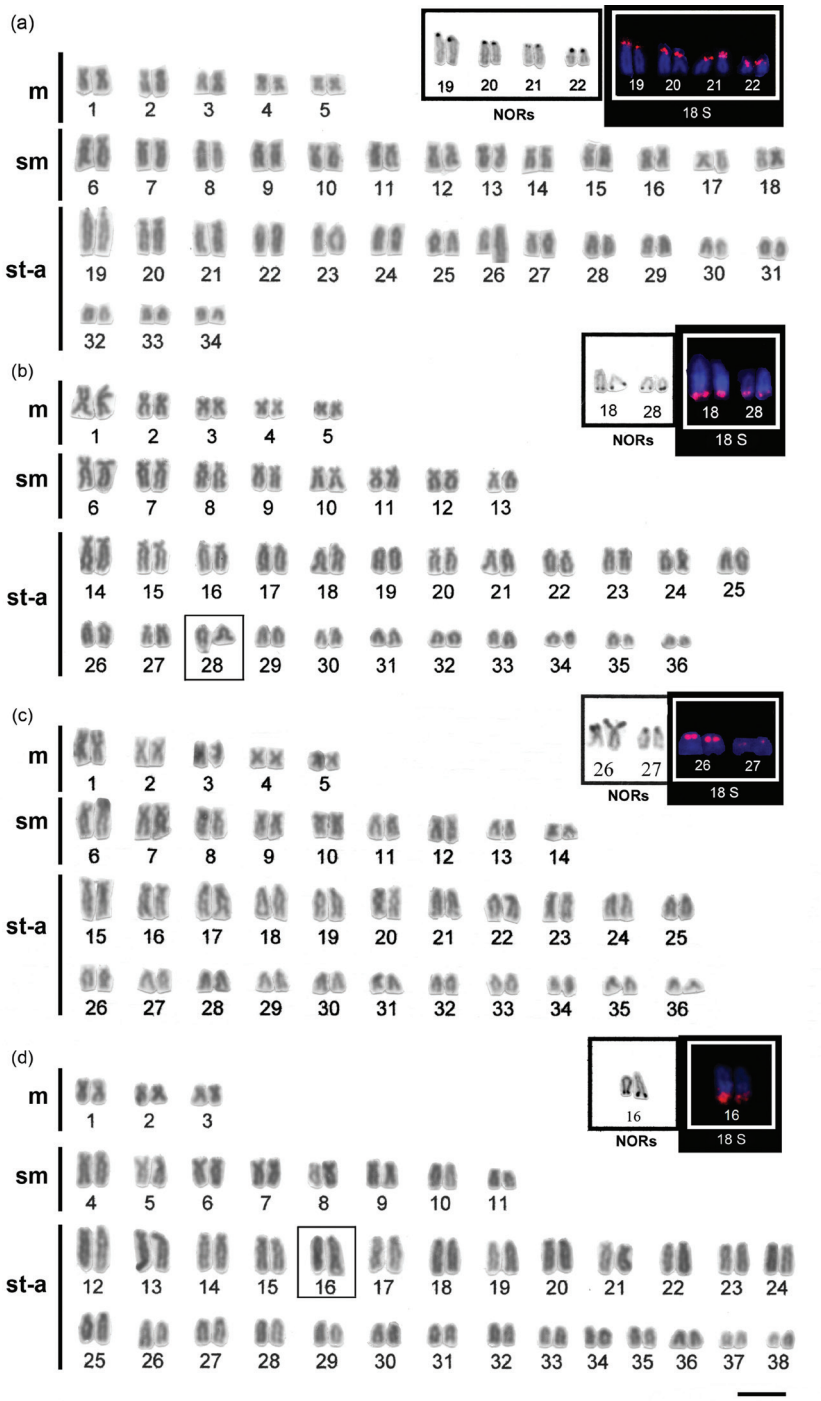

Figure 1. Karyotypes of **a** *H. ancistroides* **b** *H. strigaticeps* **c** *H. regani* **d** *H. paulinus* arranged from Giemsa-stained chromosomes. In the insets, partial karyotypes of the NOR-bearing chromosome pairs after Ag-staining (left) and FISH with 18S rDNA probe (right). Bar = 10 μm.

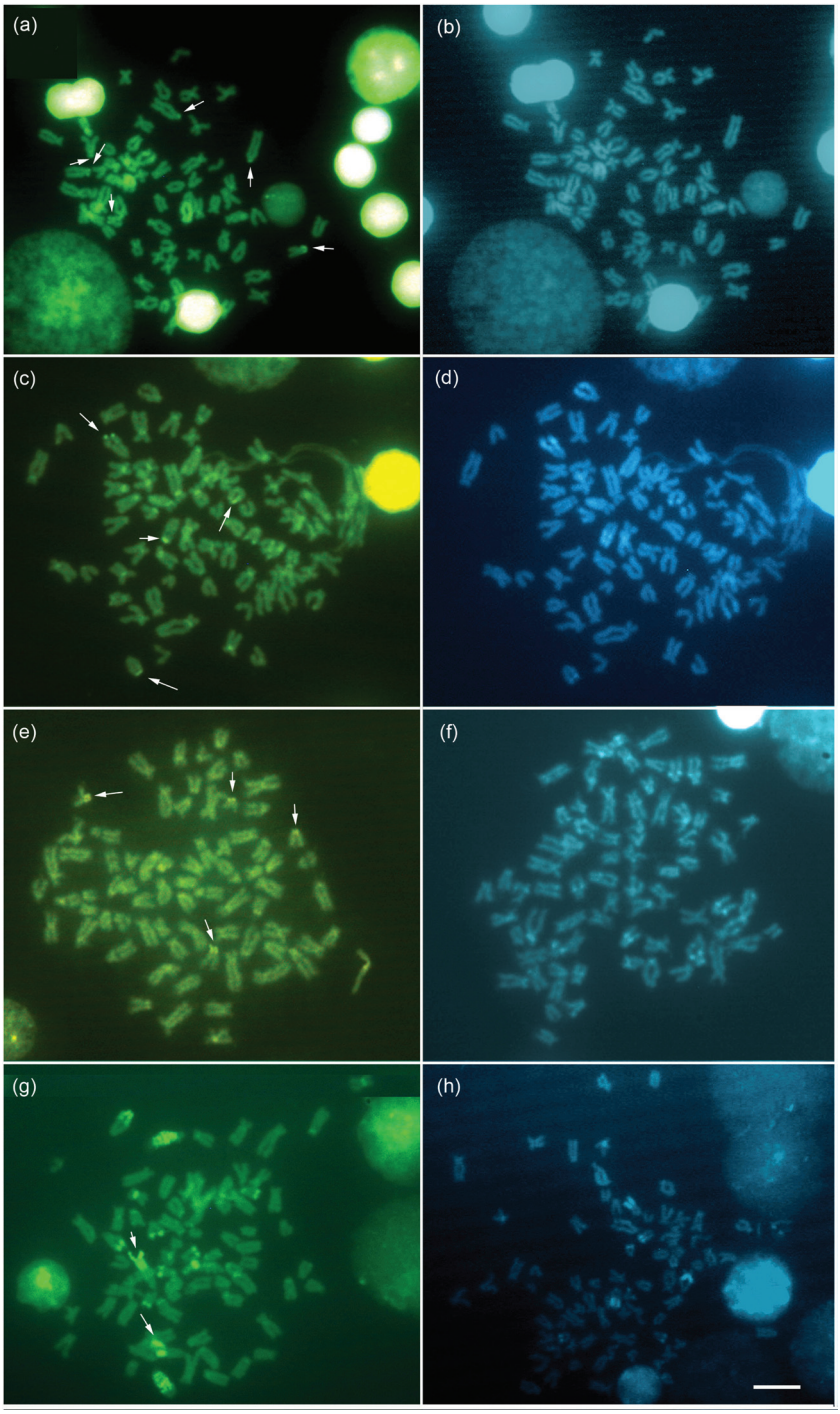

Figure 2. Metaphases stained with CMA_3 (left) and DAPI (right), of *H. ancistroides* **a, b** *H. strigaticeps* **c, d** *H. regani* **e, f** *H. paulinus* **g, h**. The arrows indicate the NOR-bearing chromosomes. Bar = 10 μm.

subtelocentric/acrocentric chromosomes, one of which was the NOR-bearing pair. In this pair, a heterochromatin block was located at the proximal portion of the secondary constriction, whereas three heterochromatin blocks, which occupied almost the entire long arm, were observed in a pair of subtelocentric/acrocentric chromosomes (pair 12) (Fig. 3d).

Discussion

All species differed with respect to their diploid chromosome number and/or karyotype, as follows: 2n=68 (10m+26sm+32st-a) in *H. ancistroides* (Fig. 1a), 2n=72 (10m+16sm+46st-a) in *H. strigaticeps* (Fig. 1b), 2n=72 (10m+18sm+44st-a) in *H. regani* (Fig. 1c), and 2n=76 (6m+16sm+54st-a) in *H. paulinus* (Fig. 1d). This variability is consistent with the chromosomal data previously reported in the genus *Hypostomus*, which showed a wide variation in 2n (from 52 to 84) (Table 1). The available cytogenetic studies showed that the species that possess the same 2n have different karyotypes. In the same way as the features observed in *H. ancistroides* (2n=68) but with different fundamental numbers (FN) among different populations, i.e. 106, 102 and 96 (Michele et al. 1977, Artoni and Bertollo 1996, Alves et al. 2006) and the characteristics found in *H. regani*, the cytogenetic analysis showed the same diploid number (2n=72) and a FN of 102 and 116 (Artoni and Bertollo 1996, Alves et al. 2006, Mendes Neto 2008), also differing from those analyzed herein (Table 1). On the other hand, studies conducted by Michele et al. (1977) in *H. paulinus* and *H. strigaticeps* showed differences in both 2n and FN. This difference may be ascribed to the existence of different cytotypes in these species, the occurrence of cryptic species, problems with the species identification or with chromosomal classification.

According to Artoni and Bertollo (2001), 2n=54 is considered as a basal condition for the family Loricariidae. In a phylogenetic study of Loricariidae using morphological data, the genus *Hypostomus* was considered the most derived (Armbruster 2004), representing a group with more derived karyotypic forms, consisting mostly of st-a chromosomes with a high diploid number. It seems that there was a divergent karyotypic evolution among the *Hypostomus* species; on the other hand, two main chromosome rearrangements appear in the evolution of the genus: i) an increase in the diploid number (2n) in several species, probably due to centric fissions and ii) the same 2n but with a difference in the karyotype formula, probably accounted by pericentric inversions.

