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RESEARCH ARTICLE

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Cytogenetics of the Porthole Shovelnose Catfish, *Hemisorubim platyrhynchos* (Valenciennes, 1840) (Siluriformes, Pimelodidae), a widespread species in South American rivers

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Abstract

Hemisorubim platyrhynchos is a medium- to large-sized pimelodid catfish distributed along several river basins of the Neotropical Region, noteworthy for representing an important fishery source. In this work, *H. platyrhynchos* from three isolated populations were cytogenetically analyzed. The karyotype shows a diploid number of 2n=56 chromosomes comprising 22m, 16sm, 10st, 8a (FN=104). NORs detected by $AgNO_3$ were located in the terminal regions of the short arm of a st chromosome pair, as confirmed by CMA_3 and FISH using an 18S rDNA probe. C-banding revealed a small amount of heterochromatin in chromosomes, including the NORs, and one biarmed pair that showed conspicuous positive bands on both arms. This fact was also evidenced when using other banding techniques, such as RE (*Alu*I), and indicates that this pair constitutes a species-specific cytogenetic marker.

Keywords

Hemisorubim platyrhynchos, Pimelodidae, Cytogenetics

Introduction

Hemisorubim platyrhynchos (Valenciennes, 1840), popularly called "jurupoca" or porthole shovelnose catfish, is a pimelodid fish inhabiting the deeper and slow-moving sections of large South American rivers (Burgess 1989, Froese and Pauly 2012). This species is the sixth largest pimelodid of the Pantanal region/Brazil (Penha et al. 2004) and is considered an important species in the fisheries of the Paraná River basin (Agostinho et al. 1995). Its body shape and color pattern are adapted to the muddy bottom where it stays. It feeds on benthic organisms and fish (Froese and Pauly 2012). Breeding and sexing information have not been reported. The extensive exploitation of its populations due to overfishing could affect its genetic variability, which is still poorly known.

The family Pimelodidae represents one of the most specious catfish groups, however relationships among species of this group still remain as unanswered questions; however, it seems self-evident that they share certain characteristics (Nelson 2006, Ferraris 2007). Some authors has been divided this family in "groups" i.e. *Calophysus* Müller and Troschel in Müller 1843, *Pimelodus* LaCépède, 1803 and "Sorubiminae" (de Pinna 1998).

From a cytogenetic point of view some reports show that these groups could also share cytogenetic characteristics, supporting additionally the classification above mentioned (Swarça et al. 2007, Sanchez et al. 2010, Carvalho et al. 2011).

Hemisorubim platyrhynchos is a monotypic species that belongs to the family Pimelodidae, however, it is considered one of the "sorubimine catfishes", an informal group of catfish that comprises other genera such as *Sorubim* Cuvier, 1829, *Pseudoplatystoma* Bleeker, 1862, and *Brachyplatystoma* Bleeker, 1862 (Lundberg and Akama 2005).

Until now only one population of *H. platyrhynchos* of the Parana River (Brazil) has been cytogenetically studied and has had its diploid number, AgNORs location and C-banding reported (Martins-Santos et al. 1996).

The objective of the present study was to describe the karyotypic structure of specimens from three populations of *H. platyrhynchos* aiming to characterize and compare the obtained results with the available cytogenetic data on this and other related species.

Material and methods

Fifteen specimens of *Hemisorubim platyrhynchos* consisting of 8 males (m) and 5 females (f), caught in the Parana River (Corrientes State, Argentina) and 2 specimens of undetermined sex from the Miranda River (Mato Grosso do Sul State, Brazil) were cytogenetically analyzed. The sampling sites in the Paraná River were: Ituzaingó (2 m), Itá Ibaté (2 f - 3 m), Yahapé (1 m), Puerto Abra (1 f), and Corrientes (2 f / 2 m) (Corrientes Province). Mitotic chromosome preparations were obtained according to the technique described by Foresti et al. (1993) for some specimens of Paraná River/Argentina and from blood culture for specimens of the Miranda River/Brazil (Fenocchio and Bertollo 1988). The specimens were deposited in the collection of the Laboratory of

the Instituto de Ictiología del Nordeste, Universidad Nacional del Nordeste/Argentina. Nucleolus organizer regions (Ag-NORs) were revealed by the silver-staining method (Howell and Black 1980) and C-banding was performed according to Sumner (1972). Restriction endonucleases were used according to Sánchez et al. (1990) with some modifications, i.e., *Alu.* I was used a concentration of 0.3 U/μL and the chromosome preparations were treated at 37°C for 4h. Chromosome staining with Chromomycin A_3 (CMA_3), a C-G specific fluorochrome, was applied according to the description provided by Verma and Babu (1995). Fluorescent *in situ* hybridization (FISH) was carried out by means of biotinylated 18S rDNA probes (segment with 1700 pb) obtained from the nuclear DNA of the fish *Oreochromis niloticus* (Linnaeus, 1758) labeled with biotin-14-dATP by nick translation (Gibco cat Nº 18247-015), according to the manufacturer's instructions. The hybridization technique, post-hybridization washes and visualization were carried out as reported by Swarça et al. (2001). The chromosomes were classified according to their arm ratios as metacentrics (m), submetacentrics (sm), subtelocentrics (st), and acrocentrics (a) (Levan et al. 1964, Guerra 1986) with modifications. The m, sm, st were considered as biarmed chromosomes and acrocentrics were considered as uniarmed chromosomes.

Results

All three populations of *H. platyrhynchos* presented the same results. The diploid number obtained was 2n=56 and the karyotype was composed of 22m+16sm+10st+8a (NF=104) without chromosomal differences between sexes (Fig. 1).

The AgNORs were located in the terminal position on the short arm of a subtelocentric (st) pair (Figs 2a, b). The bright signals correspond to the zones evidenced by argentic impregnation after FISH with the 18S rDNA probe and staining with CMA_3 (Figs 2c, d). This chromosome pair is clearly identified due to its size, shape and exclusive secondary constriction. C-banding revealed positive bands in the pericentromeric

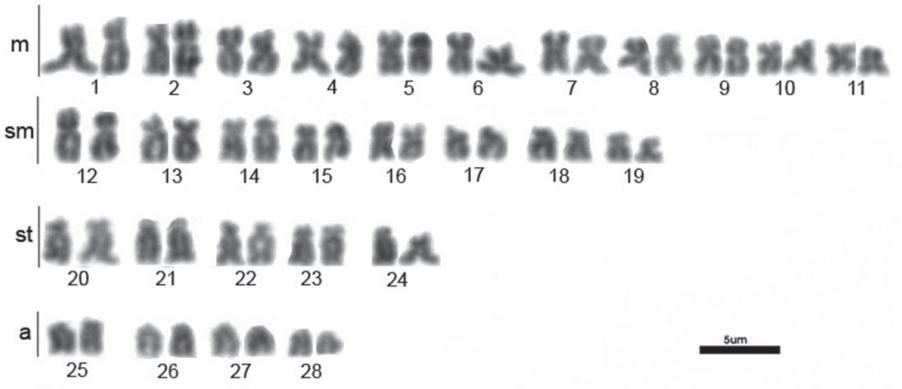

Figure 1. Karyotype of *Hemisorubim platyrhynchos*. Bar = 5mm.

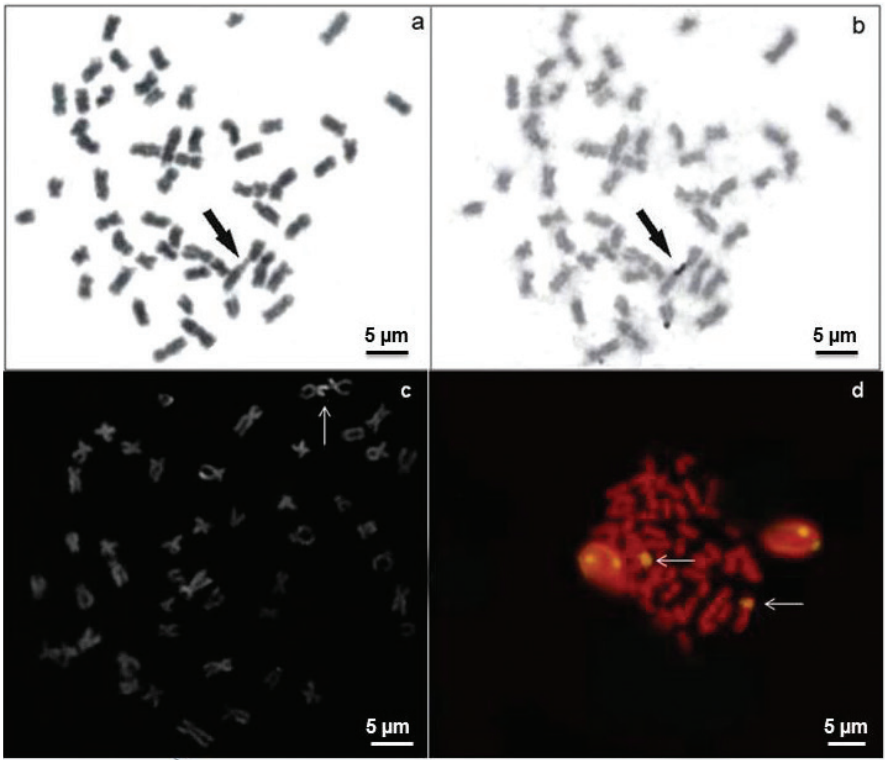

Figure 2. Metaphases of *Hemisorubim platyrhynchos* showing sequential Giemsa-$AgNO_3$ staining (**a, b**) CMA_3 banding (**c**) FISH with 18S rDNA probe (**d**). Arrows indicate the NOR- bearing chromosomes.

regions of some chromosome pairs and on the short arms of a st chromosome pair, coincident with positive C-bands and allowed the identification of a large biarmed marker pair with positive bands on almost the entire short and long arms (Fig. 3b). The *Alu*I restriction enzyme shows bands that resemble C-banding, principally on the biarmed chromosome, producing a reverse pattern (Fig. 3c). The mentioned chromosome could be considered a species-specific cytogenetic marker, since it has not been reported in other species of this group of fish.

Discussion

The karyotype of *H. platyrhynchos* was composed of 22m+16sm+10st+8a (NF=104), however, despite having the same diploid chromosome number 2n=56, *H. platyrhynchos* from the Paraná River/Brazil reported by Martins-Santos et al. (1996) presented 22m + 18sm + 6st + 10a (NF=102, recalculated in the present paper). These variations could be ascribed to chromosome rearrangements, although when alternatively using parsimony criteria, differences could be attributed to the condensation of the chromosome arms and/or to technical artifacts. In the family Pimelodidae, 23 of the 27

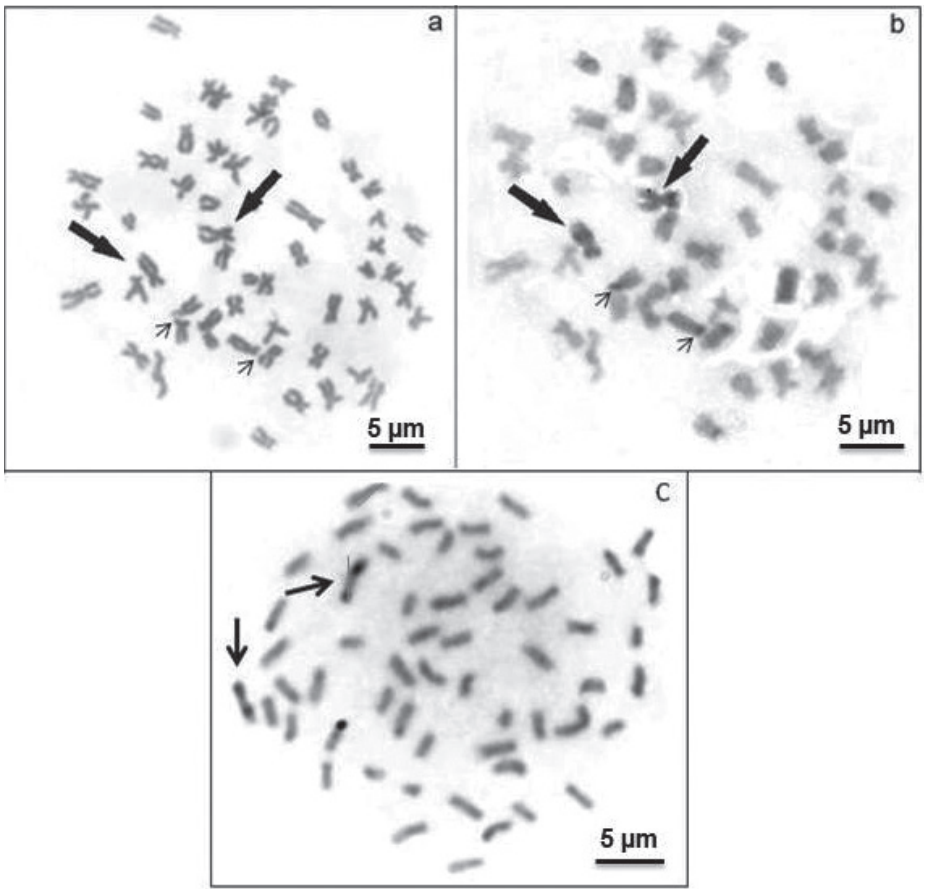

Figure 3. Metaphases of *Hemisorubim platyrhynchos* showing sequential Giemsa-C banding (**a, b**) and after *Alu*I treatment (**c**). The arrows indicate the biarmed chromosome pair (marker) with positive bands on the short and long arms, the thin arrows show the NOR-bearing chromosomes.

karyotyped species have a diploid number of 2n= 56 chromosomes, except for *Calophysus macropterus* Lichtenstein, 1819, *Luciopimelodus pati* (Valenciennes, 1840), and *Pinirampus pirinampu* (Spix & Agassiz, 1829) with 2n=50 and *Megalonema platanum* (Günther, 1880) with 2n=54, which seem to share other characteristics (Swarça et al. 2007).

One point worth emphasizing is the homogeneity of the karyotypes of species belonging to the "Sorubiminae group" with a clear prevalence of biarmed chromosomes, showing a high fundamental number. A cytogenetic feature shared by all species of this group is the AgNORs localized in the terminal position on the short arm of one pair of st/a chromosomes that also could be evidenced by C-banding, as observed in the present study and in other studied species, such as *Sorubim lima* (Bloch & Schneider, 1801) (Fenocchio and Bertollo 1992, Martins-Santos et al. 1996), *P. corruscans* (Spix & Agassiz, 1829) and *P. tigrinus* (Valenciennes, 1840) (Fenocchio and Bertollo 1992, Martins-Santos et al. 1996, Swarça et al. 2005a), *Zungaro zungaro* (Humboldt, 1821)

(Martins-Santos et al. 1996, Swarça et al. 2001), *Steindachneridion scriptum* (Miranda-Ribeiro, 1918) (Swarça et al. 2005b) and *Steindachneridion sp* (=*melanodermatum*) (Garavello, 2005) (Swarça et al. 2006).

CMA_3 staining and FISH with 18S rDNA exhibited fluorescent signals that correspond to the AgNOR sites (Fig. 2c, d). This correspondence between AgNORs, C-banding, FISH and CMA_3 staining has already been observed in almost all species of the Pimelodidae family (Swarça et al. 2001, 2008).

The relatively low amount of heterochromatin in chromosomes of *H. platyrhynchos* and in other species of the Pimelodidae catfishes suggests that this may be a characteristic of this family. On the other hand, C-banding allowed the identification of a large biarmed pair with positive bands on almost the entire short arm and on the long arm. The *Alu*I restriction enzyme on fish chromosomes produces a C-banding-like pattern (Maistro et al. 2000) and this was also observed in *H. platyrhynchos* chromosomes (Fig. 3c). This chromosome pair could be considered a species-specific cytogenetic marker, as it has not been reported in other Pimelodidae (Fig. 3b).

According to cytogenetic traits, this family could be divided into two: the "*Pimelodus* group" and the "Sorubiminae group" (=Sorubinae), and the cytogenetic data confirm that the analyzed species belongs to the second group, because it has 2n=56 chromosomes, a high NF and the NORs localized on one single chromosome pair in the terminal position of the short arms, as it occurs with the other species of this group (Swarça et al. 2007). Thus, despite its wide geographic distribution, it is evident that *H. platyrhynchos* shows a marked conservatism in its basic karyotype macrostructure, differing from many species of the Pimelodidae family, i.e., the "*Pimelodus* group", which presents a wide karyotypic variability, even within the same hydrographic basin and within the same river.

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The significance of cytogenetics for the study of karyotype evolution and taxonomy of water bugs (Heteroptera, Belostomatidae) native to Argentina

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Abstract

Male meiosis behaviour and heterochromatin characterization of three big water bug species were studied. *Belostoma dentatum* (Mayr, 1863), *B. elongatum* Montandon, 1908 and *B. gestroi* Montandon, 1903 possess 2n = 26 + X_1X_2Y (male). In these species, male meiosis is similar to that previously observed in *Belostoma* Latreille, 1807. In general, autosomal bivalents show a single chiasma terminally located and divide reductionally at anaphase I. On the other hand, sex chromosomes are achiasmatic, behave as univalents and segregate their chromatids equationally at anaphase I. The analysis of heterochromatin distribution and composition revealed a C-positive block at the terminal region of all autosomes in *B. dentatum*, a C-positive block at the terminal region and C-positive interstitial dots on all autosomes in *B. elongatum*, and a little C-positive band at the terminal region of autosomes in *B. gestroi*. A C-positive band on one bivalent was DAPI negative/CMA_3 positive in the three species. The CMA_3-bright band, enriched in GC base pairs, was coincident with a NOR detected by FISH. The results obtained support the hypothesis that all species of *Belostoma* with multiple sex chromosome systems preserve NORs in autosomal bivalents. The karyotype analyses allow the cytogenetic characterization and identification of these species belonging to a difficult taxonomic group. Besides, the cytogenetic characterization will be useful in discussions about evolutionary trends of the genome organization and karyotype evolution in this genus.

Keywords
Heteroptera, holokinetic chromosomes, karyotype evolution mechanisms, multiple sex chromosomes, rDNA-FISH

Introduction

Belostomatidae include some of the largest heteropteran species, which are general predators that play an important role as biological agents in aquatic environments (Menke 1979, Smith 1997, Saha et al. 2010). This family has a cosmopolitan distribution in tropical and subtropical areas in the world (Schnack 1976, Polhemus and Polhemus 2008). In South America, the genus *Belostoma* Latreille, 1807 is the most diverse and includes 61 species mainly distributed from Colombia and Brazil to Argentina and Chile (Heckman 2011). Nevertheless, cytogenetic reports in *Belostoma* from South America comprise the male chromosome complement of 15 species and male meiosis analysis of 13 species (Table 1). Ten of these species show a modal diploid chromosome number 2n = 29 = 26 + X_1X_2Y (male) and five species possess reduced chromosome numbers and a simple sex chromosome system XY/XX (male/female) (Table 1).