The same variability found in 2n and in karyotypes was also detected in NORs. Our data showed different phenotypes among the *Hypostomus* species, observed after silver staining and FISH. All species showed Ag-NORs and 18S rDNA sites located in the terminal regions of st-a chromosomes, but with a significant variation in number and location among them. *H. ancistroides* showed up to 8 NOR sites, all located on the short arms (Fig. 1a left and right boxes, respectively). *H. strigaticeps* showed NORs on the long arms and *H. regani*, NORs located on the short arms, and both species with up to 4 sites (Fig. 1b and 1c left and right boxes, respectively), and *H. paulinus* evidenced

only two NOR-bearing chromosomes located on the long arm (Fig. 1d left and right boxes), which could be considered as species-specific characteristics.

The presence of one pair of NOR-bearing chromosomes, and also its interstitial location seems to be a widespread condition for Loricariidae fish, since this occurs among the Neoplecostominae and Hypoptopomatinae species (Alves 2000). However, in Hypostomini, the occurrence of multiple NORs and their location in the terminal position is most common, as observed here and recorded by other authors (Artoni and Bertollo 1996, Kavalco et al. 2005, Alves et al. 2006). But the exact location and number of ribosomal sites are confirmed only by the FISH technique. With regard to the genus *Hypostomus*, the available molecular cytogenetic data on the location of ribosomal genes are few and restricted to 18S rDNA sites of *H. affinis* (Kavalco et al. 2005). These data are very important to prompt more discussions about the evolution of ribosomal DNA in this group.

In the four species presently studied, the NORs were positive for CMA_3 staining (Fig. 2), a feature that has been conserved among all Neoteleostei (Ráb et al. 1999). In addition, some other chromosomal regions were also considered GC-rich in the four species, mainly in *H. ancistroides* (Fig. 2a) and *H. paulinus* (Fig. 2g). *H. strigaticeps*, *H. regani*, and *H. paulinus* (Fig. 2d, f, h respectively) are three species that also showed several positive markers for DAPI staining, indicating AT-rich regions that were not found in *H. ancistroides* (Fig. 2b).

Some other studies carried out in *Hypostomus* (Artoni and Bertollo 1999, Kavalco et al. 2004, Cereali et al. 2008) also showed that this fish group may possess different classes of GC and/or AT-rich repetitive DNA families, as observed in the species analyzed in the present report. AT-rich regions are also rare among fishes, and have been reported mainly in some Hypostomini species (Artoni and Bertollo 1999, Kavalco et al. 2004, Rubert et al. 2008), some zebrafish species (Gornung et al. 1997, Phillips and Reed 2000), and gobiid fishes (Canapa et al. 2002).

The chromosome banding performed in all species analyzed showed a variation in the heterochromatin distribution pattern. However, the presence of heterochromatin in some chromosomes was constant, as observed in the pericentromeric region of a metacentric pair in *H. ancistroides* (pair 2), *H. strigaticeps* (pair 3), and *H. paulinus* (pair 1) (Fig. 3a, b, d, respectively), also reported in *H. nigromaculatus* by Rubert et al. (2008). An additional characteristic is the presence of some conspicuous blocks in the terminal regions of some st-a chromosomes of the karyotype. The same banding profile, organized in blocks, was also observed by others researchers: in *Hypostomus* sp. B from the Mogi Guaçu River (Artoni and Bertollo 1999), *H. affinis* (Kavalco et al. 2004), *H. cochliodon* (Cereali et al. 2008), and *H. nigromaculatus* (Rubert et al. 2008). Interestingly, in *H. paulinus*, pair 12 proved to be well differentiated, with the long arm almost entirely heterochromatic, a feature observed only in this species. On the other hand, *H. regani* showed a more distinct heterochromatin distribution in relation to the other species, with a preferential location in the interstitial regions of st-a chromosomes (Fig. 3c).

The presence of a marker chromosome that seems conserved for most *Hypostomus* species, corresponding to the NOR-bearing chromosome pair, which shows a heterochromatin block adjacent to this site (e.g. Artoni and Bertollo 1999, Kavalco et al.

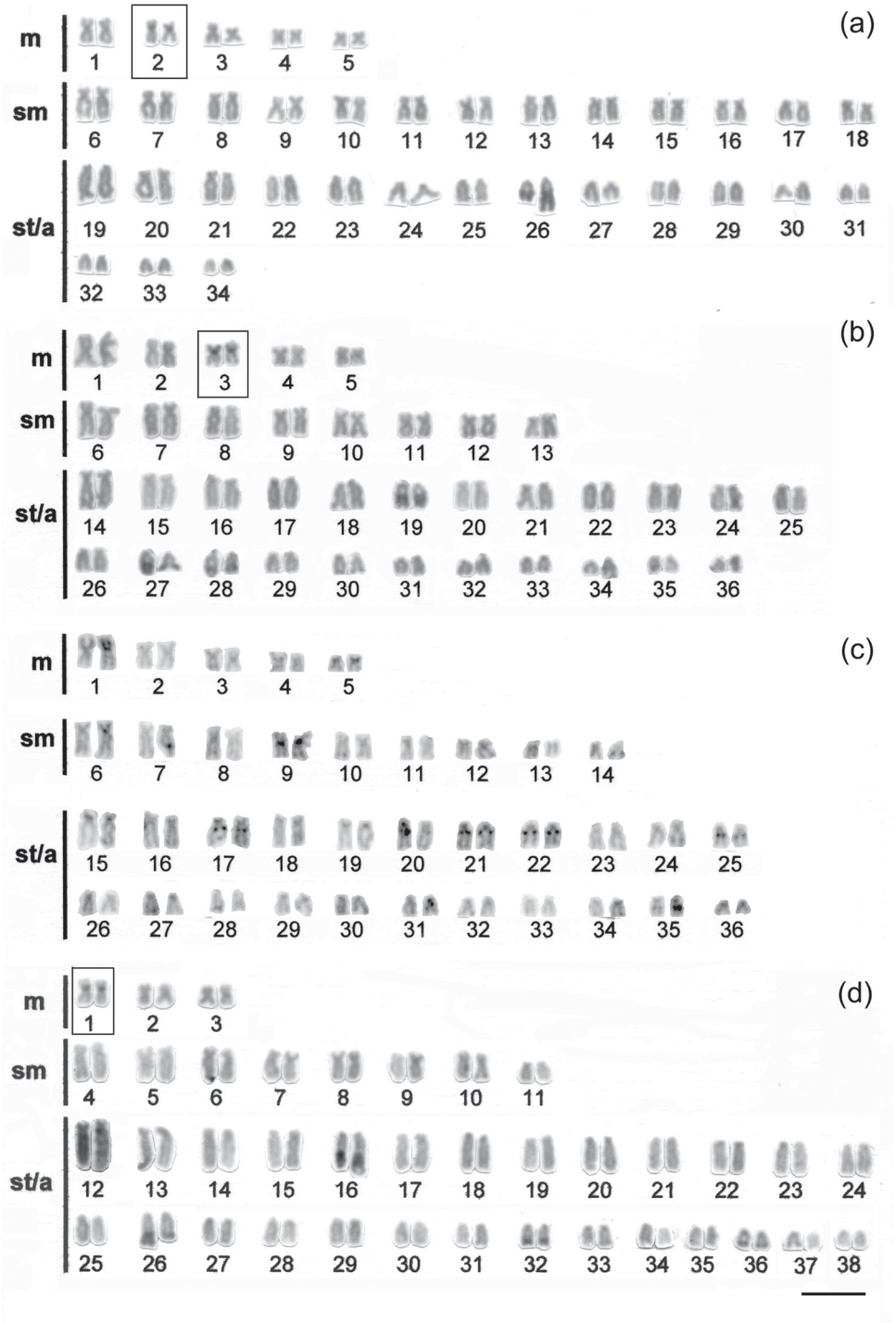

Figure 3. Karyotypes of **a** *H. ancistroides* **b** *H. strigaticeps* **c** *H. regani* and **d** *H. paulinus*, arranged from C- banded chromosomes Bar = 10 μm.

2004, Rubert et al. 2008), was also observed. It can be inferred from all data on the heterochromatin composition and distribution that each species has its own peculiarities, i.e., each species has a unique banding pattern.

Karyotypes, banding patterns, number and location of ribosomal DNA sites, and repetitive DNA are important tools for the cytotaxonomy of *Hypostomus* species. Since these characteristics do not vary among the different populations of the same species, they are significant cytogenetic markers at the species level.

Further data on other *Hypostomu*s species from different rivers, as well as detailed studies of satellite DNA sequences may clarify important issues of genome organization, be used as genetic markers, and provide interesting insights for the comprehension of the evolution of this genus.

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RESEARCH ARTICLE

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Comparative cytogenetics of two of the smallest Amazonian fishes: *Fluviphylax simplex* Costa, 1996 and *Fluviphylax zonatus* Costa, 1996 (Cyprinodontiformes, Poeciliidae)

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Abstract

The genus *Fluviphylax* Whitley, 1965 is comprized of five valid species (*F. pygmaeus* Myers et Carvalho, 1955, *F. zonatus*, *F. simplex*, *F. obscurus* Costa, 1996, and *F. palikur* Costa et Le Bail, 1999), which are endemic to the Amazon region. These fishes are the smallest known South American vertebrates and among the smallest know vertebrates on Earth. All species but the type *F. pygmaeus* have been described in late 1990's, and much remains unknown about the biology, taxonomy and systematics of this group of fishes. The aims of the present study were to establish the diploid and haploid number of *F. zonatus* and *F. simplex,* and to find species-specific markers for the discrimination of taxa. The diploid number for both species was 48 chromosomes, with no sex chromosome heteromorphism. *Fluviphylax zonatus* exhibited the karyotypic formula 4m+8sm+22st+14a and FN=82, and *F. simplex* exhibited 4m+16sm+18st+10a and FN=86. The determination of the total mean length of the chromosomes and their grouping into five size classes demonstrated different chromosome composition of the two species. This difference was further supported by the distribution of constitutive heterochromatin. The meiotic analysis revealed 24 bivalents in both species, but *F. zonatus* exhibited chromosomes with late pairing of the telomeric portions in the pachytene. These data reveal that cytogenetic characterization is useful and important for the discrimination of these species. Our study further indicates that this method could be employed in the analysis of other species of small fishes that are difficult to distinguish using traditional morphological traits or are morphologically cryptic.