All species of *Belostoma* analyzed possess holokinetic chromosomes, i.e. chromosomes without a primary constriction and therefore without a localized centromere. Autosomal bivalents are synaptic and chiasmatic, whereas sex chromosomes are asynaptic and achiasmatic, and behave as univalents in first male meiotic division. However, at metaphase II sex chromosomes associate end-to-end through the so called touch-and-go pairing, forming a pseudo-bivalent or pseudo-multivalent. In the first meiotic division, autosomal bivalents segregate reductionally while sex chromosomes divide equationally (Ueshima 1979, Papeschi and Bidau 1985, Suja et al. 2000, Papeschi and Bressa 2006, Bardella et al. 2012). During meiosis, the kinetic activity is restricted to the chromosome ends and the chromosomes can be regarded as telokinetic (Motzko and Ruthmann 1984).

Most hypotheses on karyotype evolution in Heteroptera include both autosomal and sex chromosome fusions and fragmentations (Ueshima 1979, Manna 1984, Thomas 1987, Papeschi 1994, 1996, Pérez et al. 2004). The cytogenetic data available for *Belostoma* allow to hypothesize that current karyotypes with a multiple sex chromosome system (X_nY/X_nX_n, male/female) are derived through fragmentation of the ancestral X from an XY sex chromosome system. On the other hand, reduced chromosome complements with simple sex chromosome system (XY/XX, male/female) have probably originated through several chromosomal fusions (Papeschi 1996, Papeschi and Bressa 2006, Bardella et al. 2012).

The aim of this study was to perform a detailed comparison of male meiosis behaviour and examine the structure of the holokinetic chromosomes by means of C- and fluorescent bandings, and fluorescent *in situ* hybridization (FISH) with 18S rDNA probes in *Belostoma dentatum* (Mayr, 1863), *B. elongatum* Montandon, 1908 and *B. gestroi* Montandon, 1903. The female complement and the male meiosis of *B. elonga-*

Table 1. Diploid chromosome number, chromosome bandings and nucleolar organizer region (NOR) detected by FISH in South American *Belostoma* species. *A: autosomal bivalent, **X, Y: sex chromosomes

Species	2n (male)	C bands	DAPI/ CMA_3 bands	rDNA by FISH	References
Belostoma bergi (Montandon),1899	26 + X_1X_2Y	no	no	--	Papeschi and Bressa 2004
B. bifoveolatum Spinola, 1852	26 + X_1X_2Y	yes	yes	--	Papeschi 1991, Chirino and Bressa 2011
B. candidulum Montandon, 1903	14 + XY	yes	yes	--	Bardella et al. 2012
B. cummingsi De Carlo, 1935	26 + X_1X_2Y	no	no	--	Papeschi and Bidau 1985
B. dentatum (Mayr, 1863)	26 + X_1X_2Y	yes	yes	A*	Papeschi and Bidau 1985, Papeschi 1991, this study
B. dilatatum (Dufour, 1863)	26 + X_1X_2Y	yes	no	--	Papeschi 1992
	26 + X_1X_2 X_3Y	yes	yes	--	Bardella et al. 2012
B. discretum Montandon, 1903	26 + X_1X_2Y	yes	yes	--	Papeschi 1992, Chirino and Bressa 2011
B. elegans (Mayr, 1871)	26 + X_1X_2Y	yes	yes	A*	Papeschi 1988, 1991, Papeschi and Bidau 1985,
					Papeschi and Bressa 2006
B. elongatum Montandon, 1908	26 + X_1X_2Y	yes	yes	A*	Papeschi and Bressa 2006, this study
B. gestroi Montandon, 1903	26 + X_1X_2Y	yes	yes	A*	Papeschi and Bressa 2006, this study
B. martini (Montandon, 1899)	26 + X_1X_2Y	yes	no	--	Papeschi 1991
B. micantulum (Stål, 1860)	14 + XY	yes	yes	X, Y*	Papeschi 1988, Papeschi and Bressa 2006
B. orbiculatum Estévez & Polhemus, 2001	14 + XY	yes	yes	--	Papeschi 1996, Chirino and Bressa 2011
	14 + X_1X_2Y				Papeschi 1996
B. oxyurum (Dufour, 1863)	6 + XY	yes	yes	X,Y**	Papeschi 1988, 1995, Papeschi and Bressa 2006
B. plebejum (Stål, 1858)	14 + XY	no	no	--	Papeschi 1994
	13 + XY				
	14 + X_1X_2Y				

tum and *B. gestroi* are described for the first time. These results allowed us to distinguish morphologically similar species and, also, led us to propose a scenario of karyotype evolution in the genus *Belostoma*.

Material and Methods

Insects

For meiotic analysis, adults and nymphs of *B. elongatum* (9 males and 8 females) and *B. gestroi* (4 males and 12 females) were collected from 1988 to 1990 in several fields

Table 2. Species, provenience, geographical coordinates, and number of adults' collected and examined of *Belostoma* for chromosomal analyses discriminated by gender.

Species	Chromosomal analyses	Localities from Argentina	Coordinates	N° of individuals
B. dentatum	C- and DAPI-CMA$_3$ bandings	San Pedro, Buenos Aires	33°40'33"S, 59°39'47"W	3 males
	FISH technique	Corrientes, Corrientes	27°28'16"S, 58°50'22"W	1 female
B. elongatum	Chromosome complement	Arroyo Cuay Grande, Corrientes	28°28'16"S, 58°50'22"W	1 female
	Male meiotic behaviour	Lagos de Stieler, Misiones	26°34'2"S, 54°45'57"W	1 male
		Valle Hermoso, Misiones	26°23'10"S, 54°27'58"W	8 males, 7 females
	C- and DAPI-CMA$_3$ bandings FISH technique	Corrientes, Corrientes	27°28'16"S, 58°50'22"W	3 males
B. gestroi	Chromosome complement	Río San Pedro, Buenos Aires	33°40'33"S, 59°39'47"W	1 male
	Male meiotic behaviour	Rincón Norte, Santa Fe	31°36'4"S, 60°34'12"W	3 males, 11 females
		Santa Rosa, Santa Fe	31°26'00"S, 60°22'00"W	1 female
	C- and DAPI-CMA$_3$ bandings FISH technique	Corrientes, Corrientes	27°28'16"S, 58°50'22"W	2 males, 1 female

from Buenos Aires, Santa Fe, Entre Ríos, Corrientes and Misiones provinces, all in Argentina (Table 2). For chromosome bandings and fluorescent *in situ* hybridization (FISH) technique, adults of *B. dentatum* (3 males and 1 female), *B. elongatum* (3 males) and *B. gestroi* (2 males and 1 female) were collected from 2010 to 2011 in Corrientes province (Argentina) (Table 2). Collected adults were identified according to the keys provided by Schnack (1976) and Heckman (2011).

Chromosome preparations

The captured specimens were brought alive to the laboratory and reared until their gonads were dissected out. For meiotic analysis, the adults and nymphs were fixed for 15–30 min in freshly prepared fixative (ethanol:glacial acetic acid, 3:1). Afterwards, gonads were dissected out and kept at 4º C in 70% ethanol. Slides were prepared by the squash technique in a drop of 2% iron-propionic haematoxylin following conventional procedures (Sáez 1960). For C- and fluorescent bandings, and FISH technique, gonads were dissected in a physiological saline solution for *Ephestia* Guenée, 1845 (Glaser 1917: cited by Lockwood 1961), swollen for 15 min in a hypotonic solution (0.075 M KCl), and fixed for 15-30 min in freshly prepared Carnoy fixative

(ethanol:chloroform:glacial acetic acid, 6:3:1). Spread chromosome preparations were made in a drop of 60% acetic acid with the help of tungsten needles and spread on the slide using a heating plate at 45° C as described in Traut (1976). The preparations were dehydrated in an ethanol series (70, 80 and 96%, 30 sec each) and stored at -20º C until use.

Chromosome bandings

Heterochromatin content, distribution and nucleotide composition were analysed by means of C- and sequential fluorescent DAPI and CMA_3 bandings. C-banding was performed according to Papeschi (1988), and the pre-treated slides were stained with 4'6-diamidino-2-phenylindole (DAPI; Fluka BioChemika, Sigma Aldrich Production GmbH, Buchs, Switzerland) for a better resolution of C-bands (Poggio et al. 2011). Fluorescent banding with AT-specific DAPI and GC-specific chromomycin A_3 (CMA_3; Fluka BioChemika) was carried out following Poggio et al. (2011).

Fluorescence *in situ* hybridization

Unlabelled 18S ribosomal DNA (rDNA) probes were generated by polymerase chain reaction (PCR) using universal arthropod primers: forward 5′-CCTGAGAAACG-GCTACCACATC-3′ and reverse 5′-GAGTCTCGTTCGTTATCGGA-3′ (Whiting 2002). Total genomic DNA of *Dysdercus albofasciatus* Berg, 1878, obtained by standard phenol-chloroform-isoamylalcohol extraction, was used as a template. PCR was done following the procedure described in Fuková et al. (2005). The PCR product showed a single band of about 1,000 bp on a 1% agarose gel. The band was recovered from the gel and purified by using a QIAquick Gel Extraction Kit (Quiagen GmbH, Hilden, Germany). The 18S rDNA fragment was re-amplified by PCR and then labeled with biotin 14-dATP by nick translation using a BioNick Labeling System (Invitrogen, Life Technologies Inc., San Diego, CA, USA). FISH with a biotinylated 18S rDNA probe was carried out following the procedure in Sahara et al. (1999) with several modifications described by Fuková et al. (2005) and Bressa et al. (2009).

C-positive heterochromatin and DNA measurements

Data of C-positive heterochromatin percentage and the haploid DNA content in *B. dentatum*, *B. elongatum* and *B. gestroi* are part of the results obtained by Papeschi in her Ph.D. Thesis (1992). The procedures of C-positive heterochromatin percentage and the haploid DNA content were published by Papeschi in 1991 and 1988, respectively. Hence, these results were mentioned only in this paper to analyze and discuss the extent of karyotype uniformity in these three species. Briefly, the study of the C-

positive heterochromatin measurements was performed on meiotic cells. For each species at least 10 C-banded cells at diakinesis and without superimposed bivalents were photographed. The percentage of C-positive heterochromatin was calculated as the C-positive area divided by the total chromosome area. The DNA content was carried out by Feulgen microdensitometry (Papeschi 1988) in individuals fixed similar time ago (1–3 months) (Papeschi 1991).

Statistical analysis

The total chromosome length measurements (TCL) were performed with Micro Measure for Windows, version 3.3. The TCL of all bivalents and sex chromosomes were performed in metaphase I. Differences in TCL among species were compared by using one-way analysis of variance (ANOVA), with Fisher adjusted a posterior contrast. Statistical analyses were done using Statview software (SAS Institute Inc., 1992-1998).

Microscopy, photographs and image processing

Preparations were observed in epifluorescence microscopes: Zeiss Laborlux (Carl Zeiss, Germany) equipped with an analogue camera and Leica DMLB equipped with a Leica DFC350 FX CCD camera and Leica IM50 software, version 4.0 (Leica Microsystems Imaging Solutions Ltd., Cambridge, UK). Photomicrographs from meiotic chromosome preparations were taken using Kodak colour Supra print film 400 ASA. Black-and-white images of chromosomes from C- and fluorescent bandings and FISH technique were recorded separately for each fluorescent dye with the CCD camera. Images were pseudo-coloured (light blue for DAPI, green for CMA_3, and red for Cy3), and processed with an appropriate software.

Results

Male chromosome complement and meiosis

Male meiotic karyotypes based on metaphase I autosomal bivalents (II) and sex univalents of *B. dentatum*, *B. elongatum* and *B. gestroi* show a male diploid chromosome number 2n = 13II + X_1X_2Y (Fig. 1). In the three species, the autosomes decrease gradually in size, both X chromosomes differ slightly in size and the Y chromosome is the smallest of the complement. The chromosome complement and male meiotic behaviour of *B. dentatum* have already been described (Papeschi and Bidau 1985). The three species of *Belostoma* show statistical differences in total chromosome length (TCL) ($F_{2, 93} = 8.484$; P = 0.0004), which is higher in *B. dentatum* (39.43 ± 3.72 μm), intermediate in *B. elongatum* (37.03 ± 2.96 μm) and lower in *B. gestroi* (33.31 ± 3.64 μm).

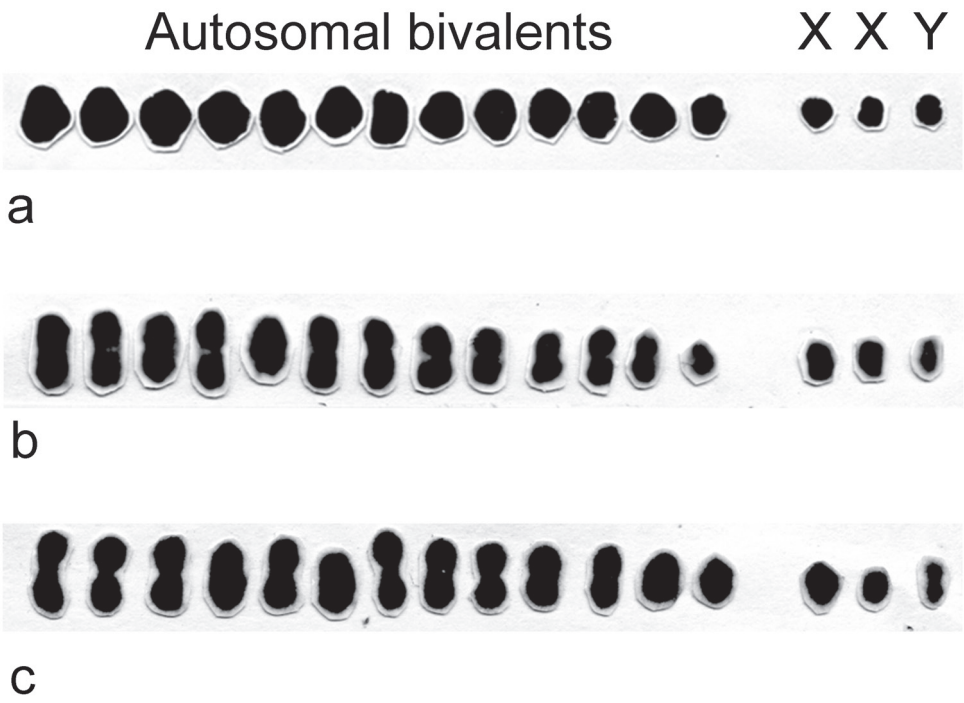

Figure 1. Male meiotic karyotypes of *B. dentatum* (**a**), *B. elongatum* (**b**) and *B. gestroi* (**c**), 2n = 13II + X_1X_2Y, stained with 2% iron-propionic haematoxylin.

Analysis of spermatogonial prometaphase of *B. elongatum* and *B. gestroi* revealed a diploid number of 29 chromosomes; both karyotypes were as described by Papeschi (1992) (Fig. 2a). Male meiotic behaviour in *B. elongatum* and *B. gestroi* was similar and followed the same pattern as previously described for other *Belostoma* species. Thus, we showed a single and combined Figure 2 with meiotic stages from both species. At synizesis, the first meiotic identifiable stage of meiosis, chromatin condenses eccentrically in the nucleus (Fig. 2b). At pachytene, an autosomal bivalent is associated with the nucleolus and the 13 autosomal bivalents are joined through their positive heteropycnotic terminal regions. The condensed sex chromosomes, close to each other, may be distinguished (Fig. 2c). In this cell, the two X chromosomes have a secondary constriction, but these constrictions are observed in only one specimen of *B. elongatum*. During the diffuse stage, all bivalents decondense completely, except for some chromocentres (Fig. 2d). In *B. gestroi*, at early diakinesis, both X chromosomes are negative heteropycnotic, and the Y chromosome is positive heteropycnotic (Fig. 2e, f). At late diakinesis, the three sex univalents and the 13 autosomal bivalents becomes isopycnotic in both species (Fig. 2g, h). Each bivalent has a single chiasma in either subterminal or terminal positions (Fig. 2e–h). At metaphase I, autosomal bivalents arrange in a ring, but the Xs and Y univalents do not show a defined position (Fig. 2i). During anaphase I, the bi-

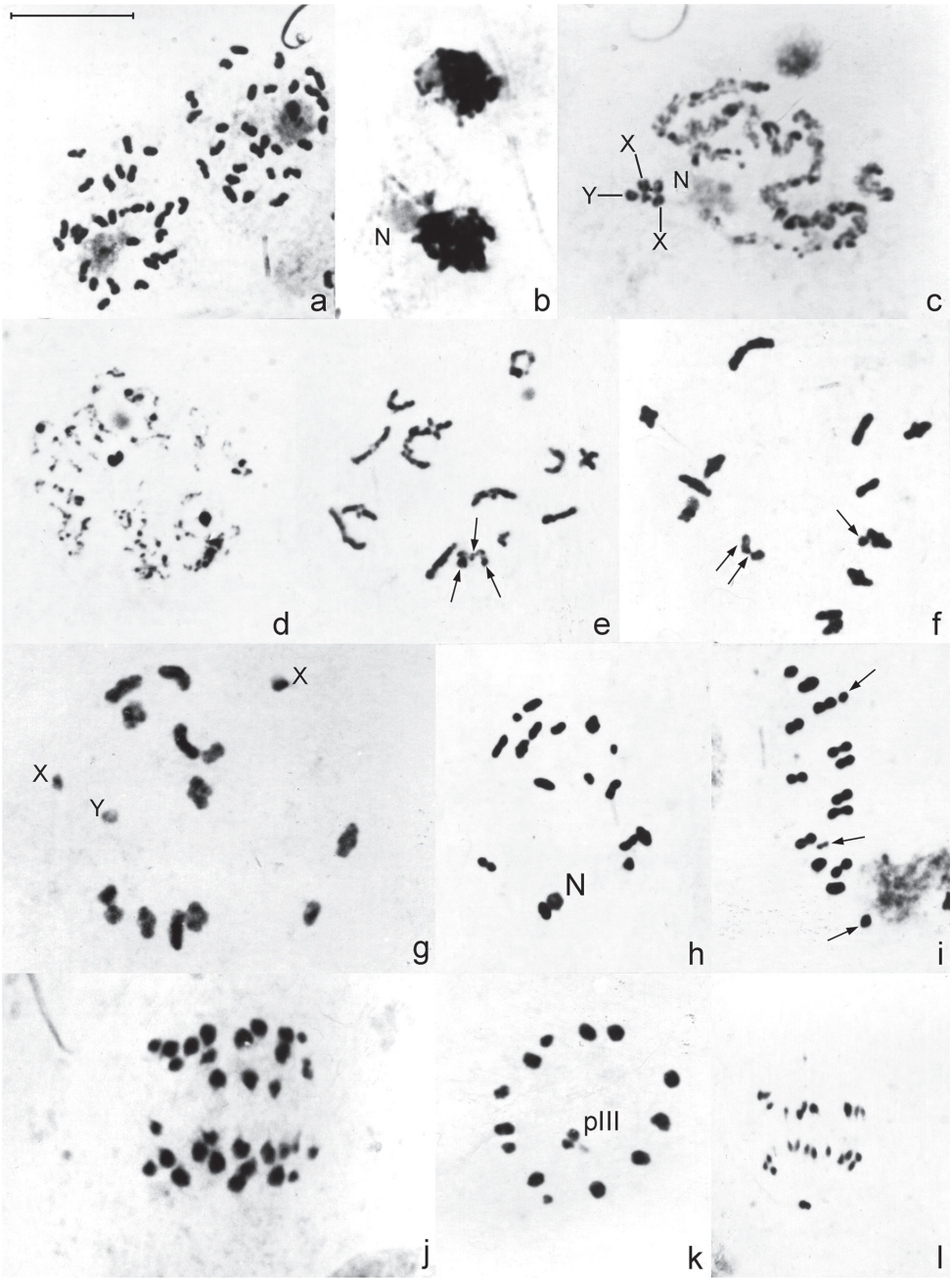

Figure 2. Male meiosis in *B. elongatum* (**b, c, g, j, k**) and *B. gestroi* (**a, d, e, f, h, i, l**) stained with 2% iron-propionic haematoxylin. **a** Spermatogonial prometaphase **b** Synizesis **c** Pachytene, X and Y = sex chromosomes **d** Diffuse stage **e–f** Early diakinesis **g–h** Diakinesis **i** Metaphase I **j** Anaphase I **k** Metaphase II, Y sex chromosome is negatively heteropycnotic **l** Anaphase II. Arrows indicate sex chromosomes. pIII = pseudo-trivalent. N = nucleolus. Bar = 10 μm.

valents divide reductionally, whereas the sex chromosomes do so equationally (Fig. 2j). All telophase I nuclei exhibit 16 chromosomes in each pole (13 + X_1X_2Y). The second meiotic division follows without an interkinesis stage. At metaphase II, the 13 autosomes dispose forming a ring and in the centre of it, the sex chromosomes are associated in a pseudo-trivalent. The Y chromosome is negatively heteropycnotic and is oriented towards the opposite spindle pole to that of X_1 and X_2 (Fig. 2k). At anaphase II, 14 chromosomes migrate to one pole (13 + Y) and 15 to the opposite one (13 + X_1X_2) (Fig. 2l).