Keywords
Chromosomes, heterochromatin, killifish, meiosis, mitosis

Introduction

The Amazonian region has the most diverse freshwater fish fauna in the world, which, although only imperfectly known (Santos and Ferreira 1999). In general, cytogenetic studies of freshwater Neotropical fishes have resulted in the analysis of approximately 1040 species, of which more than 70% correspond to the orders Characiformes and Siluriformes (Oliveira et al. 1988, 2007, 2009). Cyprinodontiform fishes comprise approximately 850 species, mostly Neotropical fishes, of which only 67 neotropical species have cytogenetic information (Costa 1998, Oliveira et al. 2007, 2009). This dearth of information from cytogenetic data is mainly due to the low commercial importance and the small size of specimens that make up the cyprinodontiforms, limiting our understanding of their chromosomal organization and karyotype evolution.

The cyprinodontiforms are a large and diverse group of teleostean fishes comprising the family Poeciliidae. The fishes of the family Poeciliidae are small and laterally compressed, widely distributed in American and African continent. The Poeciliidae include the subfamilies Poeciliinae, Aplocheilichthyinae and Procatopodinae, a group composed of the South-American *Fluviphylax* Whitley, 1965 and the African procatopodines (Ghedotti 2000, Reis et al. 2003). The cyprinodontiform genus *Fluviphylax* comprises five species: *F. pygmaeus* Myers et Carvalho, 1955, *F. zonatus* Costa, 1996, *F. simplex* Costa, 1996, *F. obscurus* Costa, 1996 and *F. palikur* Costa et Le Bail, 1999 (Myers 1955, Costa 1996, Costa and Le Bail 1999). These fishes are commonly known as killifish and are the smallest South American vertebrates, reaching a maximal size of 22 mm. The genus is endemic to the basins of the Amazon and Orinoco Rivers and Atlantic drainages of the state of Amapá, Brazil (Myers 1955, Costa 1996, Costa and Le Bail 1999, Arrington and Winemiller 2003, Hoeinghaus et al. 2004, Lasso et al. 2004). Species *F. simplex* and *F. zonatus* are endemic to the central portion of the Amazon River Basin. The geographic distribution of *F. simplex* is in the Amazonian floodplain (Várzea) from the Amanã Reserve to the city of Santarém, whereas *F. zonatus* is restricted to the lower Negro River (Costa 1996, Souza 2008).

The taxonomic history of the genus *Fluviphylax* is relatively recent. The first species was discovered and scientifically described by Myers and Carvalho in 1955. The genus *Fluviphylax* has paucity of systematic, taxonomic and genetic information, with our knowledge being almost entirely restricted to the information published in the original description. Therefore, cytogenetic studies significantly expand our knowledge base of this group, especially in the realm of understanding of chromosome evolution of *Fluviphylax*.

Cytogenetic studies have contributed significantly to the identification of fishes (Nakayama et al. 2001, 2008, Teixeira et al. 2006) as well as the understanding of

chromosome evolution in Amazonian ichthyofauna (Benzaquem et al. 2008, Gross et al. 2009). However, such studies have been restricted to larger species that are more easily handled. The present study reports a cytological characterization of two species of *Fluviphylax,* obtained by modifications of the technique described by Moreira-Filho and Bertollo (1990) and karyotype comparison with other species Poeciliidae.

Materials and methods

Specimens of *F. simplex* were collected from Lua Beach (3°07'31.7''S, 60°10'38.9''W) near the confluence of the Negro and Solimões Rivers, Amazonas, Brazil. Specimens of *F. zonatus* were collected from a small lake (3°00'19.2''S/60°03'22.6''W) located near Manaus that gathers water from the Tarumã River, which is a tributary of the Negro River (Figs 1, 2). We analyzed 24 specimens of the *F. simplex* and 37 of the *F. zonatus.* The gender determination was made only for adults specimens of each species being 6 males, 8 females and 10 indeterminated for *F. simplex*, and 7 males, 6 females and 24 interminated for *F. zonatus*. Collections were performed under a license from the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA n. 11325-1/2007). Following the chromosome preparation, some specimens were fixed in 95% alcohol. Voucher specimens were deposited in the Fish Collection at the Instituto Nacional de Pesquisas da Amazônia (INPA) in Manaus, State of Amazonas, Brazil (number 25527), and in the Animal Genetics Tissue Collection of the Laboratory of Animal Evolution and Genetics of the Institute of Biological Sciences of the Universidade Federal do Amazonas (Brazil).

Due to the small size of the specimens (less than 20 mm in total length), the cell preparations were obtained through the maceration of the each individual in a cuvette containing 6 ml of hypotonic KCl solution with the aid of two pairs of tweezers. Eyes and intestines were removed prior to maceration. The cell suspension was infused with 0.3 ml of 0.0125% colchicine solution. This preparation was incubated for 40 minutes at 37°C. The subsequent fixation of cells was carried out following the method of Moreira-Filho and Bertollo (1990). C-banding was used to characterize the constitutive heterochromatin distribution (Sumner 1972).

The chromosome preparations were analyzed under an optical microscope with an immersion objective. Selected cells were photographed with a Canon Power Shot A650 IS digital camera. The mounting of karyotypes was carried out with mitotic metaphase chromosomes, which were cut out and tentatively paired. The chromosomes were measured using the free ImageJ program and organized in decreasing order of size. Chromosome morphology was determined taking into account the position of the centromere, based on the method proposed by Levan et al. (1964). Chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st) and acrocentric (a) (Levan et al. 1964). The fundamental number (FN) was determined based on the number of chromosome arms, considering metacentric, submetacentric

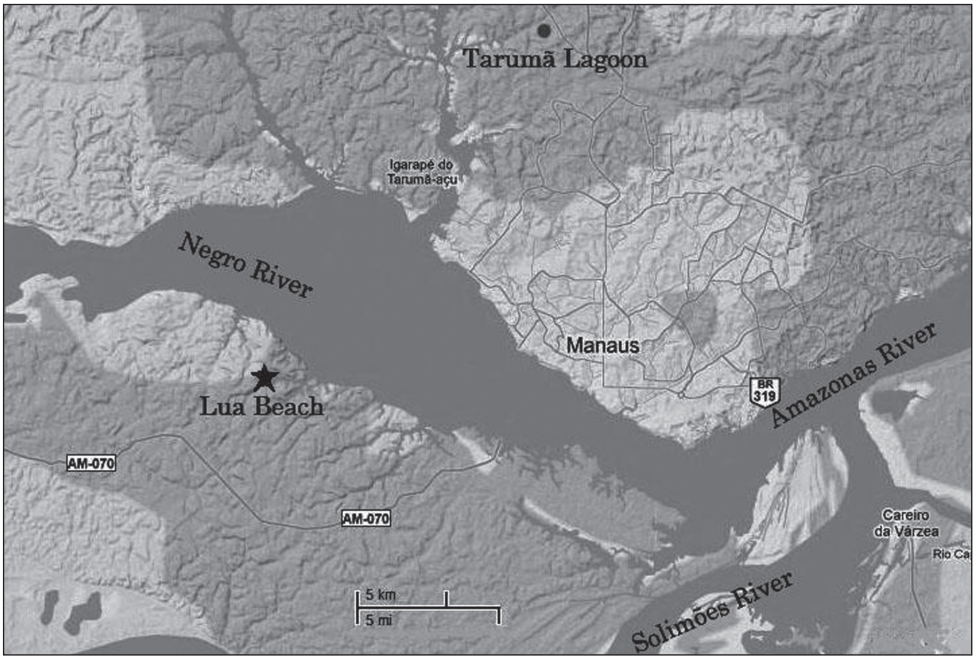

Figure 1. Sampling locations circle and star indicate sampling points for *F. zonatus* and *F. simplex*, respectively

Figure 2. a *Fluviphylax zonatus* with 20.0 mm SL **b** *Fluviphylax simplex* with 18.4 mm SL.

and subtelocentric chromosomes as having two arms and acrocentric chromosomes as having only one arm. Using the data of the mitotic chromosome measurements, of all karyotype for both species, a comparative analysis between *F. simplex* and *F. zonatus* was performed based on the length frequency of chromosome pairs by size class. Sturges' formula was used for determining the ideal number of classes: n = 1+3.32*LogN, in which "n" is the number of classes and "N" is the number of chromosomes in the haploid complement (Fonseca and Martins 1982).

Results

In this study we analyzed 428 cells of the *F. zonatus,* 16% corresponded to mitotic metaphase cells and the others were to meiotic cells, of which 46% leptotene/zygotene, 24% pachytene, 24 % diplotene/diakinesis/metaphase I and 6% in metaphase II. For *F. simplex* were obtained 384 cells corresponded to 36% mitotic cells in metaphase and the others were to meiosis cells, of which 12% leptotene/zygotene, 67% pachytene, 16 % diplotene/diakinesis/metaphase I and 5% in metaphasee II.

In the mitotic analysis, both species had a diploid number of 48 chromosomes, with symmetrical karyotypes and no sex chromosome heteromorphism. *Fluviphylax zonatus* karyotype consists of 2n=4m+8sm+22st+14a (Fig. 3a), and *F. simplex* 2n=4m+16sm+18st+10a (Fig. 3f). Constitutive heterochromatin was detected in the pericentromeric region in the majority of chromosomes in the two species (Figs 3b, g). However, in *F. zonatus* the constitutive heterochromatin occupied entire short arms of all chromosomes, with the exception of 1st and 6th pairs. Also, in the 1st pair the constitutive heterochromatin was bitelomeric and in the 6th additional marks was found in long arms (Fig. 3b). In *F. simplex* the heterochromatin blocks were less evident and in the 20th pair were found additional interstitial marks on the long arms (Fig. 3g). The mean total length of the chromosomes ranged from 1.47 to 3.06 μm in *F. zonatus* and from 1.46 and 3.28 μm in *F. simplex* (Table 1). The grouping of chromosomes into five size classes, also revealed the different length chromosome composition between the two species (Fig. 4). In *F. zonatus,* there was a greater frequency of chromosomes in Class III, which encompasses pairs ranging in size from 2.21 to 2.57 μm, and heterogeneity in chromosomal frequencies among other classes. Moreover *F. simplex* also had greater frequency of chromosomes in Class III, however, the distribution of chromosomal frequencies among other classes were homogeneous.

Gonadal cells of *F. zonatus* and *F. simplex* at interphase and prophase I had no heteropicnotic regions that indicated the presence of sex chromatin (Fig. 3c, h). The chromosomal behavior in some meiotic phases of both species was similar, but differences were detected. In pachytenic cells, both species had 2n=24 bivalents, but *F. zonatus* showed chromosomes with late pairing in the telomeric portions

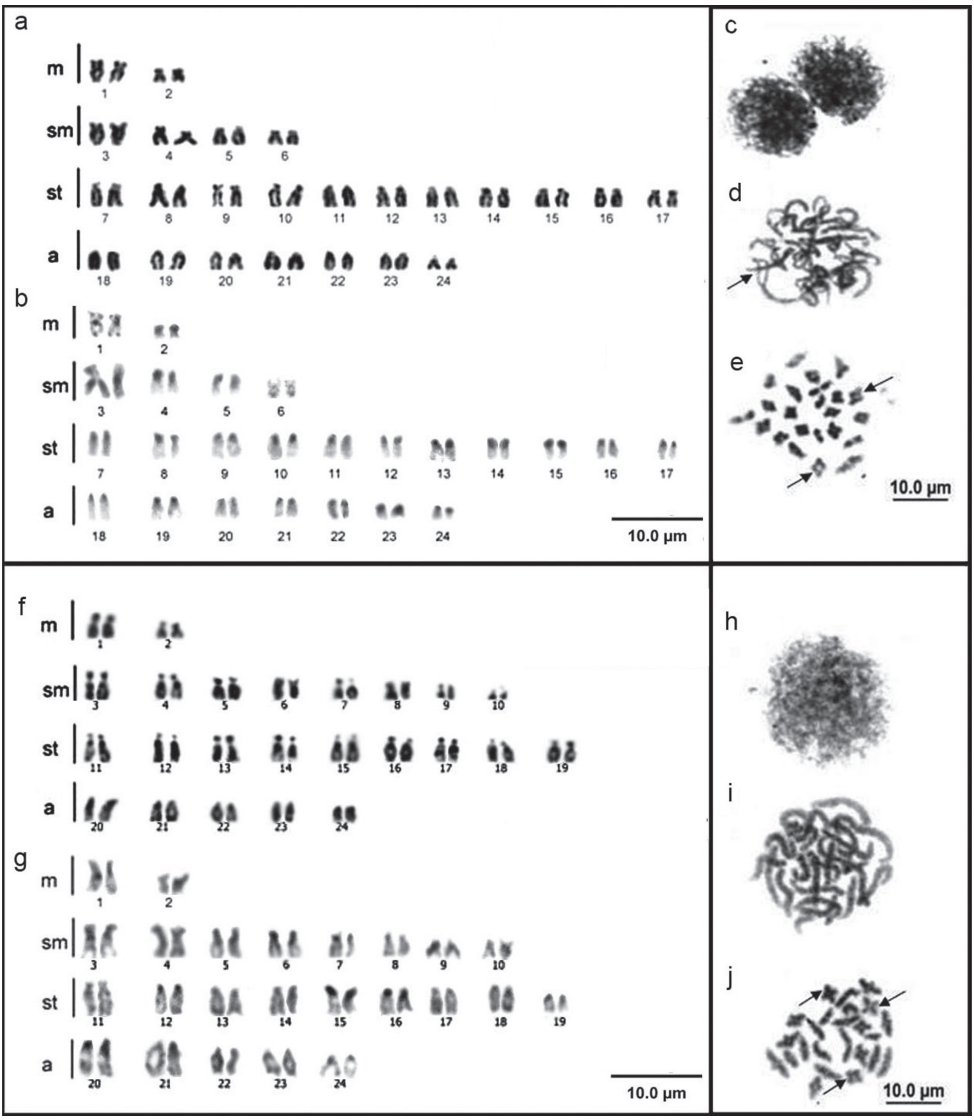

Figure 3. Data on *Fluviphylax zonatus* and *F. simplex*: with conventional staining **a, f** C-banding **b, g** initial leptotene stage **c, h** pachytene stage revealing 2n=24II **d, i** arrow indicates late pairing in some telomeric regions **d** diplotene stage with 2n=24II, arrows indicate bivalents with interstitial chiasma; arrow indicates bivalents with terminal chiasma **e, j**

(Fig. 3d), which did not occur in *F. simplex* (Fig. 3i). The analysis of diplotene cells also revealed 2n=24 bivalents in both species, but *F. zonatus* had 10 bivalents with a terminal chiasma and 14 with an interstitial chiasma (Fig. 3e), and *F. simplex* had 12 bivalents with a terminal chiasma and 12 with an interstitial chiasma (Fig. 3j). For both species, metaphases I had 2n=24 bivalents and metaphases II had n=24 chromosomes (data not shown).

Table 1. Average chromosome measurements (µm) and classifications in *F. zonatus* and *F. simplex* (Ch. Pair: Chromosome Pair; LA: Long arm; SA: Short arm; TL: Total length; AR: Arm ratio; CT: Chromosome type; m: metacentric; sm: submetacentric; st: subtelocentric; a: acrocentric). The LA, SA, TL are average values obtained from the measure of all karyotypes analyzed.

F. zonatus						*F. simplex*					
Ch. Pair	LA	SA	TL	AR	CT	Ch. Pair	LA	SA	TL	AR	CT
1	1.41	1.08	2.55	1.30	M	1	1.64	1.08	2.91	1.52	M
2	0.89	0.56	1.68	1.58	M	2	1.01	0.63	1.63	1.61	M
3	2.04	0.74	2.79	2.76	SM	3	2.14	0.82	3.13	2.61	SM
4	1.55	0.53	2.40	2.92	SM	4	2.12	0.71	2.84	2.97	SM
5	1.49	0.57	2.15	2.63	SM	5	1.79	0.79	2.54	2.26	SM
6	1.22	0.51	1.75	2.40	SM	6	1.68	0.68	2,33	2.46	SM
7	2.33	0.64	3.06	3.65	ST	7	1.57	0.66	2.31	2.39	SM
8	2.32	0.55	1.66	4.23	ST	8	1.30	0.53	1.93	2.43	SM
9	1.95	0.54	2.57	3.58	ST	9	1.12	0.44	1.65	2.54	SM
10	1.75	0.46	2.39	3.79	ST	10	0.72	0.31	1.46	2.33	SM
11	1.62	0.40	2.22	4.04	ST	11	2.57	0.49	3.28	5.20	ST
12	2.34	0.52	2.98	2.53	ST	12	2.66	0.49	3.21	5.38	ST
13	2.32	0.38	2.72	6.05	ST	13	2.26	0.57	2.98	3.93	ST
14	2.05	0.33	2.55	6.24	ST	14	2.29	0.60	2.92	3.80	ST
15	2.15	0.62	2.77	3.47	ST	15	2.44	0.45	3.08	5.40	ST
16	1.82	0.58	2.51	3.12	ST	16	1.99	0.61	2.72	3.27	ST
17	1.80	0.47	2.37	3.85	ST	17	2.13	0.47	2.68	4.57	ST
18	2.22	0.14	2.32	16.20	A	18	1.93	0.51	2.53	3.81	ST
19	2.28	0.15	2.50	15.16	A	19	1.78	0.55	2.34	3.26	ST
20	1.97	0.13	2.26	15.32	A	20	2.24	0.09	2.62	25.77	A
21	2.04	0.21	2.25	9.82	A	21	2.00	0.18	2.35	11.21	A
22	1.78	0.12	2.07	14.89	A	22	1.74	0.17	2.09	10.51	A
23	1.79	0.09	2.11	20.34	A	23	2.06	0.27	2.37	7.58	A
24	1.25	0.07	1.47	17.04	A	24	1.41	0.14	1.71	10.32	A

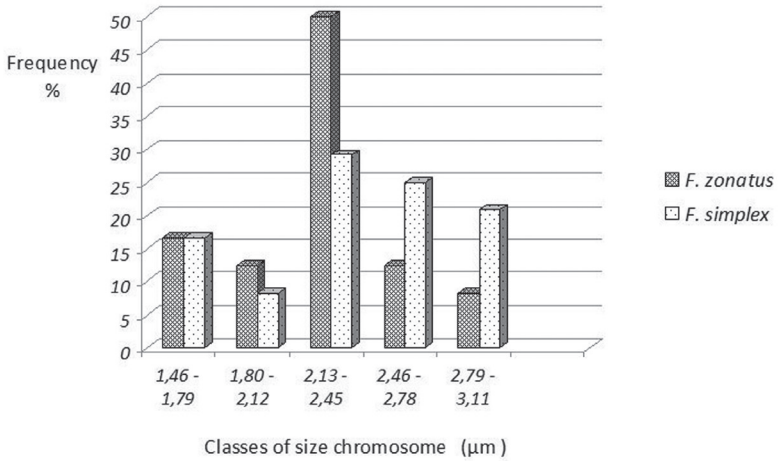

Figure 4. Analysis of chromosome size in *F. zonatus* and *F. simplex*; Y axis gives frequency of chromosomes with pair sizes in classes informed on X axis.

Discussion

The Procatopodinae and their sister sub-family Poeciliinae belong to the family Poeciliidae within order Cyprinodontiformes (Ghedotti 2000). Most of the Neotropical Poeciliidae species are diploid with 48 chromosomes (Ohno and Atkin 1966, Ojima et al. 1976, Ráb 1984, Oliveira et al. 2007). This diploid number has been found in around 51% of the species currently described and it is considered modal number for the order Cyprinodontiformes (Scheel 1972, Oliveira et al. 1988, García et al. 2001). However, variations at the ploidia level have been reported, especially in the genus *Poecilia* (Oliveira et al. 1988, Sola et al. 1990, Galetti Jr and Rasch 1993, Arkhipchuk 1999). Phylogenetic and biogeographic studies of the poeciliid fishes (Hrbek et al. 2007) report *Fluviphylax* as basal group for Poeciliidae family, corroborating the modal diploid number found in this study.