Chromosome bandings

C-banding reveals differences in the amount and location of heterochromatin among the three species analysed. In *B. elongatum*, very large C-positive blocks can be detected terminally on all bivalents from prophase I to metaphase I, and interstitial dots are also observed (Fig. 3a–c). In *B. gestroi*, in contrast, C-positive bands are very small and are always located terminally (Fig. 3d, e). The results observed in *B. dentatum* matched data previously described by Papeschi (1991) with C-positive bands terminally located in all bivalents (Fig. 3f, g). Furthermore, the two X chromosomes in the three species show terminally located bands, whereas the Y chromosome is C-negative (Fig. 3a, c–g).

All chromosomes stain homogenously with both fluorochromes on mitotic and meiotic metaphase cells in the three species, except for one of the medium-sized autosomal bivalents in *B. dentatum* (Fig. 4a–c) and *B. elongatum* (Fig. 4d–f), and one of the large-sized in *B. gestroi* (Fig. 4g–i), which show a DAPI negative/CMA_3 positive band at one terminal position.

Location of rDNA

In chromosome preparations of *B. dentatum*, FISH experiments with the 18S rDNA probe show a cluster of rDNA genes located at one end of two homologous chromosomes each (Fig. 5a). A single cluster of signals is observed in an autosomal bivalent at pachytene (Fig. 5b). During diffuse stage, hybridization signals are observed in the decondensed mass of autosomal chromatin, whereas the sex chromosomes remain condensed forming a conspicuous DAPI bright chromatin body without any signals (Fig. 5c). At diakinesis-metaphase I, one medium-sized autosomal bivalent show hybridization signals at both ends (Fig. 5d). In concordance with the results of *B. dentatum*, in mitotic metaphases of *B. elongatum* and *B. gestroi*, hybridization signals are detected in two homologous autosomes (Fig. 5e, g). At diakinesis-metaphase I, a single cluster of rRNA genes is located at both ends of a medium-sized autosomal bivalent of *B. elongatum* (Fig. 5e–f) and of a one large-sized of *B. gestroi* (Fig. 5h).

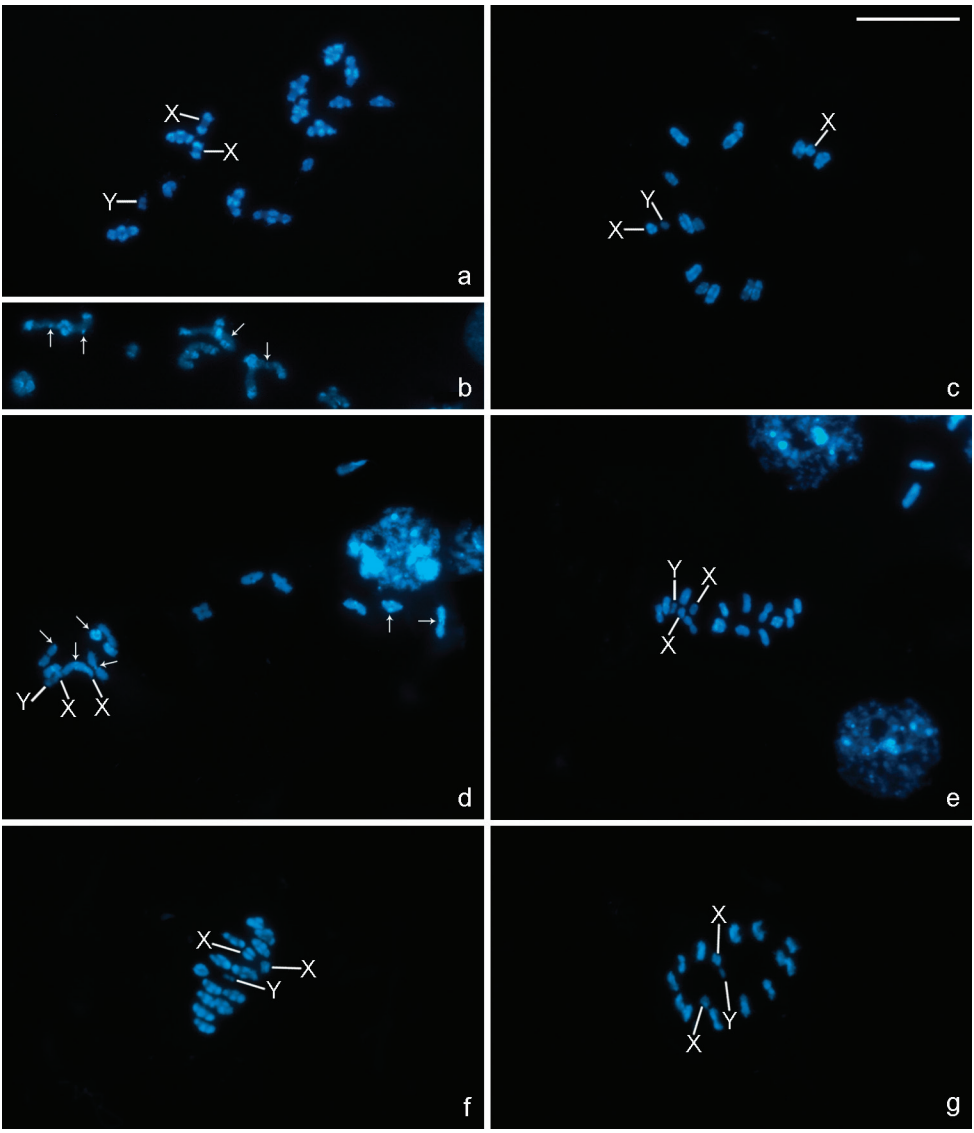

Figure 3. C-banding in chromosomes of *B. elongatum* (**a–c**), *B. gestroi* (**d, e**) and *B. dentatum* (**f, g**) stained with DAPI. **a** Diakinesis, conspicuous terminal C-positive blocks are observed in all autosomal bivalents and both X chromosomes **b** A detail of autosomal bivalents with interstitial C-positive dots (arrows) at early diakinesis **c** Late diakinesis **d** Diakinesis, small terminal C-positive bands in some autosomal bivalents (arrows) **e** Metaphase I **f** Late diakinesis, terminal C-positive bands in all autosomal bivalents and both X chromosomes **g** Metaphase II. **a, c–g** The Y chromosome is C-negative. X, Y = sex chromosomes. Bar = 10 μm.

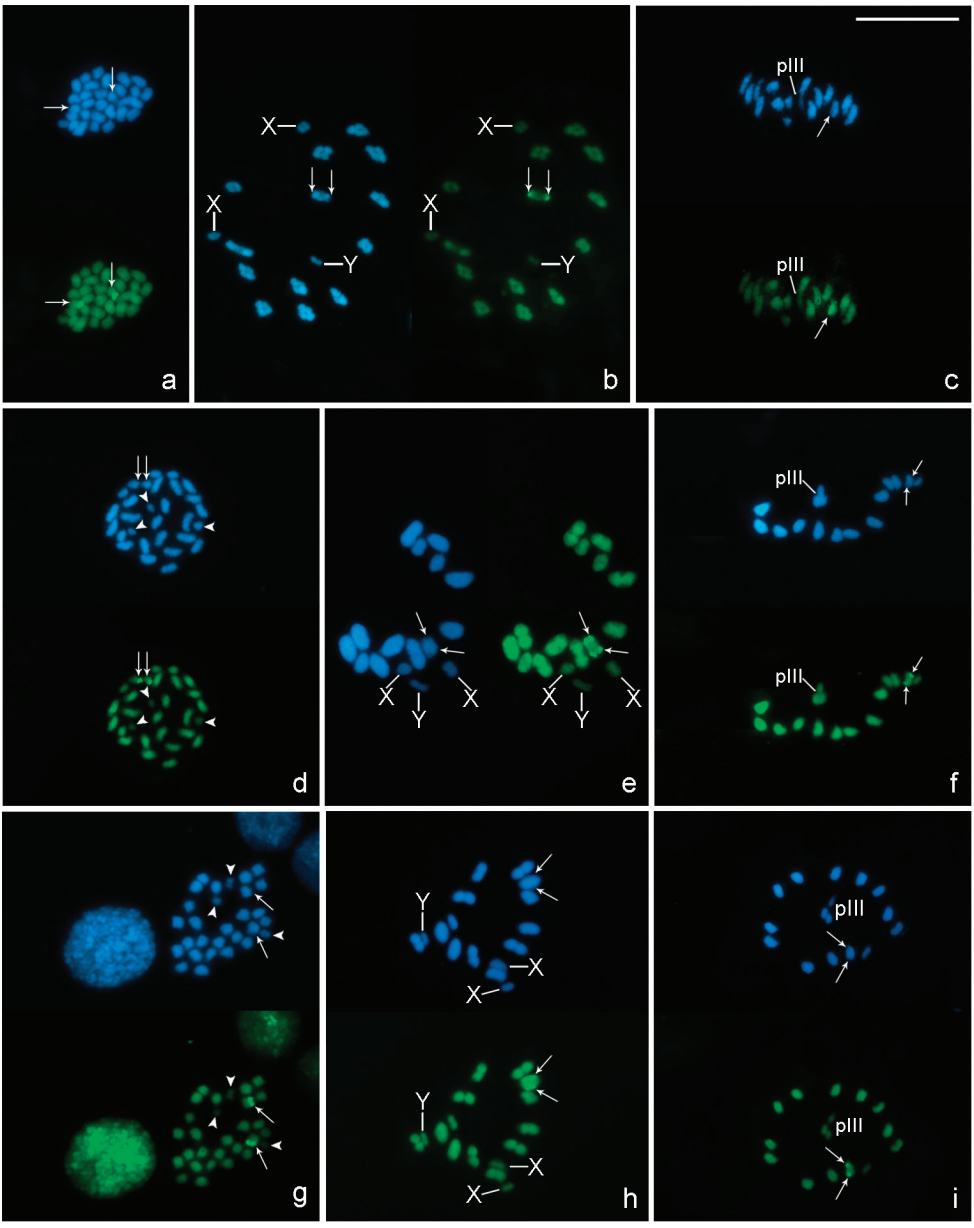

Figure 4. DAPI (blue) and CMA_3 (green) fluorescent banding in chromosomes of *B. dentatum* (**a–c**), *B. elongatum* (**d–f**) and *B. gestroi* (**g–i**). **a** Oogonial metaphase (2n = 30 = 26 + $X_1X_1X_2X_2$) **b** Diakinesis **c** Metaphase II **d** Spermatogonial metaphase (2n = 29 = 26 + X_1X_2Y) **e** Diakinesis **f** Metaphase II **g** Spermatogonial metaphase (2n = 29 = 26 + X_1X_2Y) **h** Diakinesis **i** Metaphase II. Arrows indicate DAPI negative/CMA_3 positive bands. Arrowheads show sex chromosomes (**d, g**). X, Y = sex chromosomes. pIII = pseudo-trivalent. Bar = 10 μm.

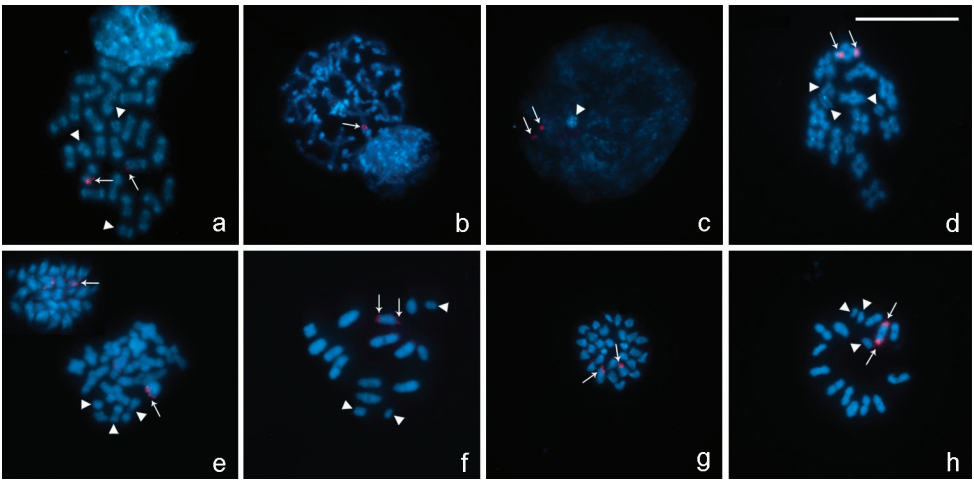

Figure 5. Location of rDNA genes in chromosomes of *B. dentatum* (**a–d**), *B. elongatum* (**e, f**) and *B. gestroi* (**g, h**) by FISH with 18S rDNA probes (red signals, arrows). Chromosomes were counterstained with DAPI (blue). **a** Spermatogonial anaphase (2n = 29 = 26 + X_1X_2Y) **b** Pachytene **c** Diffuse stage **d** Diakinesis **e** Spermatogonial metaphase and diakinesis **f** Metaphase I **g** Spermatogonial metaphase (2n = 29 = 26 + X_1X_2Y) **h** Diakinesis-Metaphase I. Arrowheads show sex chromosomes. Bar = 10 μm.

Discussion

The *Belostoma* species analyzed here shared apparently similar karyotypes, since they possess the same chromosome complement (2n = 29 = 26 + X_1X_2Y, male), with chromosomes progressively decreasing in size. In Belostomatidae, this 2n is the modal diploid chromosome number and is present in 10 species of *Belostoma* (Papeschi and Bressa 2006, Chirino and Bressa 2011). On the other hand, in *B. elongatum* and *B. gestroi* the male meiotic behaviour followed a similar pattern as previously described for other species of this genus (Papeschi and Bidau 1985, Papeschi 1996, Bardella et al. 2012). Both *B. elongatum* and *B. gestroi*, as well as *B. dentatum*, showed the following cytogenetic characteristics: a) synizesis observed, b) a multiple sex chromosome system (X_1X_2Y, male), c) Y chromosome negatively heteropycnotic at metaphase II, d) variation in the TCL and in the C-banding pattern, and e) a single pair of NOR-autosomes. Within Heteroptera, the synizesis stage was described in a few species of *Belostoma* (Papeschi and Bidau 1985, Papeschi 1992) and *Dysdercus* Guérin-Méneville, 1831 (Bressa 2003, Bressa et al. 2003). In this stage the chromatin condenses eccentrically in the nucleus and chromosome pairing begins.

Heterochromatin characterization in the three species revealed differences in the amount, distribution and location of the constitutive heterochromatin in autosomes and both X chromosomes: i) terminal C-positive bands in *B. dentatum*, ii) conspicuous C-positive bands at terminal and interstitial positions in *B. elongatum*, and iii) very scarce C-positive bands terminally located in *B. gestroi*. This variation in the constitutive heterochromatin of these three species could imply changes in the DNA content

in the karyotype evolution in the genus, which could modify the size of the chromosome complement. In accordance with this suggestion, the analysis of TCL showed a significant variation among the three species, which means that certain chromosomal changes, must have taken place during their evolution.

Papeschi (1992) found a great interspecific variation in DNA content as well as differences in C-positive heterochromatin percentage among *B. dentatum* (1.93 pg, 58.54 %), *B. elongatum* (1.75 pg, 59.47 %) and *B. gestroi* (1.13 pg, 37.24 %). Taking into account the data previously described by Papeschi (1992), together with the results obtained from the analysis of the TCL in these three species, we propose the existence of positive relationships between TCL and DNA content and between TCL and C-positive heterochromatin percentage (Fig. 6). Thus, it is apparent that differences in the TCL in the three species might represent changes or variations in DNA content since the accumulation/addition of satellite DNA in all chromosomes of the complement. The comparison between the DNA content and the C-positive heterochromatin percentage of the three species shows a general trend, i.e. an increase in the DNA content is accompanied by an increase in the amount of C-positive heterochromatin. However, *B. dentatum* and *B. elongatum* have a very different DNA content and a similar percentage of C-positive heterochromatin. On the other hand, *B. gestroi* shows the lowest DNA content and the lowest C-positive heterochromatin percentage. In accordance with the earlier reports on six other species of *Belostoma* (Papeschi and Bidau 1985, Papeschi 1988, 1991, 1992), the genome size differences between *B. dentatum* and *B. elongatum* could be due to a proportionate variation of both C-positive heterochromatin and C-negative chromatin occurred during evolution. The chromosomes of *B. gestroi* could have gained low amount of C-positive heterochromatin, or else during evolution some C-positive bands became lost.

In Heteroptera, the classical distribution pattern of C-positive heterochromatin is terminal in some or all chromosomes. Interstitial C-positive bands are described in a few species and some of them correspond to secondary constrictions and NORs. In concordance with these cytogenetic features, the C-banding pattern observed in *B. elongatum* with respect to both terminal and interstitial C-positive regions agrees with most previous reports within *Belostoma* (Papeschi 1995) and Heteroptera (Camacho et al. 1985, Panzera et al. 1995, Grozeva and Nokkala 2001, Angus et al. 2004, Ituarte and Papeschi 2004, Bressa et al. 2005, 2008, Franco et al. 2006).