Comparative analysis of chromosome size between *F. zonatus* and *F. simplex* revealed differences in the organization of the genome, that is reflected in difference of karyotype formulae, due occurrence of pericentric inversion rearrangements, which alter the karyotype formula without altering the diploid number.

Chromosomal rearrangements are considered an important mechanism of karyotypic differentiation in Aplocheiloidei and Cyprinodontiformes in general (Scheel 1972) and the fixation of chromosomal rearrangements can occur by different processes, such as genetic drift or meiotic drive (Völker et al. 2006). Currently, it is widely accepted that diversity in the size and organization of genomes is influenced by non-coding repetitive DNA, such as pseudogenes, retrotransposons, transposons and satellite DNA, the most part found in the heterochromatin. The characteristics of an actual genome of an organism is determined by differential epigenetic activity of mechanisms that cause either an increase or decrease in the amount of DNA in response to the surrounding environment (Leitch 2007). *Fluviphylax zonatus* and *F. simplex* inhabit waters with different physiochemical characteristics, which in theory can influe via epigenetic mechanisms the organization of the karyotype. *Fluviphylax zonatus* is found in black waters from the Guiana Shield while *F. simplex* occurs in white-water rivers (Costa 1996, Souza 2008). Geological and ecological differences between the habitats of the species analyzed may have driven their speciation, as they are subjected to different types of selective pressure, which may have allowed the fixation of rearrangements that resulted in the different karyotype formulas and specie-specific pattern of heterochromatin distribution.

Although not commonly performed, meiotic analyses are an extreme powerful tool for chromosomal characterization (Gross et al. 2009). Analysis of meiotic chromosomes was also of fundamental importance in the study of the species of *Fluviphylax*, as it resulted in more a thorough characterization of their chromosomes. The success of the meiotic analysis was likely due to the fact that the species analyzed reproduce throughout a large portion of their life cycle and thus are continuously producing gametes. Continuous reproduction was also reported by Roberts (1970) when analyzing populations of *F. pygmaeus.* Moreover, late pairing was observed in the telomeric portions of some chromosomes in the pachytene stage in *F. zonatus*. Late pairing is

a species-specific marker in meiotic analyses and may either occur randomly or as a result of epigenetic mechanisms (Grewal and Jia 2007). As the species did not exhibit heterochromatin in the telomeric portions, this type of chromosome behavior is likely the result of gene regulation.

Some species of Poeciliidae have visible sex chromosome, such as the ZW/ZZ sex determining system in *Gambusia puncticulata* (Ráb 1984), *Poecilia latipinna* (Sola et al. 1990), *P. formosa* (Sola et al. 1993) and *P. sphenops* (Haaf and Schmid 1984) and the XX/XY system in *P. reticulata* (Feichtinger 1988), or both systems in *Xiphophorus maculatus* (Gordon 1950). However, the two species of *Fluviphylax* analyzed here did not exhibit differentiated sex chromosomes.

Organisms with differentiated sex chromosomes generally display positive heteropycnotic corpuscles in the early stages of prophase I and differentiated meiotic behavior in these chromosomes (John 1990). In *F. zonatus* and *F. simplex,* the lack of atypical chromosome behavior in both confirms the absence of sex chromosomes. This lack of differentiated sex chromosomes is found in approximately 95% of Neotropical teleosts. The most striking characteristic with respect to the occurrence of sex chromosomes in fishes is their apparent random distribution across the phylogenetic tree of fishes, as different systems are found in closely related species of the same genus or even in different populations of the same species (Almeida-Toledo and Foresti 2001).

As evident from our and other studies (Benzaquem et al. 2008, Gross et al. 2009, Oliveira et al. 2009), chromosomal characterization is an important tool for taxonomic and karyotype evolution studies in fishes. Moreover, the comparative description of karyotype characteristics in *F. zonatus* and *F. simplex*, which are among the smallest known vertebrates, may be considered an innovative, pioneering approach for fishes of the Amazon region. The methodology employed in the present study could be used in the analysis of other species of small Amazonian fishes which abound in the Negro River basin, many of which are miniaturized and with unclear taxonomic boundaries, as well as assist in the understanding of karyotype evolution.

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Three new karyotypes extend a Robertsonian fan in Ethiopian spiny mice of the genus *Acomys* I. Geoffroy, 1838 (Mammalia, Rodentia)

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Abstract

Three new karyotypes (2n=40, 44, 52) are described revealing what are probably new cryptic species of Ethiopian spiny mice. Two other diploid numbers have already been reported for the country (2n=36 and 68) and, overall, the five known karyotypic forms constitute a common lineage differentiated by a Robertsonian process. Such arrays of karyotypic forms are known as a 'Robertsonian fan'. This view of the situation in Ethiopian *Acomys* I. Geoffroy, 1838 is based on standard chromosomal morphology that reveals a constant FN (68) and needs further investigation of chromosome homology by differential staining and/or molecular cytogenetic techniques as well as further molecular phylogenetic analysis.

Keywords

mammalian karyotype, Robertsonian variation, *Acomys*

Introduction

Karyotypic studies on mammals of Ethiopia play an important role in species identification and in the interpretation of phylogenies of taxonomic groups (Lavrenchenko 2009). This in particular concerns the rodent taxa where karyotypic data constitute a powerful tool discriminating for an inventory of species diversity. Although the spiny mice of the widespread Afro-Asian genus *Acomys* I. Geoffroy, 1838 were in the focus of cytogenetic interests since the very beginning of current chromosome preparation era, i.e. the use of colchicine/hypotonic method (Wahrman and Zahavi 1953), the karyotype description of existing taxa in this group is far from complete. New data on

karyotypic evolution are coming from the studies based on the chromosome banding techniques (Giagia-Athanasopoulou et al. 2011), and even the initial descriptions of chromosome numbers and morphology may be informative due to revealed wide chromosome variability and corresponding expectations of multiple chromosomal races or/and sibling species over wide generic distributional range (Volobouev et al. 1991).

It became clear from early descriptions of the Middle East populations that a special type of chromosomal variation, known as Robertsonian translocations, is to be regarded as the principal way of karyotypic evolution both between and within *Acomys* taxa. The differences in diploid numbers that was defined initially in representatives of *A. cahirinus* (É. Geoffroy, 1803) and *A. russatus* (Wagner, 1840) from Israel (2n=38 and 66, respectively), have shown almost exactly the 2n limits in the whole genus (Wahrman and Zahavi 1953). Further discoveries revealed even lower chromosome number (2n=36) in populations of *A. cahirinus* in the southern Sinai and had led to the discovery of a hybridization zone between the two intraspecific karyotypic forms (2n=36, 38) differed by a single whole-arm Robertsonian translocation (Wahrman and Goitein 1972). Data collected from species and populations of African *Acomys* since Matthey's pioneer studies (Matthey 1956) up to now and in other parts of the generic range (for cytotaxonomic survey see Macholán et al. 1995, Volobouev et al. 1996, Zima et al. 1999, Dobigny et al. 2001, 2002, Granjon and Dobigny 2003, Corti et al. 2005, Sözen et al. 2008) show that diploid numbers for this genus vary from 2n=36 to 2n=68 via a series of intermediate 2n and the karyotypic constitution may be transformed from a totally acrocentric (2n=68) to almost fully metacentric (2n=36) complement.

The variation in chromosome arm numbers (the Fundamental Number, otherwise FN) is relatively low, from 66 to 78 (Fadda et al. 2001). The presence of FN numbers higher than 68 indicate that structural chromosome variations other than Robertsonian rearrangements, such as pericentric inversions and heterochromatin additions or deletions, occur in *Acomys* karyotypes.

In Ethiopia, only karyotypes with the extremal 2n values were previously found which have been reported for the two species inhabited the Lower Omo River Valley in the very south (2n=36 in *A. percivali* Dollman, 1911 and 2n=60 in *A. wilsoni* Thomas, 1892, according to Matthey 1968) and two species from the Rift Valley in the centre of the country (2n=36 in *A. cahirinus* and 2n=68 in *Acomys* sp., following Sokolov et al. 1993). Most close findings from Tanzania (Corti et al. 2005) show three different karyotypes with 2n ranged from 36 for *A. ignitus* Dollman, 1910 to 60 and 62 for *A. spinosissimus* Peters, 1852 and *A. wilsoni*, respectively. The different karyotype characteristics which were reported for the last taxon (2n=62 versus 60) were not considered in that publication.

In this communication, three new karyotypes of *Acomys* are presented which adds substantially to the 2n range known for taxa inhabiting this country (40, 44 and 52 versus 36 and 60, 68) and thus extends the series of Robertsonian transformations,

known as a 'Robertsonian fan', performed in the related karyotypes. This study covers also three new geographic parts of Ethiopia where the spiny mice were never examined.

Materials and methods

Animals were collected during the 2008 and 2010 field seasons in the course of the Joint Ethio-Russian Biological Expedition (JERBE). Chromosome preparations were obtained from five *Acomys* specimens collected in two lowland regions on opposite sides of the Rift Valley. Among them, two specimens of both sexes were from a geographic site in the east (Babille Elephant Sanctuary, 9.0601°N; 42.2699°E, 1200 m a.s.l.) and three specimens from two sites of the Alatish National Park, north-western Ethiopia (Amjale, 12.3875°N; 35.7309°E, 533 m a.s.l., 2 males, and Bermil, 12.4958°N; 35.6382°E, 575 m a.s.l., 1 female).

For the karyotype analysis, bone marrow cell suspensions were prepared according to the standard colchicine, hypotonic solution and air-dried technique (Ford and Hamerton 1956) and stained on microscopic slides with Giemsa; each individual was preserved for further molecular analyses, following to a current research trends (Bulatova et al. 2009, Lavrenchenko 2009). Collected specimens were not specifically identified in the field, but deposited for further morphological or DNA examination.

Results

Three different karyotypes are found which characterize the Ethiopian *Acomys* of lowland savanna from three geographic samples in two different parts of the country. A higher chromosome number (2n=52) is detected in a male from Amjale. Its chromosome set contains lesser number of metacentric pairs than acrocentric ones (8 and 18, respectively; FN=68). Sex chromosomes cannot be reliably identified without differential staining, however, a larger acrocentric element with a distinguished short arm was recognized as the X chromosome, when one of the smaller acrocentrics may represent the Y chromosome. The autosomal arm number (FNa) characteristic for this karyotype is 66.