The results with fluorescent banding indicate that all C-positive bands in the species analysed were not enriched with AT or CG base pairs, as all chromosomes were stained homogeneously with both DAPI and CMA_3 fluorochromes, except for the C- positive band observed in the medium-sized autosomal bivalent of *B. dentatum* and *B. elongatum* and in one of the large-sized of *B. gestroi*, which was DAPI negative/CMA_3 positive. Therefore, the CMA_3 bright band is enriched in GC base pairs and could represent an NOR (see below). The presence of a CMA_3 bright band was also reported not only in other species of *Belostoma* (Papeschi and Bressa 2006) but also in other heteropteran species, at interstitial or terminal position, either on autosomes or sex chromosomes, and they are generally associated to NORs (González-García et al.

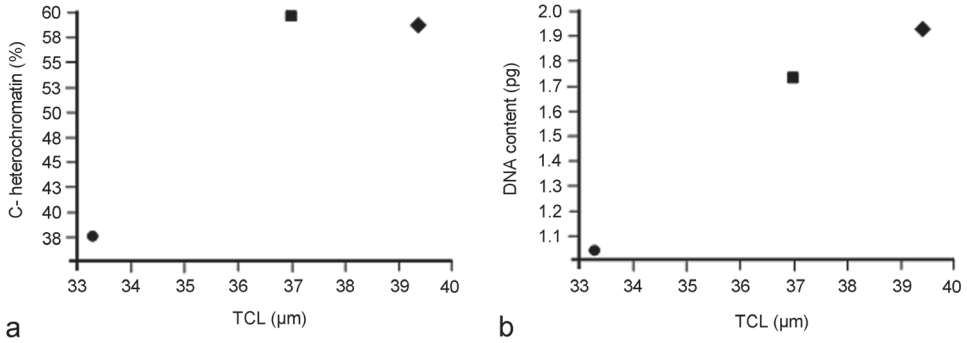

Figure 6. a Comparison between the total chromosome length (TCL) and the percentage of C-positive heterochromatin at diakinesis in *B. dentatum* (58.54 ± 1.27 %; circle), *B. elongatum* (59.47 ± 0.78 %; rectangle) and *B. gestroi* (37.24 ± 1.50 %; diamond) **b** Comparison between the total chromosome length (TCL) and the haploid DNA content (pg) in *B. dentatum* (1.93 ± 0.16 µm; circle), *B. elongatum* (1.75 ± 0.05 µm; rectangle) and *B. gestroi* (1.13 ± 0.13 µm; diamond). Data of percentage of C-positive heterochromatin and the haploid DNA content were obtained from Papeschi (1991, 1992).

1996, Papeschi et al. 2001, 2003, Rebagliati et al. 2003, Cattani et al. 2004, Grozeva et al. 2004, Poggio et al. 2011).

In Belostomatidae, the location of NORs was previously analysed by FISH with 18S rDNA probe in *B. oxyurum* (Dufour, 1863) (2n = 6 + XY, NOR in sex chromosomes), *B. micantulum* (Stål, 1860) (2n = 14 + XY, NOR in sex chromosomes), *B. elegans* (Mayr, 1871) (2n = 26 + X_1X_2Y, NOR in a pair of autosomes) (Papeschi and Bressa 2006), and *Lethocerus patruelis* (Stål, 1854) (2n = 22 + 2m + XY, NOR in sex chromosomes) (Kuznetsova et al. 2012). The present paper presents information about the number and chromosomal location of ribosomal clusters in *B. dentatum*, *B. elongatum* and *B. gestroi*, which have a single cluster located in an autosomal pair. In these three species the NOR is associated with a CMA_3-bright band. The results of the fluorescent banding and FISH in these species agree with those described for *B. oxyurum*, *B. micantulum* and *B. elegans*, in which the NOR regions are colocalized with a CMA_3-positive band and, therefore, the repeating unit of rDNA is GC-rich (Papeschi and Bressa 2006). Taking into account the data on the number and location of rDNA clusters along with the type of sex chromosome systems in Belostomatidae, we can observe two different patterns of rDNA distribution. The NOR is located at terminal position on both sex chromosomes in species that have a simple sex chromosome system (XY), or, in contrast, the NOR is placed at terminal position on an autosomal pair in the species with a multiple sex chromosome system (X_1X_2Y). Hence, our present results led us to propose that in Belostomatidae the location of rDNA genes could be associated with variants of the sex chromosome systems. Moreover, this relationship between the number and location of the NOR and the sex chromosome system has only been observed in this family of Heteroptera.

Previous cytogenetic data on worldwide Belostomatidae species allowed Papeschi and Bressa (2006) to propose an ancestral male karyotype 2n = 28 = 26 + XY, from

which the karyotypes with multiple sex chromosome systems (2n = 26 + X_1X_2Y) and those with a low 2n (6 + XY, 14 + XY, 13 + XY, 22 + XY) would have originated by fragmentation of the ancestral X chromosome and chromosomal fusions, respectively. It is generally accepted that multiple sex chromosome systems in Heteroptera are the result of fragmentation(s) of the X and/or Y chromosome(s) of an ancestral simple sex chromosome system (Heizer 1950, Hughes-Schrader and Schrader 1961, Ueshima 1979, Manna 1984, Papeschi 1996, Papeschi and Bressa 2006). The holokinetic nature of heteropteran chromosomes and the achiasmatic male meiosis of sex chromosomes are the main facts that support this hypothesis and may account for the variability (Ueshima 1979, Manna 1984, Thomas 1987). In most cases of multiple sex chromosomes, the increase in the number of sex chromosomes is not accompanied by a reduction in the number of autosomes. The analysis of different populations of *B. orbiculatum* Estévez and Polhemus, 2001 (Papeschi 1996), *B. plebejum* (Stål, 1858) (Papeschi 1994), *B. dilatatum* (Dufour, 1863) (Bardella et. al 2012), *Oechalia pacifica* (Stål, 1859) (Heizer 1950) and *Banasa zeteki* Sailer, 1959 (Pentatomidae) (Schrader and Hughes-Schrader 1958), polymorphic for the sex chromosome systems, lend support to the hypothesis of a fragmentation origin of a multiple sex chromosome systems. In all these examples one chromosome of the simple system (XY) was replaced by two chromosomes of smaller size in the mutant individuals. Therefore, these interspecific polymorphisms of sex chromosomes represent a direct evidence of the origin of multiple sex chromosome system through fragmentation in *Belostoma*. On the other hand, the species of this genus with reduced chromosome numbers are characterized by a larger chromosome size, a low DNA content and very scarce C-positive heterochromatin (Papeschi and Bressa 2006). These karyotypes probably originated from the ancestral chromosome complement through chromosome fusions. The possibility of their occurrence is supported by the fact that the autosomal fusions have been found in heterozygous condition in natural populations of *B. plebejum* (Papeschi 1994), *Triatoma infestans* (Klug, 1834) (Poggio et al. 2013) and *Mepraia gajardoi* Frías, Henry and González, 1998 (Pérez et al. 2004) (Reduviidae).

Published data on karyotype evolution in species of this genus (Papeschi and Bressa 2006) along with the present results of rDNA-FISH support the hypothesis that in the ancestral male karyotype (2n = 28 = 26 + XY) the NOR would have been located in a pair of autosomes (Fig. 7). A fragmentation of the single X chromosome in the ancestral karyotype resulted in multiple X chromosomes and led to a karyotype with 2n = 29 = 26 + X_1X_2Y, but keeping the ancestral NOR-autosome pair, as represented by *B. dentatum*, *B. elegans*, *B. elongatum* and *B. gestroi* (Fig. 7). On the other hand, autosomal fusions and the fusion of the ancestral sex chromosome pair (XY) with the autosomes carrying the NOR would result in the reduction in the diploid chromosome (2n = 8, 16), increasing the chromosomes size and led to the presence of rDNA genes in both X and Y chromosomes. Alternatively, the rDNA gene cluster could have been translocated from an autosomal location to the X and Y chromosomes, as it has been proposed in *Dysdercus albofasciatus* Berg, 1878 (Bressa et al. 2009). Furthermore, future studies within *Belostoma* about this regular pattern will shed light on the karyotype evolution within the genus and also support the mechanisms involved in the karyotype evolution.

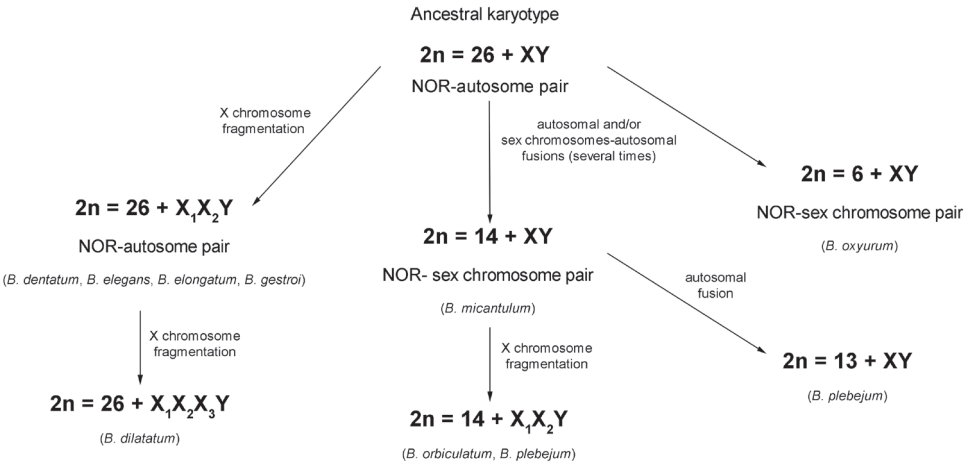

Figure 7. Hypothetical scheme of karyotype evolution in species of the genus *Belostoma*. See text and Table 1 for details.

Conventional taxonomy of water bugs has focused almost entirely on adult specimens. There are relatively few publications on interspecific differences among the larvae, and fewer still concern South American species. The literature of the genus *Belostoma* includes much confusion because, in many cases, the species are very similar in coloration and appearance and only males or rarely only females can be identified (Heckman 2011). In *B. dentatum*, *B. elongatum* and *B. gestroi*, the karyotype analyses allow us to get a detailed characterization and a better knowledge of their chromosomal structure. Hence, we conclude that the cytogenetic studies provide valuable features that can be used to solve problems on taxonomic identification, at least for this genus.

Acknowledgements

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RESEARCH ARTICLE

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New method for visualization of C-heterochromatin in synaptonemal complex spreads

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Abstract

DAPI staining of the metaphase chromosomes pretreated with barium hydroxide generates a C-like banding pattern. In this work a protocol for visualizing similar pattern at the synaptonemal complex (SC) spreads after immunostaining is suggested. This method was used to visualize centromeric and sex heterochromatin at the SC spreads of guppy fish (*Poecilia reticulata* Peters, 1859). The efficiency of this method was further confirmed at SC spreads of the northern red-backed vole (*Myodes rutilus* (Pallas, 1779)), the guinea pig (*Cavia porcellus* (Linnaeus, 1758)), and the pigmy shrew (*Sorex minutus* Linnaeus, 1766).

Keywords

Synaptonemal complexes, pachytene, DAPI, C-banding

Introduction

Immunofluorescent analysis of synaptonemal complexes (SC) is widely performed in medical and comparative cytogenetics to study chromosome synapsis and recombination in humans with chromosomal abnormalities (Solari 1999) as well as in various plant and animal species (Anderson et al. 1999; Basheva et al. 2010; Borodin et al. 2008). Specific antibodies are used to visualize lateral and central elements of SC, double strand breaks and crossovers. These antibodies are also used to reveal specific meiotic histone modifications and other important characteristics of meiotic cells.

Centromeres are used as an important marker in SC karyotyping. For instance, centromeres are commonly detected by human autoantibodies to human centromere proteins (CREST serum from human scleroderma patients). These proteins are conservative and human antibodies may be used to study various species (Basheva et al. 2010, Del Priore and Pigozzi 2012, Moens 2006). However, their affinity decreases as phylogenetic distance increases. For example, CREST serum from human scleroderma patient can detect centromeres in zebrafish only at concentrations that are five times higher than the concentrations used in mammals (Moens 2006). Centromeres in guppy have not been detected even at higher concentration of human antibodies to human centromere.

In this study a simple protocol was designed to obtain C-like banding on SC spreads and visualize centromeres without the use of antibodies. This method is cheaper and applicable if antibodies fail to detect centromeres because of a large phylogenetic distance. This method is a modified DAPI-staining technique combined with pretreatment commonly used for C-banding. The proposed method was tested on guppy fish. The efficiency of this method was confirmed in three of five examined mammal species. Previously, the DAPI staining after barium hydroxide treatment was shown to visualize C-like banding on the metaphase chromosomes of fish (Russo, Rocco et al. 1999), plants (Barros e Silva and Guerra 2010), insects (Bella and Gosálvez 1991) and human (Heng and Tsui 1993). However, in this study the proposed technique was used for the first time to analyze SCs.

Materials and methods

Mammal spermatocytes were prepared according to the prescribed method of (Peters et al. 1997).Testicular fragments were immersed in a hypotonic extraction buffer containing 30 mMTris, 50 mM sucrose, 17 mM trisodium citrate dihydrate, 5 mM EDTA, pH 8.2 for 30 to 60 min. A suspension was made in a 40 µl drop of 100 mM sucrose, pH 8.2 (pH was adjusted using NaOH), on a clean glass slide. First, the tubules, approximately 2 cm in length, were torn to pieces between the tips of two fine watchmaker forceps in 20 µl of sucrose solution. Thereafter, the volume was increased to 40 µl and a slightly cloudy suspension was made. The tubular remnants were removed and the remaining suspension was divided between two new clean glass slides that had been dipped just before in a freshly made and filtered (0.2 µM) 1% paraformaldehyde (PFA), pH 9.2, solution containing 0.15% Triton X-100. Nuclei were dried for at least 2 h in a closed box with high humidity. Finally, the slides were washed twice for 2 min in 0.4% Photo Flo (Kodak) and dried at room temperature.

This method also yielded satisfactory results for guppy spermatocytes. However, more accurate results were obtained using the method of (Moens 2006). In brief, 30 µl drops of 1/3×PBS hypotonic solution were placed on the dry slide. The testes of the guppy males were macerated in 50µl to100 µl of PBS, and 1 µl portions of suspension were injected into the drops. The cells were fixed by 1% paraformaldehyde after 20

min of hypotonic treatment. The slides were washed twice for 2 min in 0.4% Photo Flo (Kodak) and dried at room temperature.

The slides with guppy spermatocytes were permeabilized by the incubating in 10 mM sodium citrate solution with 0.1% Tween-20 at 95° C for 20 min before immunostaining was performed. The slides were then cooled to room temperature for 20 min and rinsed in PBS with 0.1% Tween-20 for 2 min twice. Immunostaining was performed according to the protocol described in (Anderson et al. 1999) with slight modifications. SC was detected by rabbit polyclonal antibodies to SC axial element protein SYCP3 (1:500, Abcam, Cambridge) and goat anti-rabbit Cy3-conjugated secondary antibodies (1:500, Jackson, West Grove).The centromeres were detected by human anti-centromere antibodies (ACA) (1:100 for mammals to 1:20 for guppies, Sigma-Aldrich) and goat anti-human FITC and Cy3 (for the vole) conjugated secondary antibodies (1:100, Vector Laboratories). After washing, the antifade solution with DAPI (Vectashield, Vector Laboratories) was mounted on the slides.

The preparations were photographed using Axioplan 2 Imaging microscope with a CCD camera (CV M300, JAI Corporation, Japan), CHROMA filter sets, and an ISIS4 image processing package (MetaSystems GmbH, Germany). The coverslips were carefully removed after the photographs were taken. The preparations were washed in 2×SSC for 5 min to remove the antifade solution and then dehydrated in ethanol series 70%, 80% and 100% for 3 minutes in each. The preparations were then air-dried and stored in 0,2 N HCl at room temperature for 20 min to 30 min. The slides were transferred to saturated barium hydroxide solution at 55°C for 1 min to 10 min. Afterward, the preparations were incubated in 2×SSC at 55°C to 60°C for 60 min. The preparations were re-mounted in the antifade solution with DAPI and the cells were re-photographed.

Results and discussion

In vole, guinea pig and pigmy shrew, the centromeres were marked by antibodies and DAPI signals. In vole, DAPI-stained pachytene cells untreated with barium hydroxide produced a unique banding pattern for each chromosome. This banding pattern is visible even without image contrast adjustment, and bright bands correspond to centromere antibody signals (Fig. 1a). However, the centromeric bands of most chromosomes were indistinguishable by their brightness from the interstitial ones. After the incubation in barium hydroxide for 5 minutes, all the bands except the centromeric bands and the sex heterochromatin faded and disappeared (Fig. 1b).

In guinea pig, DAPI-stainied pachytene chromosomes untreated with barium hydroxide produced a weak differential pattern that only became visible after image contrast was adjusted, and the centromeres were indistinguishable (Fig. 2a). After barium hydroxide treatment for 5 minutes, a distinct DAPI signal at the centromeres and over the sex bivalent was observed (Fig. 2b).

In pigmy shrew the DAPI-stained pachytene chromosomes untreated with barium hydroxide produced a unique pattern for each bivalent. This result agrees with that of

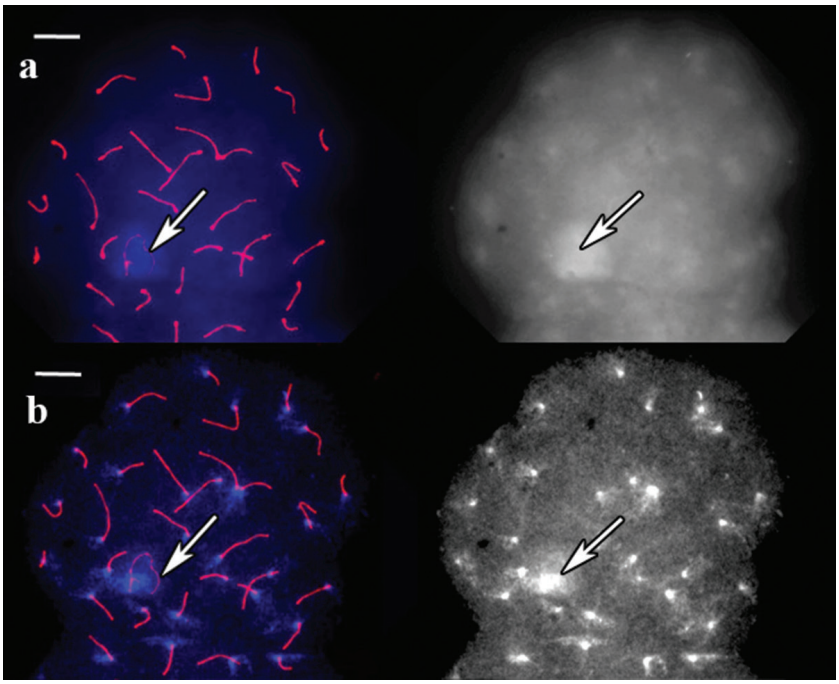

Figure 1. SC spread in red-backed vole. **a** A cell after immunostaining, prior to barium hydroxide treatment. Left: Red shows SCs (long lines) and centromeres (dots), blue represents DAPI. Right: the same image, DAPI channel separately **b** The same cell after 5 min of barium hydroxide treatment. Left: Red shows to SCs (long lines) and centromeres (dots), blue represents DAPI. Right: the same image, DAPI channel separately. Arrows indicate sex bivalent. Scale bars = 20 μm.