The lesser chromosome numbers are found in two other karyotypes (Figure 1). 2n=44 is characteristic for animals from the Babille Elephant Sanctuary. In this karyotype 12 metacentric and 10 acrocentric pairs are present (FN=68). The acrocentric chromosomes apparently include the XX pair in females and XY in males. NFa is 66 in this case. 2n=40 is reported from Bermil, and only 6 pairs of acrocentrics are identified in this karyotype (FN=68), one of which may be determined as the XX pair in a female studied, by analogy with the sex chromosomes of other *Acomys* species (Table 1). NFa is again 66.

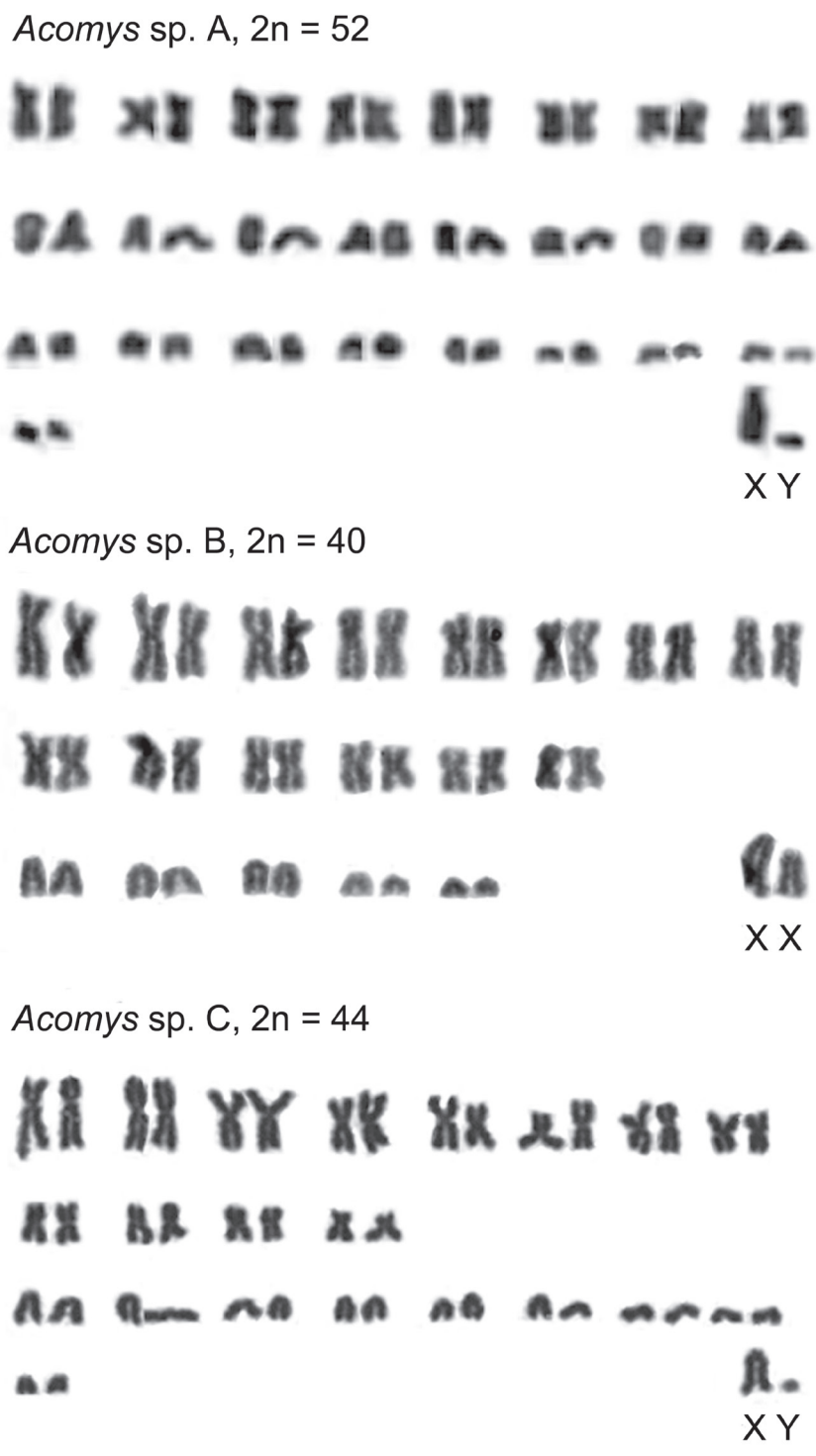

Figure 1. Karyograms of Ethiopian *Acomys* spp. A – Amjale, B – Bermil, C - Babille.

Discussion

Description of three new karyotypes of *Acomys* presented in this study indicates that there is more karyotype diversity in Ethiopian *Acomys* than it was thought previously. Three additional 2n numbers that were found (40, 44, and 52) fill in the series of 2n changes where only minimal (36) and maximal (60, 68) limits were known until now (Table 1).

The common feature for five out of six karyotypes is the same number of chromosome arms (FN). The FN value is invariably 68 for full chromosome complements and 66 for the autosomes (FNa) in the karyotypes with various 2n which were attributed to at least five species, including *A. cahirinus* (2n=36) and four unidentified taxa, probably cryptic species - *Acomys* sp. (2n=68), *Acomys* sp. A (2n=52), *Acomys* sp. B (2n=40), and *Acomys* sp. C (2n=44) (Matthey 1968, Sokolov et al. 1993, our data).

Regarding this Ethiopian group, interrelations based on Robertsonian rearrangements could be suggested. It is well known that it is usually difficult to decide whether Robertsonian fusions or fissions occurred in each given group, and the interpretation of karyotype evolution depends upon the context of the comparative analysis. It is often a working hypothesis that a chromosome set with the most numbers of acrocentrics is the primitive one (2n=68 in the case of *Acomys*) and that karyotypic evolution should pass via successive fusions of any twin acrocentrics into one metacentric. In fact, due to the bi-directional Robertsonian process and probable multiple fusions of original acrocentrics, the chromosomal sibling species may exist that should confuse a hypothetic common chain of karyotypic changes from maximal to minimal 2n, or, in our case, from 2n=68 to 2n=36 via 2ns such as 52, 44 and 40. Indeed, a clear indication on Robertsonian, or centric fission has been obtained through the karyotype comparison between the continental (Turkey) *A. cilicicus* Spitzenberger, 1978 and Mediterranean sea island (Cyprus) spiny mouse, *A. nesiotes* (Bate, 1903), which differ in 2n as 36 and 38, respectively (Zima et al. 1999).

As for the 60-chromosome karyotype of *A. wilsoni*, one of the first reported from the Omo Valley by Matthey (1968), it is to be regarded as distinguished from the group mentioned above even on the level of routine chromosome data because it has a different FN=76 (FNa=74, see Table 1 and Introduction). In reference to modern chromosome data, it should be, however, questioned to what species does each of the two initial karyotype descriptions of south Ethiopian *Acomys* - *A. percivali* and *A. wilsoni* (sensu Matthey cit.) - belong. The first of the two was attributed to *A. cahirinus* after the work of Sokolov et al. (1993) as it has the same chromosome number, 2n=36 (see Table 1). Following recent karyotype descriptions from Tanzania, another chromosome numbers, 2n=62 and FNa=76, are attributed to *A. wilsoni* which is in this karyotypic form included into the current mammal species checklists (Fadda et al. 2001, Corti et al. 2005).

The spiny mice with 2n=60 were found in central and southern parts of Africa, but the karyotype peculiarities do not allow to consider them conspecific. Comparing to Ethiopian karyotype with FNa=74, two other species with the same 2n=60 show

Table 1. Karyotypic data on *Acomys* collected from Ethiopia.

Species	2n	FN	FNa	X	Location	Reference
A. wilsoni	60	76	74	A	S Ethiopia: Omo Valley	Matthey 1968
**A. percivali*	36	68	66	A	S Ethiopia: Omo Valley	Matthey 1968
A. cahirinus	36	68	66	A	S Ethiopia: Rift Valley (Konso; Arba-Minch)	Sokolov et al. 1993
A. cahirinus	36	68	66	A	Central Ethiopia: Rift Valley (2 sites along the middle Awash valley)	Sokolov et al. 1993
Acomys sp.	68	68	66	A	Central Ethiopia: Rift Valley (Koka, upper Awash valley)	Sokolov et al. 1993
Acomys sp. A	52	68	66	A	NW Ethiopia: Alatish National Park (Amjale)	This study
Acomys sp. B	40	68	66	A	NW Ethiopia: Alatish National Park (Bermil)	This study
Acomys sp. C	44	68	66	A	E Ethiopia: Babille	This study
Total: 6 or more species	36–68	68 (76)	66 (74)	A	Rift Valley and S, NW and E lowland Ethiopia	3 publications

* Referred to *A. cahirinus* in Sokolov et al. 1993.

not only differences in FNa – 68 in *A. selousi* De Winton, 1896 and 70 in *A. spinosissimus* – but reveal serious difference in the morphological structure of X chromosome (metacentric with a heterochromatic arm in the first case and submetacentric in the second one). Moreover, they are characterized by the unique XO system in both sexes, accompanied with the atypical inter- and intraindividual autosome number variation (2n=58-62) (Barome et al. 2001, Fadda et al. 2001, Corti et al. 2005). In Ethiopian *Acomys* no other sex chromosome constitution than standard XX/XY is observed and there was no other X chromosome in their karyotypes than a moderate size acrocentric which is considered as the original type for the genus (Table 1).

There are still more examples of 2n similarity in the karyotypes of different *Acomys*. Three of four 2n values that were found in Ethiopia have been reported for *Acomys* from other territories, i.e. the first finding of 2n=68 from Burkina-Faso (see Sokolov et al. 1993) or 2n=44 from Mali (Dobigny et al. 2001), 2n=40 from Crete (Giagia-Athanasopoulou et al. 2011), and only 2n=52 is shown for this genus for the first time.