(Belonogova et al. 2006). However, the pattern did not exhibit any specific signal at the centromeres (Fig. 3a). Bright DAPI spots were observed after the specimens were treated with barium hydroxide for 5 min (Fig. 3b). Such bright spots corresponded to ACA signals (Fig. 3c). The chromosome-specific DAPI pattern faded but remained recognizable. However, these spots were absent on some bivalents.

In domestic cat (*Felis silvestris catus* (Linnaeus, 1758)) and red fox (*Vulpes vulpes* (Linnaeus, 1758)), the centromeres were successfully detected by ACA, but any centromere-specific DAPI staining was not observed on either untreated pachytene chromosomes, or barium hydroxide-treated chromosomes. The results were not improved although the treatments were modified by increasing HCl pre-incubation time to 30 min, extending barium hydroxide incubation time to 10 min, or decreasing barium hydroxide incubation time to 1 min. The failure to obtain centromeric DAPI signal on the pachytene chromosomes of domestic cat and red fox corresponds to previously published data about the absence of centromeric C-bands and centromeric satellite DNA in cat chromosomes(Matthews et al. 1980; Santos et al. 2004) and absence of centromeric C-bands on most fox chromosomes (Mäkinen 1985).

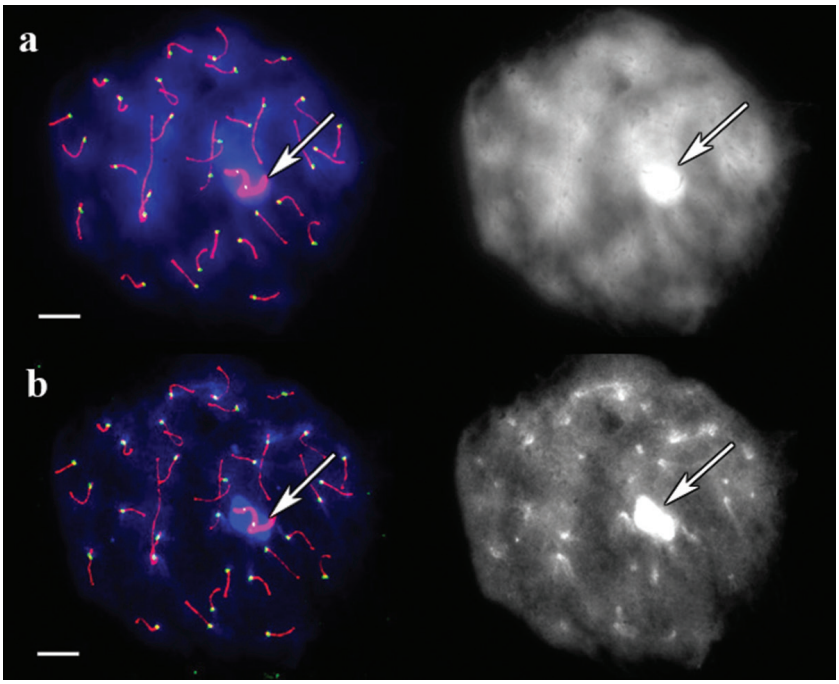

Figure 2. SC spread in guinea pig. **a** A cell after immunostaining, prior to barium hydroxide treatment. Left: Red, green and blue indicate SCs, centromeres, and DAPI, respectively. Right: the same image, DAPI channel separately **b** The same cell after 5 minutes of barium hydroxide treatment. Left: Red, green and blue indicate SCs, centromeres, and DAPI, respectively. Right: the same image, DAPI channel separately. Arrows indicate sex bivalent. Scale bars = 20 μm.

DAPI staining of untreated guppy male pachytene chromosomes produced completely uniform fluorescence without any specific signals (Fig. 4a). ACA did not also show any specific signal in the cell, although the concentration was five times higher than that recommended by the manufacturer. After 5 min of barium hydroxide treatment a bright spot appeared at the end of each bivalent, and the residual fluorescence faded (Fig. 4b). DAPI spots were mostly located at SC ends opposite to the recombination nodules. This finding was attributed to suppressed recombination near the centromeres. This effect has also been described in various vertebrate species including mammals (Basheva et al. 2008) and zebrafish (*Danio rerio* (Hamilton, 1822)) (Moens 2006).

The XY bivalent in guppy was easily distinguishable from a delayed synapsis at early and mid pachytene stages and from an excessive thickening of the axial elements at late pachytene stage. This bivalent showed one small DAPI signal at one end and a large DAPI-positive block close to its opposite end (Fig. 4c).This result is consistent with the pattern obtained by (Nanda et al. 1993) after conventional Giemsa C-banding on guppy metaphase chromosomes. The C-positive bands were detected at the centromeres and at the distal parts of both X and Y chromosomes.

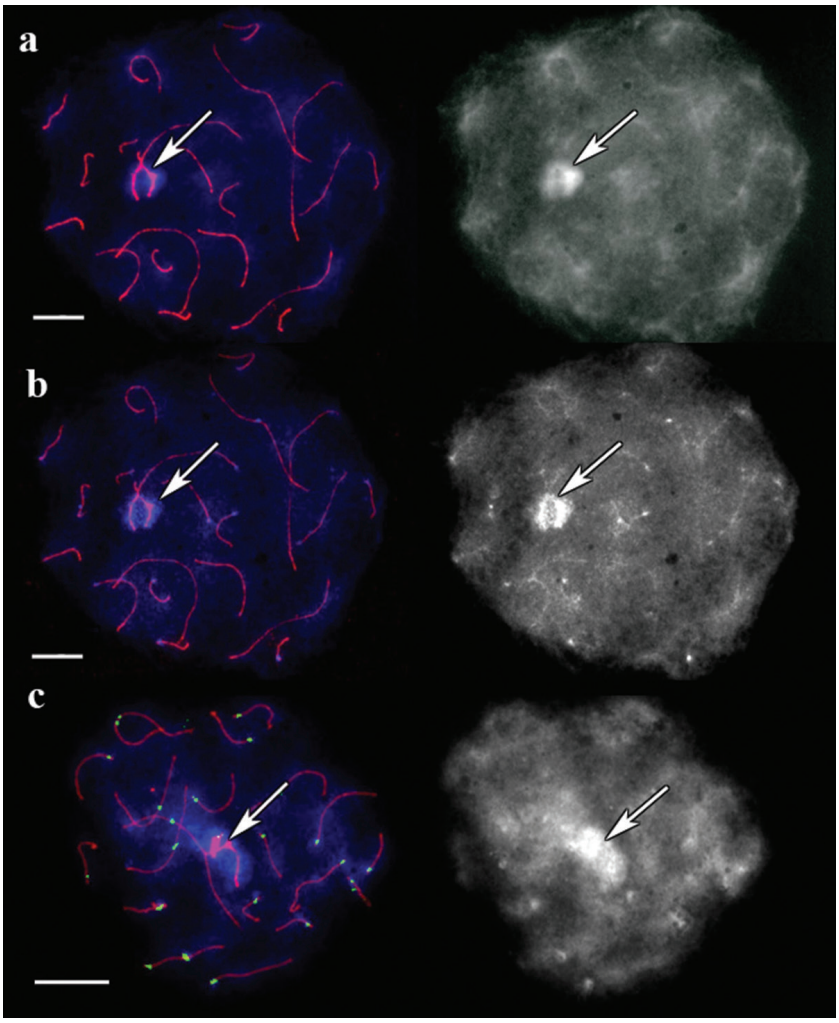

Figure 3. SC spread in pigmy shrew. **a** A cell after immunostaining, prior to barium hydroxide treatment. Left: Red shows SCs, and blue represents DAPI. Right: the same image, DAPI channel separately **b** The same cell after 5 minutes of barium hydroxide treatment. Left: Red shows SCs, and blue represents DAPI. Right: the same image, DAPI channel separately **c** Bright DAPI spots with their corresponding centromere signals. The cell after 5 min of barium hydroxide treatment. Left: Red, green and blue indicate SCs, centromeres, and DAPI, respectively. Right: the same image, DAPI channel separately. Arrows indicate sex bivalent. Scale bars = 20 μm.

In guppy, staining quality depend on the age of preparations. In this study, optimal results were obtained from two to six month old preparations. Centromeric and sex fluorescent blocks were also visible on fresh slides, but only after image brightness and contrast were adjusted. Although the time allotted for barium hydroxide treatment was extended to 10 min, optimal results from the fresh preparations were not obtained. This may occur possibly because higher level of DNA degradation in the old preparations facilitated denaturation by barium hydroxide.

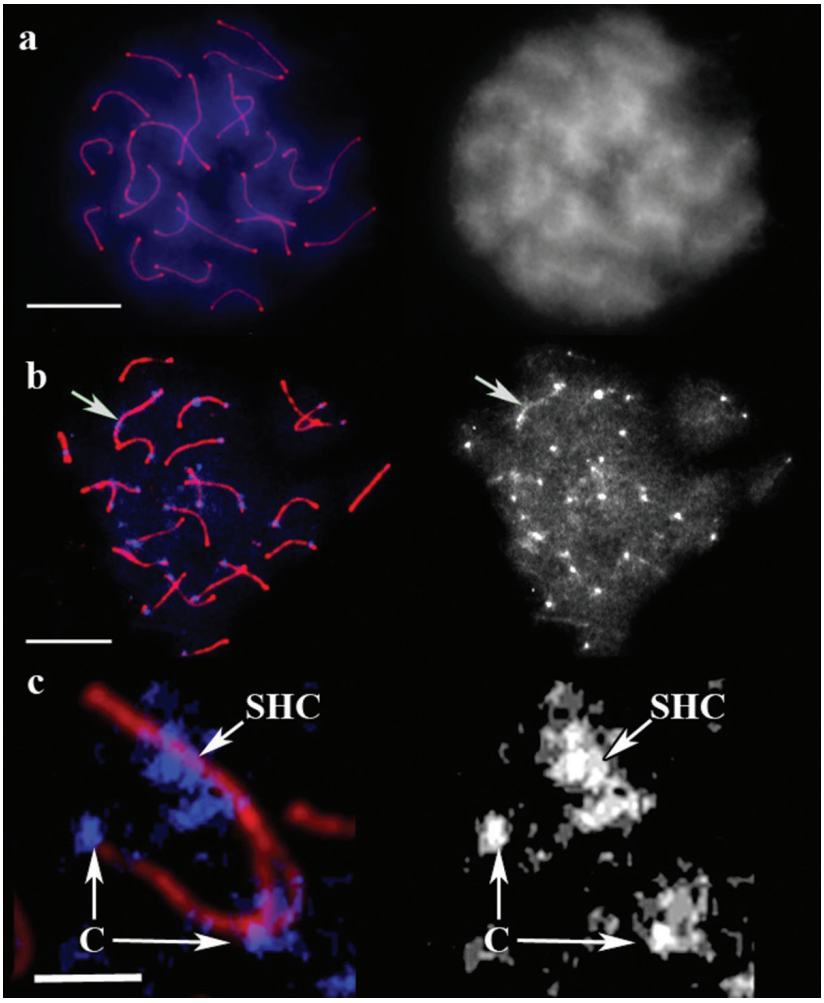

Figure 4. SC spread in guppy. **a** A cell after immunostaining, prior to barium hydroxide treatment. Left: Red shows SCs, and blue represents DAPI. Right: the same image, DAPI channel separately. Scale bar = 20 μm. **b** The cell after 5 min of barium hydroxide treatment. Left: Red shows SCs, and blue represents DAPI. Right: the same image, DAPI channel separately. Arrow indicates sex bivalent. Scale bar = 20 μm. **c** Sex bivalent during pairing. Red shows SC, blue represents DAPI. C: centromere, SHC: sex heterochromatin. Scale bar = 5 μm.

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Heterochromatin variation among the populations of *Mus terricolor* Blyth, 1851 (Rodentia, Muridae) chromosome type I

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Abstract

Twenty five to thirty specimens each from ten populations of *Mus terricolor* of the Terai and the Dooars regions of the Darjeeling foothills of West Bengal were cytogenetically analyzed using C-banding. Results showed intra- and inter- population variation of C-band positive heterochromatin ranging from very large blocks to minute amounts or even complete absence of heterochromatin. Large blocks of centromeric C-bands were found in Bidhan Nagar, Garidhura, Malbazar, Nagrakata and Maynaguri populations in most of the autosomes, while the rest of the populations had large blocks of C-bands on a few autosomes only. Such intra- and inter- population variation may be due to accumulation of C-positive heterochromatin, which has not got fixed homogeneously in all autosome pairs. X-chromosomes invariably possess a C-banded short arm a telomeric C-band at the distal end of the long arm in all populations. The entire Y-chromosome was C-band positive with slight population differences in staining intensity. The results suggest quantitative as well as qualitative variation of C-positive heterochromatin.

Keywords

Heterochromatin, C-banding, *Mus terricolor*

Introduction

The earth-colored mouse *Mus terricolor* is a common field mouse of the Indian subcontinent infesting paddy and wheat fields and was known as *Mus dunni* Wroughton, 1912 until Musser and Carleton (1993) synonymised it with the former name. This

Mus species co-exists in the same habitat with the sibling species *Mus booduga* Gray, 1837. Both the species have 2n=40 chromosomes like other species of the subgenus *Mus*. In contrast to all acrocentric chromosomes of *M. booduga*, *M. terricolor* is characterized by a large submetacentric X and a large acrocentric Y chromosome (Matthey and Petter 1968, Sharma and Garg 1975, Markvong et al. 1975, Manjunatha and Aswathanarayana 1979). The short arm of X and the entire Y chromosome are heterochromatic (Sharma et al. 1986, 1990). Compared to the conserved karyotype of *M. booduga* throughout its distribution range, *M. terricolor* shows divergent karyotypes due to a variable number of heterochromatic short arms established in homozygous condition (Sen and Sharma 1983, Sharma et al.1986, 1990) which indicates that *M. terricolor* is in an active phase of evolutionary differentiation. Cytogenetic studies by Sharma and his associates revealed that *M. terricolor* is differentiated into three distinct karyotypes (2n = 40) designated as chromosome types I, II and III. Chromosome type I has a wide distribution throughout the subcontinent except the southern peninsular region and has all acrocentric autosomes with C-band positive minute perceptible short arms. Chromosome type II, characterized by two pairs of submetacentric autosomes 1 and 3 with heterochromatic short arms, is found in Mysore and Erode in the peninsular India, while Chromosome type III distributed in Chennai, Tirupati and Madurai is characterized by three pairs of submetacentric autosomes 1, 3 and 6 with heterochromatic short arms.

Karyotype differentiation in *Mus terricolor* is due to acquisition of varying amount of constitutive heterochromatin in and around the centromere on specific autosomes. Different studies have been carried out in *M. terricolor* chromosome types and their populations covering vast regions of southern, central and western part of India (Sharma and Garg 1975, Sen and Sharma 1983, Sharma et al. 1986, Sharma 1996) but populations from West Bengal in eastern India were not included. The northern part of West Bengal, characterized by hills (Darjeeling District) and the Terai and the Dooars regions in the foothills, is well known for biodiversity and diverse ecological features.

In view of the aforesaid situation, this study has been conducted to know the extent of intra- and inter- population heterochromatin variation in *M. terricolor* chromosome type I from the Terai and the Dooars regions of foothills of Darjeeling in West Bengal.

Materials and methods

Animals

The individuals of *M. terricolor* were collected from paddy fields by digging burrows during harvesting season of the crop (November to December) from ten different locations of the Terai and the Dooars of foothills of Darjeeling in West Bengal, India. Three of the collection sites are in the Terai and seven collection sites are in the Dooars. The river Tista separates the Terai and the Dooars as a physical barrier. The name of the collection sites and their provisional geographical coordinates has been shown in

Table 1. Populations, collection sites, geographical coordinates and number of studied individuals of *M. terricolor*.

Populations	Collection sites	Geographical coordinates	No. of specimens
	Terai region		
NXL	Naxalbari	26° 41'00"N / 88° 13'00"E	30
GDH	Garidhura	26° 48'24"N / 88° 16'38"E	28
BDN	Bidhan Nagar	26° 16'00"N / 88° 12'00"E	28
	Dooars region		
APD	Alipurduar	26° 31'21"N / 89° 32'37"E	25
RBD	Rohimabad	27° 54'00"N / 80° 30'05"E	27
KGM	Kumargram	26° 36'50"N / 89° 49'30"E	29
MNG	Maynaguri	26° 33'07"N / 88° 49'26"E	25
NGK	Nagrakata	26° 54'00"N / 88° 50'00"E	29
MLB	Malbazar	27° 01'00"N / 89° 20'17"E	30
CBH	Cooch Behar	26° 32'05"N / 89° 07'12"E	26

Table 1 along with population name and number of individuals studied from each site. Animals were collected and identified by mitotic chromosome preparation. 25–30 individuals from each population were analyzed for this study. Individuals of *Mus terricolor* are abbreviated for convenience according to their collection localities. In the Terai region these are NXL (Naxalbari), GDH (Garidhura), BDN (Bidhan Nagar), and in the Dooars these are APD (Alipurduar), RBD (Rohimabad), KGM (Kumargram), MNG (Maynaguri), NGK (Nagrakata), MLB (Malbazar) and CBH (Cooch Behar).

Mitotic Chromosome Preparation

Mitotic chromosomes were prepared from bone marrow of colchicine injected mice with hypotonic treatment following air dried method after Lee and Elder (1980) and modified by Baker et al. (1982).

C-Banding

C-banding was carried out using the BSG (Barium/Saline/Giemsa) method of Sumner (1972) with slight modifications. Two to three day old slides were treated with 0.2N Hydrochloric acid for 1h at room temperature followed by 2-3 rinses in distilled water. The slides were treated in freshly prepared 5% aqueous solution of Barium hydroxide [$Ba(OH)_{2.}$] at 50°C for about 2-5 minutes, followed by thorough rinsing in distilled water.

Slides were dried and incubated for 2 h at 60°C in 2 x SSC, pH 7.2 (0.3M Sodium Chloride containing 0.03 M Tri-Sodium Citrate). SSC treated slides were rinsed in distilled water and stained in 5% Giemsa, buffered with phosphate buffer (pH 6.8) for 20–30 minutes and were differentiated in distilled water, dried and mounted in DPX medium.

Karyotype preparation

A minimum of 10 plates of metaphase spreads were scored for each specimen and karyotypes were prepared from selected metaphase plates. The chromosomes were numbered on the basis of euchromatic long arms as per recommendations of the Committee on Standardized Genetic Nomenclature for mice (1972).

Results

All the individuals of *M. terricolor* analyzed from ten populations of the Terai and the Dooars demonstrated the diploid number 2n=40 with all acrocentric autosomes and

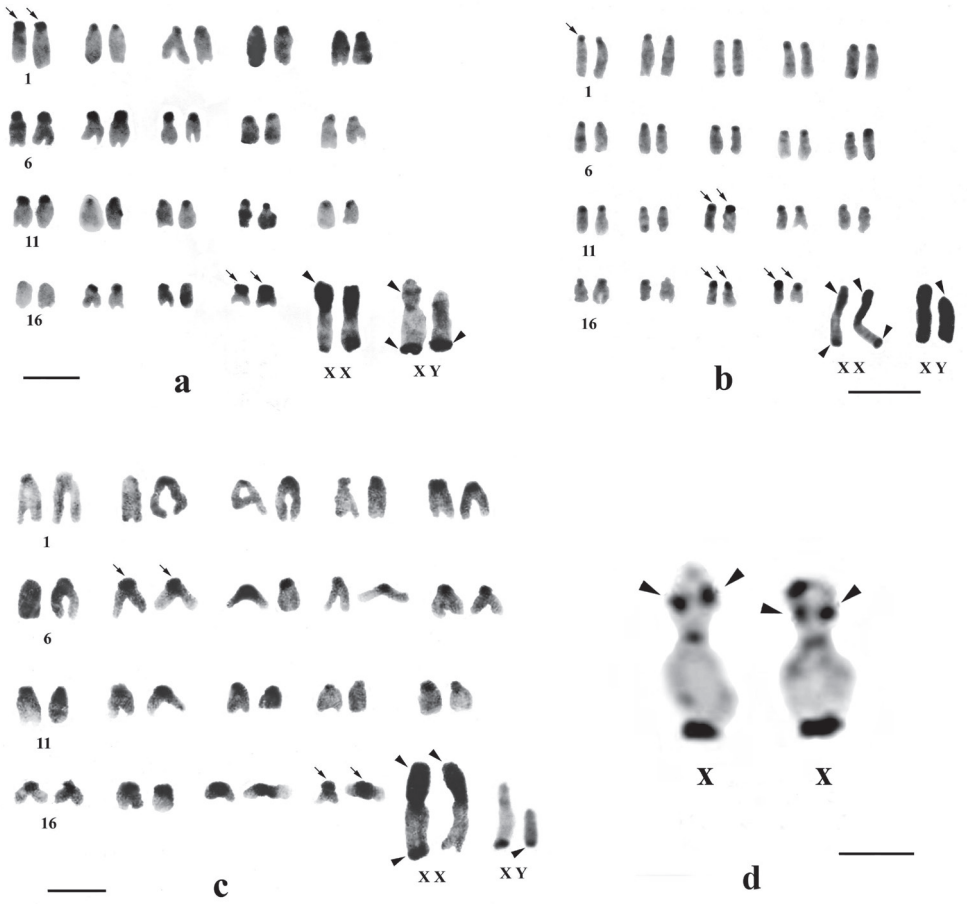

Figure 1. C-banded karyotypes of *M. terricolor* type I from Terai populations. **a** NXL **b** BDN **c** GDH population **d** segmental C-band on short arm of X chromosome in *M. terricolor* from NXL population. Centromeric C-bands are thin arrowed, C-band in short arms of X, entire Y and telomeres of X and Y are arrow headed. Bar = 4μm.

a large submetacentric X and a large acrocentric Y chromosomes in the complement as characteristic. No chromosomal polymorphisms like inversion and Robertsonian translocations were observed. Chromosomes prepared from each individual showed C-band staining, however, few metaphases in each slide either did not show C-band staining or has weak stain. Analyzable metaphase spreads always showed C-bands shown in the representative karyotypes from each population (Figs 1–3).

Autosomal heterochromatin variation

Differential C-banding revealed extensive heterochromatin variation between and within populations. C-bands were found to be localized in the centromeric region of autosomes throughout the populations varying in size. According to the size of C-bands, the heterochromatin was divided into large blocks, and small to minute C-positive

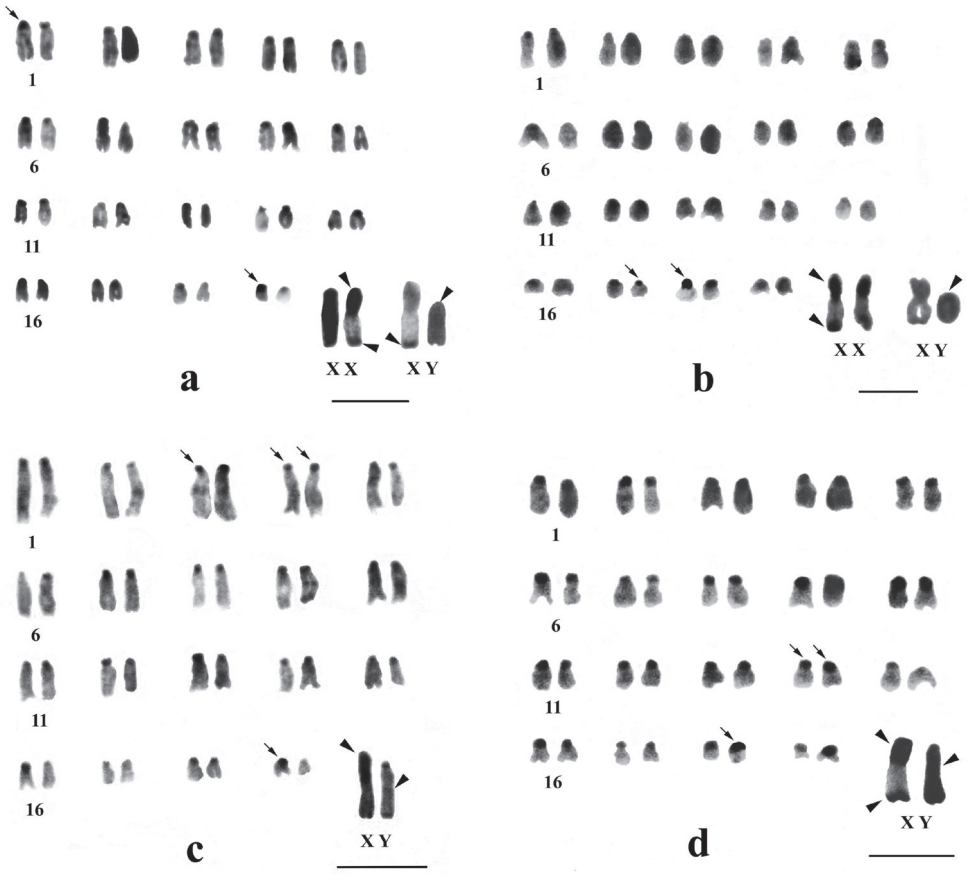

Figure 2. C-banded karyotypes of *M. terricolor* type I from Dooars populations. **a** APD **b** RBD **c** KGM **d** MNG populations. Centromeric C-bands are thin arrowed, C-band in short arms of X, entire Y and telomeres of X and Y are arrow headed. Bar = 4μm.

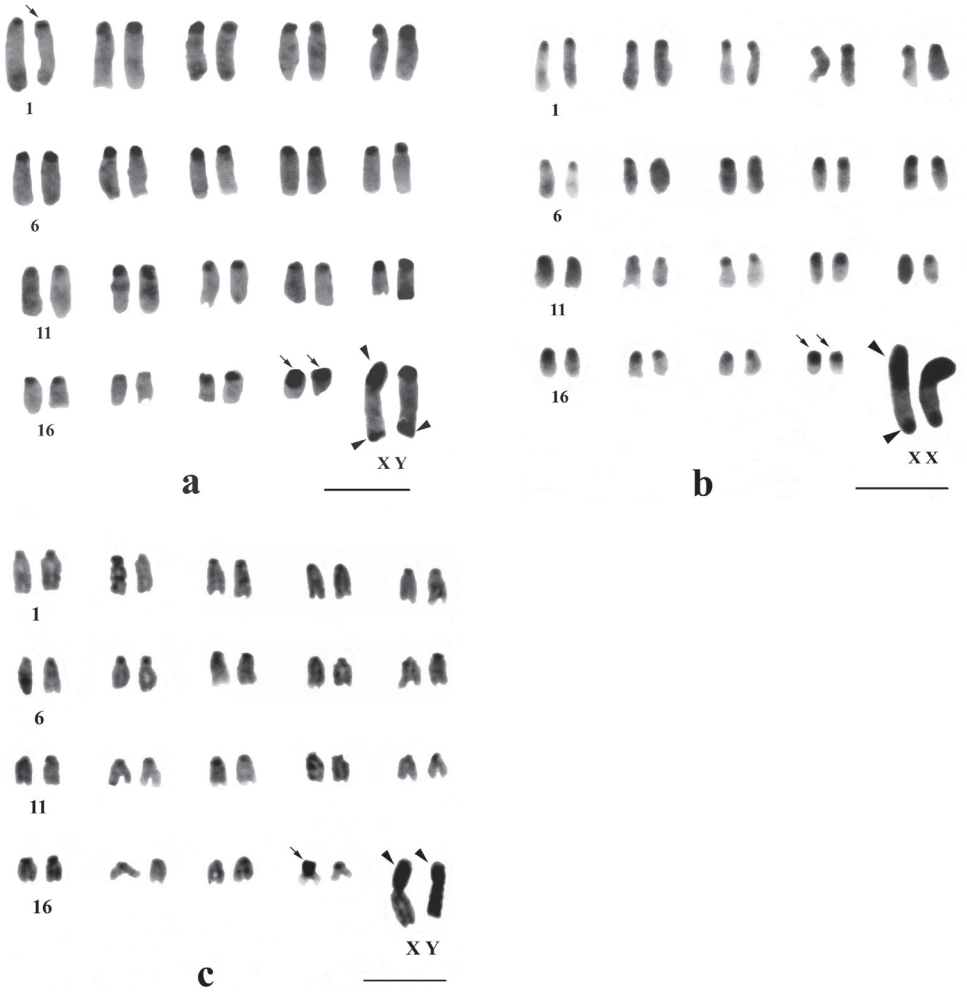

Figure 3. C-banded karyotypes of *M. terricolor* type I from Dooars populations. **a** MLB **b** NGK **c** CBH populations. Centromeric C-bands are thin arrowed, C-band in short arms of X, entire Y and telomeres of X and Y are arrow headed. Bar = 4μm.

heterochromatin (Table 2). Results showed that individuals of BDN, GDH, MLB, NGK and MNG had large blocks of centromeric heterochromatin in most of the autosomes (Figs 1b,c, 2d, 3a,b). Moreover, the distribution of C-positive heterochromatin was not found to be homogeneous in all autosome pairs. Each chromosome of such pair was stained differentially. Autosome pair 19 consistently showed a large block of C-positive heterochromatin in almost all populations of the Terai and the Dooars with variation between the homologs of the pair (Figs 1–3). In the individuals of populations GDH, BDN, RBD, MLB, NGK and MNG large blocks of heterochromatin were also observed in chromosome 18, which was fixed in homologous condition. In contrast to other populations, NXL, RBD, APD, KGM and CBH were found to have few

Table 2. C-band variation in different populations of *M. terricolor*. (s)-Heterogeneity of C-band between homologous autosome pair; SA-short arm of X; LA- long arm of X; WA- entire Y; + denotes intensity of C-band staining.

Population	Size and location of C-positive heterochromatin in autosomes		C-positive heterochromatin in sex chromosomes		
	Centromere		X		Y
	Large	Small to Minute	SA	LA	WA
NXL	1,4-8,11, 19	2,3,9,10,12-15,17,18	+++	+	+
GDH	2(s),3(s), 4(s),5-8, 10-13, 15-19	1,2(s),3(s),4(s),9, 14	+++	+	++
BDN	1-14,17(s),18,19	15,16,17(s)	+++	+	+++
APD	17(s),19(s)	1-16,17(s),18,19(s)	+++	+	++
RBD	5(s),7,10,11(s),12(s),16,18,19	1-4,5(s),6,8,9,13-15,17	+++	+	+++
KGM	17(s),19(s)	1-16,17(s),18,19(s)	+++	+	++
MLB	2-10,12-15,18,19	1,11,16,17	+++	+	++
NGK	2,4,5,8-11,14,16-19	1,3,6,7,12,13,15	+++	+	_
MNG	1-14, 16,18,19	15,17	+++	+	+++
CBH	2,13,16,19	1,3-12,14,15, 17, 18	+++	+	+++

autosomes with prominent large blocks of C-bands (Figs 1a, 2a,b,c, 3c). Interestingly, autosome 16 was found to be C-band negative in *M. terricolor* NXL of the Terai while rest of the autosomes showed moderate to prominent C-bands (Fig. 1a).

Heterochromatin variation in sex chromosomes

The X and Y chromosomes of *M. terricolor* were found to be consistently C-band positive in all populations, however, minute differences were observed in size and intensity of C-bands both at intra- and inter- population level (Table 2, Figs 1–3).

X chromosome

The short arm of X chromosomes in all populations were found to be invariably C-band positive i.e. heterochromatic while the long arms were euchromatic. The telomere of long arms revealed prominent C-band positive staining. In some individuals of NXL and BDN the C-band was found to be localized at two distinct points of short arm of X, so that the short arm was differentiated into faint and darkly stained regions with strong C-band positive distal telomere (Fig. 1a, d). One female *M. terricolor* in GDH population showed interesting result. One of the two X-chromosomes in this specimen was strongly stained at the telomeric end but the other X was totally devoid of C-band positive telomeric staining, while short arm was intensly C-band positive (Fig. 1c).

Y chromosome

The entire Y chromosome was found to be consistently C-band positive in all populations; however, some differences were noticed in the intensity of banding (Table 2). Faintly stained Y chromosome was observed in NXL, GDH, KGM and MLB populations (Figs 1a, c, 2c and 3a), while rest of the populations revealed intensely stained Y which is the characteristic of the species. Like X chromosomes, the telomeric end of the Y was also found to be C-banded with population differences.

Discussion

The mouse major satellite DNA, largely present as pericentromeric constitutive heterochromatin blocks in all chromosomes except Y, is highly repetitive (Jones 1970, Pardue and Gall 1970, Dev et al. 1973). This region has been found to be highly variable and fast evolving indicating its role in early stages of evolution (Shaw 1994). Constitutive heterochromatin has been shown to be highly polymorphic between and within species of *Mus* (Akeson and Davisson 1991, Forejt 1973, She 1990, Piálek 2005, Mitsainas et al. 2009). The studies on different rodents of the genera *Peromyscus, Mastomys, Oryzomys, Sigmodon, Rattus, Apodemus* and *Mus* revealed a common C-band pattern, i.e. large sized centromeric C-bands in the autosomes and X- chromosomes, and a completely heterochromatic small Y-chromosome (Modi 1987 and references there in).

Mus terricolor is an actively speciating incipient species complex in which constitutive heterochromatin is playing a major role in karyotype differentiation (Sen and Sharma 1983, Sharma et al. 1986, 1990, Bahadur 1995). Variation in autosomal C-positive heterochromatin in the range of populations studied, suggest that *M. terricolor* is in a dynamic state of speciation. Variation and accumulation of heterochromatin have been shown in rodents by many workers. They have agreed that the accumulation of C-heterochromatin represents a recently evolved trait in rodents (Baverstock et al. 1976, Greenbaum and Baker 1978, Gamperl 1982a, Gamperl et al. 1982, Sen and Sharma1983, Qumsiyeh et al. 1988, Gallardo 1991). C-band polymorphisms in terms of size variation in wild derived inbred strains of mice have also been reported by Akeson and Davisson (1991). In our study the presence of population specific and/or chromosome specific large blocks of C-bands, either in homozygous or in heterozygous condition suggest an increase or accumulation of C-positive heterochromatin which is consistent with above findings.

C-band polymorphism in X chromosomes of *M. terricolor* populations revealed interesting features. Two discrete heterochromatic blocks on short arms of X chromosomes in NXL and BDN (Fig. 1a, d) suggest segmental localization of heterochromatin. Balajee and Sharma (1994) have also shown the same result in *M. terricolor* by digesting the metaphase chromosomes with *Alu*I and staining with Giemsa which produces C-band like features.

The large size of the Y chromosome in *M. terricolor* is due to accumulation of C-positive heterochromatin (Sharma 1996) which shows population wise variation in banding intensity. In our study the cause of staining differentiation is not clear, though C-band polymorphism and apparent absence of C-positive chromatin in the Y chromosome has been shown in different species of rodents (*Phenacomys intermedius* Merriam, 1889, *Microtus californicus* Peale, 1848, *Microtus orchogaster* Schreber, 1842, *Clethrionomys californicus* Merriam, 1890 and *Microtus oregoni* Bachman, 1839, *Microtus arvalis* Pallas, 1778) by different workers (Zenzes and Voiculescu 1975, Gamperl 1982a,b, Vorontsov et al. 1984, Modi 1987) which suggests compositional heterogeneity of heterochromatin (Peacock et al. 1981, Patton and Sherwood 1982, Gallardo 1992) or unusual DNA sequences with different staining properties. (John and Miklos 1979, Peacock et al. 1981).

Populations of *M. terricolor* showed prominent telomeric C-band on the long arm of X and also on acrocentric Y, but telomeric C-bands were not observed in autosomes in any population. Large prominent autosomal telomeric C-bands have been shown in the common wood mouse, *Sylvaemus sylvaticus* Linnaeus, 1758 by Nadjafova et al. (1993) and Nadjafova (2008) who implicated its role in differentiation of the species. The evolution of telomeric heterochromatin has been suggested to occur due to transposition and amplification of the centromeric satellite component (Hirning et al. 1989) in case of *Sylvaemus sylvaticus*. The situation in *M. terricolor* needs to be intensively investigated for conclusive inferences.

Intra- and inter-specific karyotype evolution involving heterochromatin has been studied and discussed in many species but the evolutionary significance of heterochromatin is not established due to simultaneous involvement of chromosomal rearrangements, like inversions and Robertsonian translocations (Duffey 1972, Bradshaw and Hsu 1972, Pathak et al. 1973, Robbins and Baker 1981, Patton and Sherwood 1982, Davis et al. 1985, Modi 1987). However, evolutionary classification of the European wood mice of the subgenus *Sylvaemus* and genus *Apodemus* is based on chromosomal markers, like species specific C-positive heterochromatin (Orlov et al. 1996, Nadjafova 2008). Comparative FISH analysis of C–positive blocks of centromeric heterochromatin in different species of wood mice, *Sylvaemus* (Rubtsov et al. 2011) and three chromosomal forms of *Sylvaemus uralensis* Pallas, 1811 (Karamysheva et al. 2010) revealed variation in copy number and the level of homology of repetitive sequences as well as their localization. In the present study overall centromeric heterochromatin variation in size and intensity of bands in autosomes and heterochromosomes in *M. terricolor* populations is also suggestive of quantitative as well as qualitative variation. Chatterjee et al. (2003) have commented on the basis of their southern hybridization experiments that *M. terricolor* types differ in satellite DNA organization from that of *Mus musculus* Linnaeus, 1758, an allied species, and *Mus booduga*, the sibling species.

It can be concluded that very large to minute C-bands and even absence of C-bands in centromere of autosomes within and between populations of *M. terricolor* indicates presence of differential amount of heterochromatin which might have evolved by non-reciprocal DNA turnover mechanisms in wild populations that has also been suggested by many workers (Dover 1982, Redi et al. 1990), however, this needs more extensive studies.