The 36-chromosome karyotype is most widely distributed in Mediterranean area and presented among African taxa. The same FN characteristics of the karyotypes with the same 2n may indicate the karyotypic clusters of common origin in the related groups. It is, however, interesting that preliminary data from mt-DNA analysis (Lavrenchenko, in prep.) focused on Ethiopian – i.e. East African – karyotypic forms that we described in this paper do not support their genetic affiliation with taxa from western parts of Africa with the same 2n=40 or 44 and FNa=66 (Nicolas et al. 2009). For analysis of geographically distant taxa, high-resolution chromosome banding should be used in addition to other methods. It was already shown that externally the 'same' 36-chromosome karyotypes could be formed via the complex Robertsonian arm fusions leading to partial, i.e.monobrachial homology between the variable metacen-

trics (Volobouev et al. 2007). If so, any finding of the 2n=36 karyotype does not seem to be a reason to attribute a corresponding taxon immutably to *A. cahirinus*.

Besides the Robertsonian changes occured generally in autosomes, heterochromatin variability exists regarding the X chromosome. To avoid probable variation in FN due to the presence/absence of a short heterochromatic arm in the X-chromosome, Macholán et al. (1995) suggested that the autosomal number, FNa, is better to be used for the karyotype comparison. Whether such kind of variation presents in the X with or without clear short arm as we observed in 3 karyotypes (Fig. 1) is to be evidenced by application of differential staining of heterochromatin.

As a result of this preliminary chromosome study, we may conclude that 3 new karyotypes (2n=40, 44, 52) are to be added to at least three karyotypic forms of Ethiopian *Acomys* (2n=36, 60, 68), described previously (Matthey 1968, Sokolov et al. 1993, our data). It follows from the comparison of data that five out of six karyotypes, except the one with 2n=60, may form a Robertsonian fan, the reliability of which is to be checked by further chromosome analysis of Ethiopian populations. And, finally, wide taxonomic expertise of the genus is needed based on the complex morphological, fine karyological and, in particular, molecular investigation of spiny mice inhabiting this country.

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RESEARCH ARTICLE

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Presence of the 54-chromosome common vole (Mammalia) on Olkhon Island (Lake Baikal, East Siberia, Russia), and the occurrence of an unusual X-chromosome variant

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Abstract

We report a new finding of the 54-chromosome sibling species of the common vole in East Siberia - the first description from Olkhon Island (Lake Baikal). The karyotype of a male specimen revealed by routine staining and C-banding demonstrates the unambiguous presence of *Microtus rossiaemeridionalis* Ognev, 1924 (recently often regarded as as junior synonym of *M. levis* Miller, 1908). Comparison with conspecific specimens from the European part of the species range (from the left bank of the river Volga) shows that the vole of the island population has a smaller X-chromosome due to a reduced quantity of C-positive heterochromatin. This is just the third example of this type of X-chromosome variant with previous cases on an Arctic island (Svalbard) and the West Siberian lowland (Novosibirsk) and the only one on a lake island. Although *M. rossiaemeridionalis* is largely monomorphic in its karyotype, our data show that one specific type of X-chromosome variant is remarkably widespread, though rare.

Keywords

chromosome sibling species, common voles, *Microtus arvalis* group, *Microtus rossiaemeridionalis*, Lake Baikal, X-chromosome

Introduction

The investigation of intraspecific variability of chromosomes is one of the traditional approaches to study evolutionary processes. Comparative karyological investigations

of new and especially extreme localities of widely distributed species are of particular interest (Král et al. 1980).

Since the description of two sibling species in the common vole differing in diploid numbers (2n=46 in *M. arvalis* Pallas, 1779 and 2n=54 in *Microtus rossiaemeridionalis* Ognev, 1924) (Meyer et al. 1969, 1972, Malygin and Yatsenko 1986, Common Vole: The sibling species 1994) the vast range of the species previously known as *M. arvalis* has been revised to demarcate areas occupied by these new taxa (e.g. Shenbrot and Krasnov 2005). Among karyotypic data on *M. rossiaemeridionalis*, represented localities in its European part of the range (e.g. Král et al. 1980, Common Vole: The sibling species 1994, Meyer et al. 1996) outnumber those from Siberia, where data from no more than a dozen scattered sites are available from the huge Asian territory within the boundaries of the Russian Federation and Kazakhstan (Meyer et al. 1996, Yakimenko and Kryukov 1997).

Here we report one of the easternmost findings of the 54-chromosome karyotype for *M. rossiaemeridionalis*, from an isolated population on Olkhon Island in Lake Baikal.

Materials and Methods

Small mammal trapping was conducted in July 2008 in Irkutsk province, East Siberia, on the western shore of Lake Baikal and on Olkhon Island that was separated by a narrow channel (2-5 km in width) from the mainland. A sole adult male of the common vole was live-trapped on Olkhon Island about 800 m from the village of Khalgai (53°42'14"N; 107°31'32"E) at the edge of larch – pine forest, bounded by steppe habitat.

For the cytogenetic comparison, fresh chromosome preparations were prepared in a similar way for 3 specimens, 2 males and 1 female, collected in March 2011 in the European part of the range of the common vole (village of Dyakovka on the left bank of the river Volga, Saratov province: 50°42'54"N; 46°45'52"E). These animals were caught using live-traps on the bushy slope of the right bank of the river Yeruslan, about 400 m from the village and about 2.5 km from the Dyakovsky Forest (Fig. 1). In addition to our data, a recent Siberian collection site (Novosibirsk, West Siberia) is indicated in the distribution map of the *M. rossiaemeridionalis* taken from the official web-site of A.N. Severtsov Institute of Ecology and Evolution (http://www.sevin.ru/vertebrates/).

Materials for cytogenetic and further molecular analyses were fixed in the field following a standard protocol (Bulatova et al. 2009), while the skull and postcranial skeleton were deposited in the Laboratory of Historical Ecology of the A.N. Severtsov Institute.

Standard mitotic and meiotic chromosome preparations were obtained in the field from the bone marrow and from testes following Ford and Hamerton (1956) with some modifications (Bulatova et al. 2009) and Williams et al. (1971), respectively, and

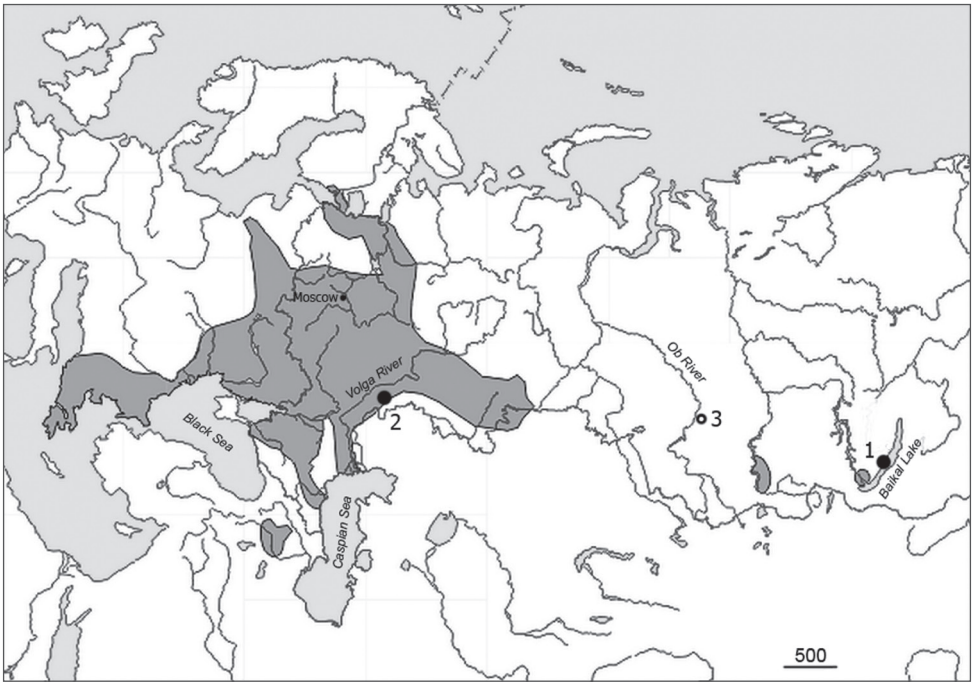

Figure 1. Map showing collection sites for the 54-chromosome (Sibling) vole under study. Black circles – our data (**1** Olkhon isl., Lake Baikal **2** Dyakovka, left bank of Volga River.) Open circle – most recent Siberian finding **3** Novosibirsk vic., right bank of Ob River. Colored are the territories where verified findings (karyotype, allozymes) were obtained from.

then analyzed in the laboratory under a light microscope. Routine Giemsa staining and the C-banding technique of Sumner (1972) were used to define the karyotype.

Results

The four voles examined had the same chromosome number 2n=54 and identical autosomal karyotype supplemented by the typical sex chromosomes complement – XX in females, XY in males. All chromosomes but the smallest pair of metacentric autosomes were acrocentric (NF=56). Routine staining was ineffectual in identifying more than two pairs of large acrocentrics and the small metacentric pair from the morphologically homogeneous group of medium to small acrocentrics. After C-banding, the centromeres of all autosomes were positively C-stained. The two largest elements with additional C-blocks of heterochromatin were classified as the pair of sex chromosomes, the Y being totally heterochromatic and the X carrying a large telomeric block (Fig. 2). The X chromosome was always the largest element in the complement and the Y the next largest, but close in size to the largest autosome.

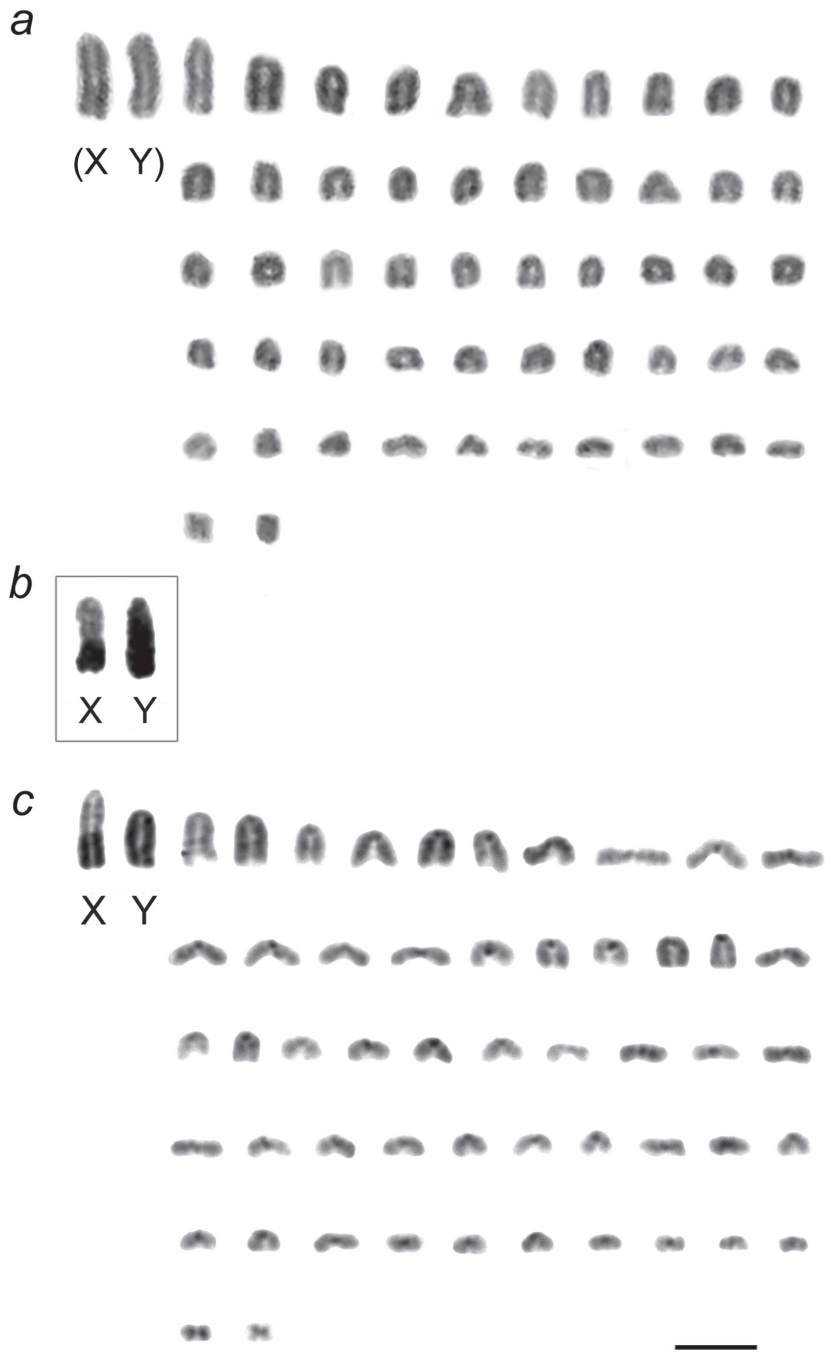

Figure 2a-b. Mitotic chromosomes of East Siberian and East European *M. rossiaemeridionalis*: **a** conventionally stained chromosomes of the male from Olkhon Island arranged by size, with provisional identification of the XY sex chromosome pair, based on chromosome length **b** C-banded sex chromosomes of the same individual **c** C-banded chromosomes of a male from the east bank of the river Volga. Bar=10 μm.

However, the length of the X chromosome varied between voles from the two geographically distant regions. In the male from Olkhon Island both sex chromosomes looked alike in routinely stained karyograms and did not exceed considerably in size the largest autosome (Fig. 2a). They differed each from the other only by C-banding, and in this case the distal heterochromatic block marking the X occupied less than a half of its total length (Fig. 2b). Similar proportions in length of the sex chromosomes were seen in meiotic plates of this individual showing X and Y stick configuration. Autosomes formed bivalents during meiosis whereas sex chromosomes remain asynaptic (Fig. 3).

In stark contrast, in voles from the European sample the X was larger than either the Y or the larger autosomes, and this can only be due to a larger amount of telomeric heterochromatin occupying the distal half of the X chromosomes in males (Fig. 2c) as well as in a female studied.

Discussion

The chromosomal characteristics of the specimens studied are consistent with the karyotypic features of the 54-chromosome sibling species of the common vole, a member of the *Microtus arvalis* group (Common Vole: The sibling species 1994). Two chromosomal sibling species of common voles, *Microtus arvalis* s. str. (2n=46) and *M. rossiaemeridionalis* (2n=54), can be recognized simply by the diploid number, wherever they occur. Localities of their separate or common distribution have been progressively sampled since the first description of the karyotypes in European Russia (Meyer et al. 1969) and have been many times updated, with the most comprehensive lists having been provided by Král et al. (1980), Common Vole: The sibling species (1994) and Meyer et al. (1996). For the 54-chromosome species, the first descriptions were obtained from the East European Plain and Caucasus, but there were few from neighboring Asian regions. In particular, Meyer et al. (1996) listed two geographic sites in Kazakhstan and two more in southern and eastern Siberia which indicated the border of species distribution eastwards. The most eastern finding was from Mount Khashkai, about 250 km to the west of Lake Baikal, Nukuty Distr., Irkutsk Prov. (ca. 53°40'N; 102°30'E, inferred from the map). Unfortunately, no details of those karyotypes were provided. Also there are no karyotypic details of the few individuals of *M. rossiaemeridionalis* previously obtained from Saratov Province (including the village of Dyakovka) for the electrophoretic analysis of haemoglobin in the blood (Tikhonova et al. 2005).

It is interesting that although the ranges of the two sibling species, *M. arvalis* and *M. rossiaemeridionalis*, significantly overlap in Eastern Europe and in their Asian parts; these species have been found, in general, to be separated in Siberia and Kazakhstan (Meyer et al. 1996). Since the 1990s, however, the 54-chromosome karyotype was detected in common voles from Novosibirsk (Yakimenko and Kryukov 1997, Mazurok et al. 1995, Fig.1), even though only 46-chromosome *M. arvalis* specimens were re-

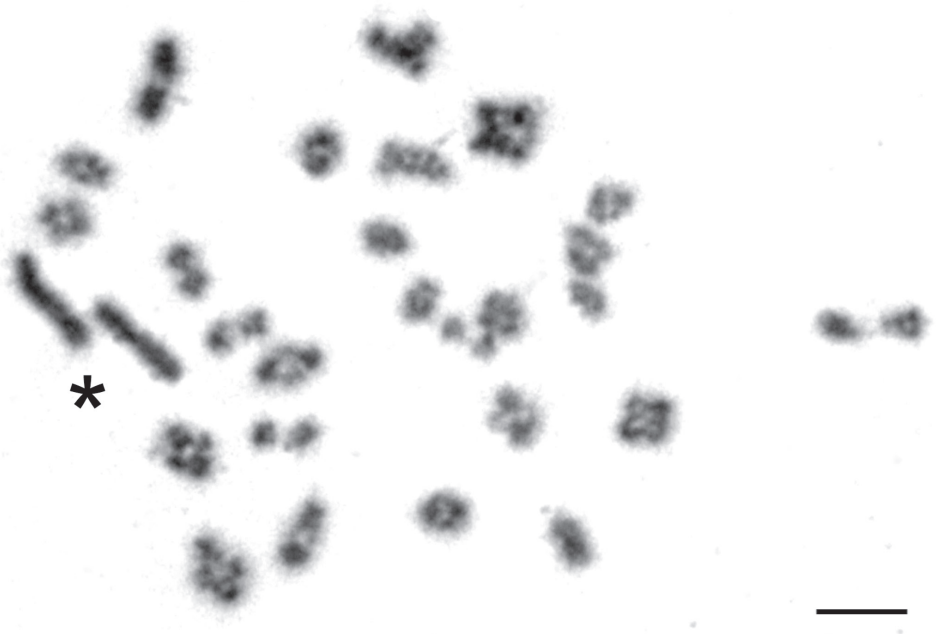

Figure 3. Meiotic spread with 26 autosomal bivalents and the characteristic asynaptic configuration of the sex chromosomes (star) from a male common vole of Olkhon Island ($2n$=54). Bar=10 μm.

ported in earlier studies from the same geographical region (Král and Liapunova 1975, Král et al. 1980)

Further chromosomal studies added only a few occurrence sites for *M. rossiaemeridionalis* in Kazakhstan (Kovalskaya 1994), Western Siberia (close to Novosibirsk; Yakimenko and Kryukov 1997), Trans-Volga (Baskevich et al. 2008), and, finally, in the Far East (vicinity of Sovetskaya Gavan city in Khabarovsk Terr.; Kartavtseva et al. 2011). Since Malygin (1983), the findings of *M. rossiaemeridionalis* were assumed to follow generally the Transsiberian transport system, thus suggesting a human-induced way of introducing this vole eastwards. The reported occurrence of *M. rossiaemeridionalis* on Olkhon Island is one of the most eastern locations of the species and of particular interest, because it represents an isolated island population. Olkhon is the largest island of Lake Baikal (71 km in length and about 12 km in width or 730 km^2) and has been geographically isolated for 0.7-0.8 million years (Galazij 2005, Agafonov and Akulov 2006). Considering the probable long autonomous existence of the vole population on the island, fixation of an unusual karyotype might have been expected, and this was in fact observed through a transformed X-chromosome (Fig. 2). Even if the voles were actually introduced within a historically short period, some dozens of years ago – coinciding with the age of the Transsiberian railway system – this observation adds to the little known intraspecies karyotypic variability in *M. rossiaemeridionalis*. Similar variation in the X-chromosome due to a reduced amount of heterochromatin has

been reported for 54-chromosome voles from two geographically distant populations, i.e. one from western Siberia in the vicinity of Novosibirsk (Yakimenko and Kryukov 1997) and the other from the Arctic island of Svalbard (Fredga et al. 1990). In both those cases, a rearrangement was detected in a single X-chromosome of a sole specimen among a few studied individuals and interpreted as the deletion of a heterochromatic part in the X-chromosome (Fredga et al. 1990, Yakimenko and Kryukov 1997).

Meiotic preparations in a male from Olkhon Island (Fig. 3) revealed that the sex chromosomes remain asynaptyc which is typical for *M. rossiaemeridionalis* and the related species (Borodin et al. 1995, Mitsainas et al. 2010).

Cytogenetically, our findings indicate that in the *M. rossiaemeridionalis* karyotype, which otherwise is being considered rather stable, there is an X chromosome predisposition to intraspecific variation. Our data indicate that the variation affects the heterochromatic part of the X chromosome and have shown the value of karyotypic investigations on new and especially extreme localities in uncovering new karyotypic variability. Even in a rather invariant species like *M. rossiaemeridionalis*, such studies are worthwhile.

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