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The first cytogenetic description of *Euleptes europaea* (Gené, 1839) from Northern Sardinia reveals the highest diploid chromosome number among sphaerodactylid geckos (Sphaerodactylidae, Squamata)

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Abstract

The karyotype of a sphaerodactylid gecko *Euleptes europaea* (Gené, 1839) was assembled for the first time in this species. It is made of 2n = 42 gradually decreasing in size chromosomes, the highest chromosome number so far acknowledged in the family Sphaerodactylidae. The second chromosome pair of the karyotype appears slightly heteromorphic in the male individual. Accordingly, FISH with a telomeric probe revealed an uneven distribution of telomeric repeats on the two homologues of this pair, which may be indicative of an XY sex-determination system in the species, to be further investigated.

Keywords

Sauria, Gekkota, karyotype, chromosomal evolution, telomeric repeats, XY male heterogamety

Introduction

The Italian Gekkotan fauna includes four species: two gekkonid species – *Mediodactylus kotschyi* (Steindachner, 1870) and *Hemidactylus turcicus* (Linnaeus, 1758), a phyllodactylid gecko *Tarentola mauritanica* (Linnaeus, 1758), and a sphaerodactylid *Eu-*

leptes europaea (Gené, 1839) (Bauer et al. 2008). *Euleptes europaea*, the focus of the present study, commonly known as the European leaf-toed gecko, the single living species of the genus *Euleptes*, which was recently resurrected from synonymy with of *Phyllodactylus* (Bauer et al. 1997). Moreover, not long ago, this monotypic genus was considered *incertae sedis*, along with few other Afro-Eurasian genera of the same clade (*Pristurus* Rüppell, 1835, *Teratoscincus* Strauch, 1863, *Quedenfeldtia* Boettger, 1883) plus neotropical *Aristelliger* Cope, 1861, because they all fall into an unresolved polytomy (Gamble et al. 2008a, 2008b). The most up to date phylogeny of Gamble et al. (2011), however, places this monotypic genus into Sphaerodactylidae, once again raised to a rank of a family, which is a sister clade to Gekkonidae and Phyllodactylidae and embraces a large range of species from both Old and New World.

In Europe, *Euleptes* Fitzinger, 1843 is described from at least the early Miocene; the single modern species, *E. europaea*, is a relic endemic of the western Mediterranean region which survived during isolation on the Corso-Sardinian microplate (Müller 2001). In contrast with the other three species widespread on the Italian territory, the current geographic range of *E. europaea* is restricted to Sardinia, Corsica, small mainland and insular areas of Liguria and Tuscany, including the isles of Elba, Gorgona, Capraia, Pianosa, Montecristo, Giglio, and Giannutri, and also to small offshore islands of southern France, Sardinia, and Corsica (Sindaco et al. 2006), as well as to three islands of the Tunisian coast (Delaugerre et al. 2011). This peculiar, largely insular, distribution indicates a relatively recent contraction of its range (Arnold and Ovenden 2002).

Euleptes europaea remains the only gecko of the Italian fauna, which has not been characterized cytogenetically. It is not surprising, since of approximately 1,000 species of Geckonids, in the broad sense, karyotypes have been described for less than 10% of them (Olmo and Signorino 2005, Trifonov et al. 2011). Cytogenetic data are very scarce in Sphaerodactylidae, as well: only 3% of approximately 196 species have been karyotyped (Ezaz et al. 2009). Accordingly, we carried out cytogenetic analyses of *E. europaea* performing a karyological description of individuals from Sardinia, supplemented by physical mapping of telomeric repeats. Molecular cytogenetic investigations on reptiles are largely lacking, but they may be beneficial to solving taxonomic problems and phylogenetic uncertainties and to comprehending evolutionary matters, such as the mechanisms of chromosome evolution and emergence of neo-sex chromosomes, especially in geckos, which are characterized by different sex-determination systems even among closely related taxa (Gamble 2010, Kawai et al. 2009).

Materials and methods

We analyzed a limited sample - one male, one female, and one juvenile - from a population of the island of Santa Maria near the north coast of Sardinia. The animals were handled according to the European Code of Practice for the housing and care of animals used in scientific procedures (Council of Europe 1986). Analyzed specimens

(voucher numbers: EUL1 male, EUL2 juvenile, EUL3 female) are preserved in 70% ethanol and are housed in the herpetological collection of the Dipartimento di Biologia e Biotecnologie "Charles Darwin" Università di Roma "La Sapienza" (CEAC).

Metaphase plates were prepared from bone marrow, intestinal, and testicular cells using standard air-drying method after injection of 1:1000 solution Vinblastine Sulphate, Velbe® (Lilly) as antimitotic solution. The slides were colored with 5% Giemsa solution. For each individual, about 20 metaphase plates were studied and photographed. The telomeric probe was commercially synthesized as two oligonucleotides $(GGGTTA)_7$ and $(TAACCC)_7$ both end-labeled with Cy3 (Bio-Fab Research). The oligonucleotides were dissolved (2 ng/μL) in a hybridization mix made up of 5% Dextran sulphate, 2XSSC, and 5 μg/μL sonicated salmon DNA. For FISH, standard procedures for the hybridization of repetitive sequences (Lichter et al. 1992) were carried out, followed by high-stringency post-hybridization washes at 42°C. As a routine, chromosomes were counterstained with DAPI (4',6-diamidin-2-fenilindolo, 1μg/mL) and propidium iodide (0.5 μg/mL). Ten metaphases per individual were analyzed under Zeiss AxioPhot epifluorescence microscope. The photographs were acquired with a SenSys 1400 CCD camera (Photometrics®). Images were processed using IP-lab software (Scanalytics®) and Adobe® Photoshop® CS3.

Results and discussion

The karyotype of *E. europaea* is composed of 21 chromosome pairs gradually decreasing in size (Fig. 1a). There is no pronounced subdivision of the chromosome complement into macro- and microchromosomes; 17 chromosome pairs may be considered telocentric chromosomes: tiny short arms, visible in some of more elongated chromosomes, are not taken into account for the fundamental number. The minute chromosomes № 20 and № 21 are telocentric, while the smallest pair of the karyotype is definitely biarmed. The largest chromosomes of the complement (pairs № 1 and № 2) are also biarmed, precisely, subtelocentric. However, both homologues of the chromosomes № 2 had short, similar in size true arms only in the female individual (Fig. 1b). In the male, one of the homologues of chromosomes № 2 showed somewhat greater overall compactness and smaller or more contracted short arms in most metaphases after conventional Giemsa staining (Fig. 1c). The degree of this heteromorphism was relevant enough to be worth noting: the average centromeric index of the two homologues of this pair was 14.7% and 8.3%.

FISH with a telomeric probe detected all ordinary telomeric sites of the chromosomes. The present results are in accordance with previously obtained data in *Gonatodes taniae* Roze, 1963, the only other sphaerodactylid species, in which chromosomal distribution of telomeric sequences has been studied so far (Schmid et al. 1994). Also, amplification of the telomeric signals characterized most of telocentric chromosomes in centromeric regions (Fig. 2). This pattern, together with DAPI counterstaining, allowed to better classify chromosomes and arrange homologues in pairs. In the obtained

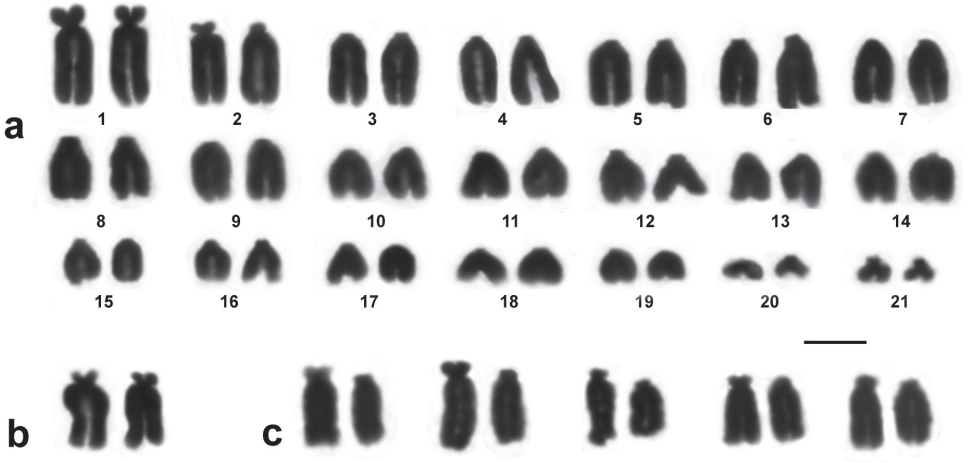

Figure 1. Chromosome complement of *E. europaea* from Sardinia. **a** 2n = 42 male karyotype **b** homomorphic chromosomes 2 (female); **c** – examples of heteromorphic chromosomes №2 (male). Bar = 5 μm.

karyotype, conspicuous interstitial pericentromeric signals are clearly separated from minor regular telomeres in the biarmed chromosomes and in several chromosomes with tiny short arms (e.g., № 8 and № 13 in Fig. 2). Furthermore, in all chromosome pairs, interstitial telomeric sites (ITS) are virtually of the same intensity and size in both homologues, whereas the two homologues of the chromosomes №2 of the male differ for the intensity of interstitial signals.

The ITS sites at centromeres have been described in many different taxonomic groups (Meyne et al. 1990). In some lineages, they were shown to result from retaining the ancestor telomeres after, for example, Robertsonian or tandem fusion/fission (Ventura et al. 2006) or more complex (Fagundes et al. 1997) rearrangements. On the other hand, telomere-like sequences are often present in chromosomes as a component of the satellite DNA (Garrido-Ramos et al. 1998). In many species, centromeric regions of chromosomes contain substantial amounts of telomeric repeats, which often constitute a major component of heterochromatin and is supposed to play an important role in evolutionary chromosomal rearrangements (Slijepcevic et al. 1997, Ruiz-Herrera et al. 2008).

In summary, the karyotype of *E. europaea* looks quite unusual if compared with other records available in the family Sphaerodactylidae, and the chromosome number is the highest among all species of the family presently studied. Since the phylogenetic position of *Euleptes* within Sphaerodactylidae is uncertain, we provide a comparative analysis of all-encompassing data. The genus *Euleptes* falls in a poorly supported assemblage of genera without clear relationships with each other, which includes the following species-poor Afro-Asian genera: *Pristurus* Rüppell, 1835, endemic to Middle East and Arabia, the Asiatic *Teratoscincus* Strauch, 1863, and the Moroccan *Quedenfeldtia* Boettger, 1883, as well as the neotropical species-rich *Aristelliger* Cope, 1861 (Gamble

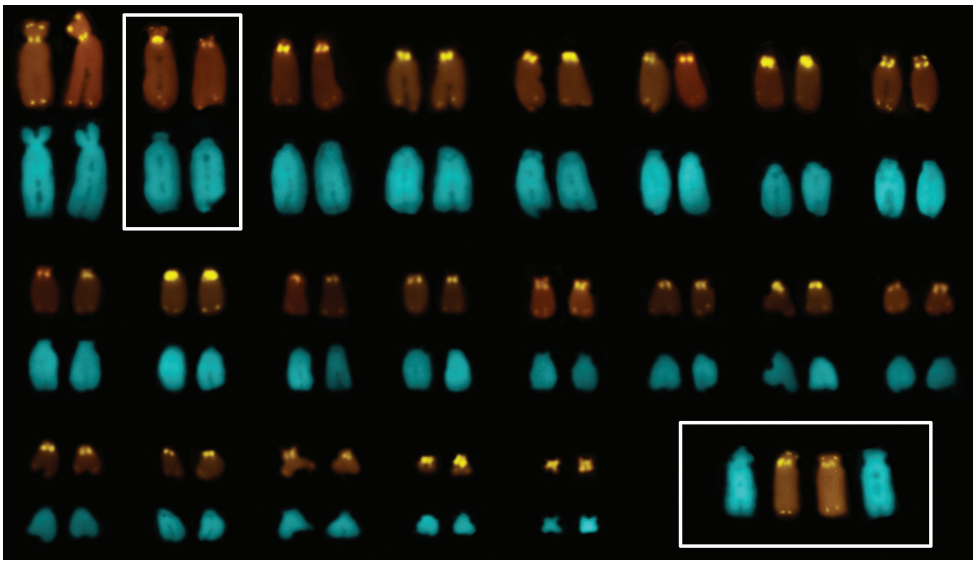

Figure 2. A karyotype of *E. europaea* after FISH with a telomeric probe (upper array) and DAPI-staining (lower array); slightly heteromorphic chromosomes № 2 are framed; the same chromosome pair of a female is shown in the insert below.

et al. 2011). Among these taxa, *Teratoscincus scincus* (Schlegel, 1858) from several Chinese populations (Zheng et al. 1998) and its two subspecies (*T. s. scincus* and *T. s. rustamowi*) from the Central Asia (Kazakhstan, Tadjikistan, and Turkmenia) (Manilo 1993, Manilo and Pisanets 1984), as well as *T. przewalskii* Strauch, 1887 (Zheng et al. 1998), all have a 2n = 36 karyotype. The results of different authors are in accordance with each other in presenting a karyotype formula of 2n = 36, with 24 macrochromosomes (6 biarmed and 18 telocentric) and 12 microchromosomes, except for a pioneer result of De Smet (1981), who reported a karyotype of 2n = 34 all acrocentric chromosomes in *T. scincus* (Schlegel, 1858). According to Branch (1980), *Pristurus carteri* (Gray, 1863) have similar, 2n = 34 all-acrocentric karyotype.

The family Sphaerodactylidae includes also one well supported major clade, which comprises five genera of the neotropical sphaerodactylid lizards (*Coleodactylus* Parker, 1926; *Gonatodes* Fitzinger, 1843; *Lepidoblepharis* Peracca, 1897; *Pseudogonatodes* Ruthven, 1915, and *Sphaerodactylus* Wagler, 1830) (see dos Santos et al. 2003). The highest diploid number of chromosomes within this cluster is 32. Thus, three species of *Gonatodes* (*G. humeralis* (Guichenot, 1855), *G. hasemani* Griffin, 1917, and *G. vittatus* (Lichtenstein, 1856)) and *Coleodactylus amazonicus* (Andersson, 1918) show 2n = 32, all telocentric karyotypes (McBee et al. 1984, 1987, Rada De Martinez 1980, dos Santos et al. 2003), but some species of *Gonatodes* have lower diploid number (2n = 22 and 26 in *G. ceciliae* Donoso-Barros, 1966) (McBee et al. 1987) or exceptionally low one (2n = 16 in *G. taniae* Roze, 1963), which is thought to be due to a series of centric fusions from an acro(telo)centric ancestral karyotype (Schmid et al. 1994).

Based on its prevalence among the neotropical sphaerodactylid geckos, the 2n = 32 all-acrocentric karyotype was proposed as ancestral, while centric fusion was assumed as the main mechanism of chromosome evolution in this latter grouping (Schmid et al. 1994). On the other hand, once, considering the family Gekkonidae, then inclusive of sphaerodactylid lizards, King (1977) suggested as ancestral a 2n = 38 karyotype with exclusively acrocentric chromosomes. Taking in account the present evidence of the 2n = 42 karyotype of *E. europaea* with mainly telo(acro)centric chromosomes, we must agree with dos Santos et al. (2003) that it is still premature to speculate on the ancestral karyotype for Sphaerodactylidae.

Another outcome of the present study is a possible male chromosome heteromorphism in *E. europaea*. However, provided the extremely limited sample presently examined, chromosome polymorphism unrelated to sex is possible, as well. If the present data in *E. europaea* actually reflect the XX/XY sex determination system, which is still to be corroborated, it would be indicative of rather new or undifferentiated sex chromosomes. The available cytogenetic data on sex chromosomes in Sauria are rare, but give an idea of how different may be the morphology and composition of sex chromosomes in different species with male (XX/XY) or female (ZZ/ZW) heterogamety (Ezaz et al. 2009). Among few karyotyped geckos of Spherodactylidae, no female heterogamety has been found yet, while male heterogamety has been reported in only one species – the Venezuelan *Gonatodes ceciliae* Donoso-Barros, 1966 (McBee et al. 1987). However, in this species, a large metacentric X and a small acrocentric Y chromosome are remarkably heteromorphic. Finally, a genetic sex determination system may be hypothesized in a lizard species, which inhabits particular environment, as very small islets and isolated rocks. Such environment possibly will not provide consistent temperature ranges, which are necessary to assure a balanced sex ratio within population (Pen et al. 2010). In fact, *Tarentola mauritanica*, which is known to have environmental sex determination, has not been found on islets so small as the ones, where the *E. europaea* is often observed (Delaugerre et al. 2011).

The main conclusions of the present analysis are: 1) the diploid chromosome number in Sphaerodactylidae may reach 2n = 42, the uppermost value so far observed in the family, as well as one of the highest diploid numbers among all Gekkotan lizards (acknowledged maximum is 2n = 46 in Thailand house gecko, *Cosymbotus platyurus* (Schneider, 1792) (classified also as *Hemidactylus platyurus* (Schneider, 1792)) according to Olmo and Signorino (2005), as well as in *Hemidactylus bowringi* (Gray, 1845) according to Nakamura (1932) and Ota (unpublished) (in Ota et al. 1989)), whereas even higher numbers of chromosomes characterize some unisexual triploid lineages, e.g., the parthenogentic gecko *Hemidactylus stejnegeri* Ota et Hikida, 1989 (3n = 56) or *H. vietnamensis* Darevsky, Kupriyanova et Roshchin, 1984 (3n = 60) or *H. garnotii* Duméril et Bibron, 1836 (3n = 70) (see Ota et al. 1989); 2) centromeric regions of all chromosomes of *E. europaea* are rich in telomeric repeats, which may play an active role in the karyotype evolution of the lineage; 3) on the base of likely heteromorphism of chromosome pair № 2, a male heterogamety may be tentatively hypothesized in *E. europaea.*

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A new form of the mole vole *Ellobius tancrei* Blasius, 1884 (Mammalia, Rodentia) with the lowest chromosome number

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Abstract

The subterranean mole vole, *Ellobius tancrei,* with a specific variability in autosomes (2n = 31–54) and unusual sex chromosomes (XX in males and females), represents an amazing model for studying the role of chromosome changes in speciation. New materials from the upper reaches of the Surkhob River in the Pamiro-Alay mountains resulted in the discovery of a new form with 2n = 30. The application of Zoo-FISH and G-banding methods allowed the detection of 13 pairs of autosomes as Robertsonian metacentrics originated after fusions of acrocentrics of an assumed ancestral karyotype of *E. tancrei* with 2n = 54. The sex chromosomes (XX, in both sexes) and one pair of acrocentric autosomes are the only acrocentrics in this karyotype, and the set with 2n = 30 possesses the lowest possible chromosome number among populations of *E. tancrei*.

Keywords

speciation, mole voles, *Ellobius*, Robertsonian rearrangements, chromosome painting, *Cricetidae*

Introduction

Naturally occurring chromosome variability is essential for understanding the disputed role of chromosome changes in speciation (White 1978, King 1993). A Robertsonian (Rb) translocation is a chromosome rearrangement involving the centric fusion of two acrocentric chromosomes to form a single metacentric chromosome, and it is one of the most frequent events in mammalian karyotype evolution (Slijepcevic 1998, Ruiz-Herrera et al. 2012). Well-studied cases of extensive Robertsonian variation in species such as the house mouse, *Mus musculus domesticus* Schwarz et Schwarz, 1943 (Gropp et al. 1969, Piálek et al. 2005), the common shrew, *Sorex araneus* Linnaeus, 1758 (Ford et al. 1957, Wójcik et al. 2003), or the mole vole *Ellobius tancrei* (Lyapunova et al. 1980, 2010) have their own specific features. All these species are widely distributed; for example, house mice are distributed all over the globe due to human activity. Mice and shrews include a lot of races within their ranges, but *E. tancrei* has 2n = 54 over most of the distribution range and a great variety in chromosome numbers (2n from 53 to 31), which is restricted to a limited area in the Pamiro-Alay, with only single non-homologous translocations (2n = 53) recorded outside, in the Tien Shan mountains (Lyapunova et al. 1985). An amazing feature of mole voles is their subterranean way of life. Living underground preserves mole voles from predators, restricts spreading, and promotes their sociality. The last two factors may contribute to a rapid fixation of chromosome translocations due to more or less permanent monogamous bonds (our unpublished data).

Thorough analysis based on G-banding revealed a complicated structure of chromosome variability in the Surkhob River valley (Pamiro-Alay), where forms with the same chromosome numbers have different sets of Rb metacentrics. It was concluded that the variability was produced by hybridisation, as well as chain fusions (Bakloushinskaya, Lyapunova 2003). New field explorations and applying a fluorescence *in situ* hybridisation (Zoo-FISH) approach have revealed a case of monobrachial homology in this region too (Bakloushinskaya et al. 2010), and raised a question of natural limits for fusions. In numerous field explorations, we were unable to find additional animals at the area where a single specimen with 2n = 31 was collected (Lyapunova et al. 1980). The surrounding areas are inhabited by the form with 2n = 32, and that is why the specimen was considered by us, for a long time, as a case of a single mutation. The main aim of this study was to investigate suitable mole vole areas in the upper reaches of the Surkhob River and determine the structure of karyotypes for discovered animals.

Material and methods

Five animals (two females and three males) from two colonies were captured by live traps (Golov 1954) on the northern bank of the Surkhob River in Pamiro-Alay (39°15.37'N, 71°20.59'E, 900 m above sea level) in April, 2010.

Chromosomes from bone marrow (Ford, Hamerton 1956) were prepared from all animals; tissues of three specimens were used for tissue culturing. Fibroblast cell

lines were prepared as previously described (Sitnikova et al. 2007). Full sets of paints derived from flow-sorted chromosomes of the field vole *Microtus agrestis* Linnaeus, 1761 (Sitnikova et al. 2007) were used. FISH was performed according to previously published protocols (Yang et al. 1999; Graphodatsky et al. 2000). G-banding was carried out for all metaphase chromosomes prior to FISH using trypsin treatments (Seabright 1971).

Images were captured using VideoTesT-FISH 2.0. and VideoTesT-Karyo 3.1. (VideoTesT) or Case Data Manager 6.0 (Applied Spectral Imaging Inc., ASI) software with either a ProgRes CCD (Jenoptik) or ASI CCD camera, respectively, mounted on an Axioskop 2 plus (Zeiss) microscope with filter sets for DAPI, FITC, and rhodamine. Hybridisation signals were assigned to specific chromosome regions defined by GTG-banding patterns previously photographed and captured with the CCD camera. All images were processed using PaintShop Photo Pro X2 (Corel).

Results

We analysed the structure of karyotypes obtained by direct methods from bone marrow and from cultures. It is known that spontaneous chromosome aberrations may appear in cell cultures (Clare 2012), so it was necessary to control the karyotype structure by a direct method. Karyotypes of all animals contained 30 chromosomes (Fig. 1), and at least 30 plates were counted for each specimen. One pair of submetacentric chromosomes (N 7) is typical for *E. tancrei* and distinguishes it from the chromosomally stable sibling species *E. talpinus* Pallas, 1770. As we reported recently, these chromosomes obtained evolutionary new centromeres (Bakloushinskaya et al. 2012). There are also 12 pairs of Rb metacentrics, one pair of acrocentric chromosomes, and the sex chromosomes XX, which are acrocentric in both sexes. Each of the fifteen chromosome painting probes of the field vole, *Microtus agrestis,* (MAG 2, 4, 6, 10–13, 15, 16, 18–24) delineated one region in the *E. tancrei*, 2n = 30 karyoform; eight probes (MAG 3, 5, 7–9, 13, 14, 17) each delineated two chromosome segments; and the MAG 1 probe delineated four chromosome segments. The only MAG X probe showed signals on both male and female X chromosomes; the MAG Y probes has not produced any specific signal. In total, the 21 MAG autosomal probes revealed 35 conserved segments in the genome, which corresponds to the genome composition of typical *E. tancrei*, 2n = 54 (Bakloushinskaya et al. 2012). The comparison revealed a complete homology between acrocentrics and corresponding Rb metacentrics. The smallest Rb metacentric [Rb(24.26), Fig. 1] has never been detected in karyotypes of other chromosomal forms, including the form with the low chromosome number, 2n = 32, which inhabits the northern bank of the Surkhob River (Bakloushinskaya, Lyapunova 1990). Acrocentric chromosomes involved in the translocation were determined by G-banding as chromosomes number 24 and 26, according to the new nomenclature developed for *E. tancrei*, 2n = 54 (Bakloushinskaya et al. 2012), and confirmed by applying Zoo-FISH probes MAG18 and MAG24 (Fig. 2).

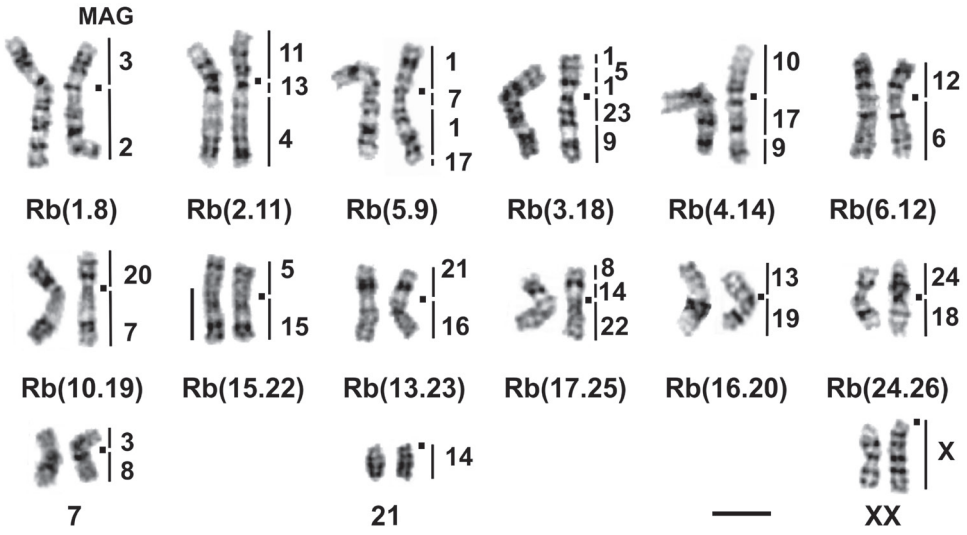

Figure 1. G-banded karyotype of *E. tancrei*, 2n = 30 (25618 ♂). The chromosome nomenclature follows Bakloushinskaya et al. (2012). Black squares mark the positions of centromeres. Vertical black bars and the numbers beside them mark the localisation of *M. agrestis* (MAG) chromosome segments. Bar = 10 μm.

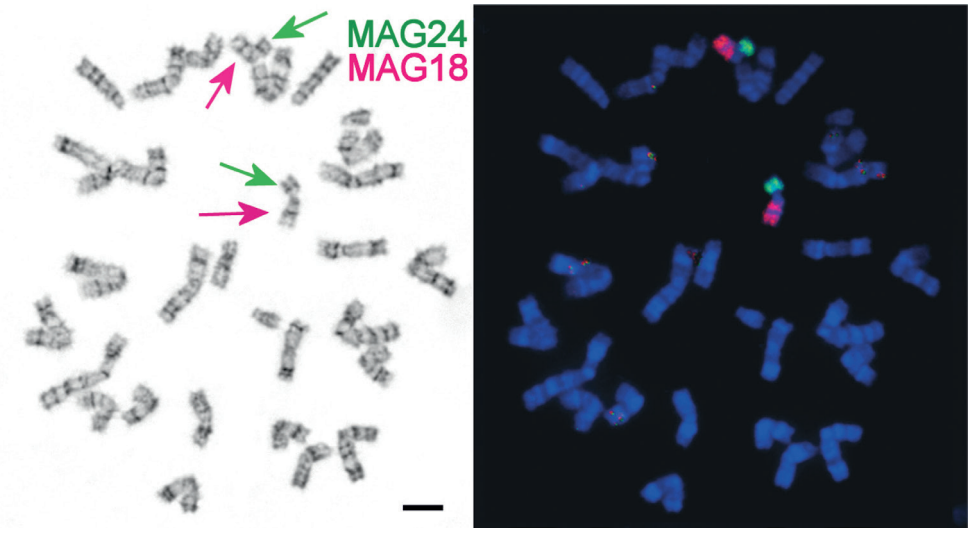

Figure 2. a G-banded *E. tancrei*, 2n = 30 (25601 ♀) metaphase spread. **b** the same spread, fluorescent *in situ* hybridisation (Zoo-FISH) of *M. agrestis* (MAG) chromosome 18 (red) and 24 (green) on Rb metacentrics (red and green arrows). Bar = 10 μm.

Discussion

The analysis of the spatio-temporal variation in the structure of a chromosomal polymorphism zone in *E. tancrei* with an interval of 25 years showed that the distribution

of chromosomal forms within the area has not changed during this period, except for a small range expansion of a low-chromosomal form (2n = 32) at the western boundary (Lyapunova et al. 2010). Discovery of the chromosome form with 2n = 30 expanded the limits for chromosome rearrangements to the maximal number of fusions in *E. tancrei*, and also moved a border of the chromosomal polymorphism zone to the East, closer to the *E. alaicus* Vorontsov et al., 1969 range. The form with 2n = 30 may be considered as the lowest possible chromosome number for *E. tancrei*, because in such a karyotype only the sex chromosomes (XX, in both sexes) and one pair of acrocentric autosomes remain not rearranged. To date, whole-arm reciprocal translocations (WARTs) have not been recorded in *E. tancrei*. The X chromosomes clearly do not tend to be involved in Rb translocations; a heterozygous 2n = 53 karyotype with an X-autosome Rb-translocation was detected only once in a single female of *E. tancrei* from the Tien-Shan (Lyapunova et al. 1985).

Applying the Zoo-FISH method allows the detection of the homology of translocations, which are only estimated by G-banding. The new karyotype with 2n = 30 contains three Rb translocations (metacentrics 2, 3, 4, Fig. 1) that are homologous to fusions recently described for the 2n = 48 populations inhabiting the northern bank of the Surkhob River, approximately 100 km to the west. Based on these data, we suggest a common origin for these populations. Independent origin may be suggested for a population from the southern bank of the Surkhob River (2n = 50), which shares monobrachial homology with the 2n = 48 form. Furthermore, a partial homology was revealed by Zoo-FISH in spite of their similar G-banding picture (Bakloushinskaya et al. 2010). This case of hidden variability requires a re-investigation of other known chromosomal forms of *E. tancrei*. The Pamiro-Alay is a mountain system with deep valleys and large rivers; mole voles have a mosaic pattern of distribution there. An existence in small demes with limited possibilities for spreading may provoke inbreeding and fast fixing of chromosome rearrangements (Bush et al. 1977). A subterranean way of living may enhance such a process. Speciation by monobrachial centric fusions is one of the well-documented models for house mice (Baker, Bickham 1986, Nunes et al. 2011). However, in the common shrew, the gene flow may be not affected by even extensive monobrachial homology in a hybrid zone between karyotypic races (Basset et al. 2006, Horn et al. 2012). Additional molecular cytogenetic studies are needed to clarify the homology of different chromosomal forms of *E. tancrei* and determine the role of different chromosome rearrangements in species evolution.

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RESEARCH ARTICLE

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Karyotypes of some medium-sized Dytiscidae (Agabinae and Colymbetinae) (Coleoptera)

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Abstract

An account is given of the karyotypes of 29 species of medium sized Dytiscidae (Coleoptera). Of the 20 species of *Agabus* Leach, 1817, 18 have karyotypes comprising 21 pairs of autosomes and sex chromosomes which are either X0(♂) or XX (♀). These species are *A. serricornis* (Paykull, 1799), *A. labiatus* (Brahm, 1791), *A. congener* (Thunberg, 1794), *A. lapponicus* (Thomson, 1867), *A. thomsoni* (J. Sahlberg, 1871), *A. confinis* (Gyllenhal, 1808), *A. sturmii* (Gyllenhal, 1808), *A. bipustulatus* (Linnaeus, 1767), *A. nevadensis* Håkan Lindberg, 1939, *A. wollastoni* Sharp, 1882, *A. melanarius* Aubé, 1837, *A. biguttatus* (Olivier, 1795), *A. binotatus* Aubé, 1837, *A. affinis* (Paykull, 1798), *A. unguicularis* (Thomson, 1867), *A. ramblae* Millan & Ribera, 2001, *A. conspersus* (Marsham, 1802) and *A. nebulosus* (Forster, 1771). However two species, *A. infuscatus* Aubé, 1838 and *A. adpressus* Aubé, 1837, have developed a neo-XY system, with karyotypes comprising 21 pairs of autosomes and XY sex chromosomes (♂). No chromosomal differences have been detected between typical *A. bipustulatus* and *A. bipustulatus* var. *solieri* Aubé, 1837, nor have any been found between the three species of the *A. bipustulatus* complex (*A. bipustulatus*, *A. nevadensis* and *A. wollastoni*). The four species of *Colymbetes* Clairville, 1806, *C. fuscus* (Linnaeus, 1758), *C. paykulli* Erichson, 1837, *C. piceus* Klug, 1834 and *C. striatus* (Linnaeus, 1758) have karyotypes comprising 20 pairs of autosomes and sex chromosomes which are X0 (♂), XX (♀). Two of the species of *Rhantus* Dejean, 1833, *R. exsoletus* (Forster, 1771) and *R. suturellus* (Harris, 1828) have karyotypes comprising 20 pairs of autosomes and X0/XX sex chromosomes, but the other three species, *R. grapii* (Gyllenhal, 1808), *R. frontalis* (Marsham, 1802) and *R. suturalis* (Macleay, 1825) have 22 pairs of autosomes and X0/XX sex chromosomes. *Agabus congener* and *Rhantus suturellus* may have one B-chromosome. Nine of the species have previously published karyotype data but for seven of these the data are wrong and are here corrected.

Keywords

Chromosomes, karyotypes, sex chromosome systems, Dytiscidae, *Agabus*, *Colymbetes*, *Rhantus*

Introduction

When Smith and Virkki (1976) compiled their list of beetles whose chromosome numbers were known, they gave data for 2120 species, including 138 named species belonging to the suborder Adephaga. Of these 110 were Carabidae, 21 Dytiscidae and 7 Gyrinidae. By 1984 the number of carabid species whose chromosome numbers were known had increased to 426 (Serrano and Yadav 1984) and the number of Dytiscidae had reached 32, though five of these were unidentified (Yadav et al., 1984). Interestingly, the total number of world species of Carabidae is given as "more than 40,000" (Wikipedia) while the number for Dytiscidae is about 4080 (Nilsson-Örtmann and Nilsson, 2010), so at this stage the proportion of species for which chromosome numbers are listed in the two families is about the same. Data have continued to accumulate, so that Galian and Moore (1994) give the number of carabid species whose chromosome numbers are known as "more than 800". Numbers for Dytiscidae have also continued to increase. Saleh Ahmed et al. (2000) gave data on 1 species of *Hydrovatus* Motschulsky, 1 *Hydroporus* Clairville and 3 *Nebrioporus* Régimbart (Hydroporinae), 1 *Agabus* Leach (Agabinae), 1 *Colymbetes* Clairville (Colymbetinae) and 1 *Eretes* Laporte and 1 *Hydaticus* Leach (Dytiscinae). Aradottir and Angus (2004) gave information on 7 species of *Ilybius* Erichson (Agabinae), Dutton and Angus (2007) described the karyotypes of 7 species of the "*Stictotarsus griseostriatus* (De Geer) group" (now in the genus *Boreonectes* Angus) (Hydroporinae), and Tatton and Angus (2011) reported on 30 species related to *Deronectes* Sharp (Hydroporinae), of which 27 had no previously published data, bringing to total number of dytiscid species with known chromosome numbers to about 82. This gives both the Carabidae and the Dytiscidae as having about 2% of their species with known chromosome numbers.

The present paper reports on 20 *Agabus* species, of which only four had previously published chromosome data (wrong for three of the species), 4 *Colymbetes*, all of which have previously published data, though for three of the species these data were wrong, and 5 *Rhantus* of which one species had published data, again wrong. This gives a net increase to over 100 in the number of dytiscid species for which information on chromosome numbers are available. The data have been gathered over more than 25 years, and include the results of research projects by three undergraduate students of Royal Holloway, University of London, supervised by R. B. Angus. D. E. Wenczek (1994) studied *Rhantus* Dejean, J. C. Carter (2001) *Rhantus* and *Colymbetes*, and M. J. Clery (2009) made a special study of the *Agabus bipustulatus* (Linnaeus) species group.

Material and Methods

The species studied, with their localities of origin, collectors and dates, as well as the number of specimens yielding successful preparations, is given in Table 1. Nomenclature and classification follow Nilsson and Hájek (2013, internet version). Where there is more than one locality for a given species the localities from which various prepara-

Table 1. Material studied.

Species	Locality	Collector, date	Material
Genus *Agabus* Leach, 1817			
Subgenus *Agabus* s. str.			
A. serricornis (Paykull, 1799)	SWEDEN: Västerbotten, Åmsele.	A. N. Nilsson, 1990	1♂
A. labiatus (Brahm, 1791)	FINLAND: Lapponia Inarensis, Inari	R. B. Angus, 2008	1♂, 1♀
Subgenus *Acatodes* C. G. Thomson, 1859			
A. congener (Thunberg, 1794)	SCOTLAND: Ayrshire, Knockewart Moss	G. N. Foster, 1986	2♂♂, 1♀
	SWEDEN: Västerbotten, Sirapsbaken	A. N. Nilsson, 1986	2♂♂, 1♀
A. lapponicus (Thomson, 1867)	SWEDEN: Västerbotten, Skörträskberget	A. N. Nilsson, 1986	3♂♂, 1♀
A. thomsoni (J. Sahlberg, 1871)	NORWAY: Finnmark east, Bugøynes	R. B. Angus, 2008	1♂
A. confinis (Gyllenhal, 1808)	SWEDEN: Västerbotten, Vindeln, Strycksele	A. N. Nilsson, 1991	3♀♀
A. sturmii (Gyllenhal, 1808)	ENGLAND: Surrey, Chobham Common	R. B. Angus, 1991	1♂, 1♀
A. infuscatus Aubé, 1838	NORWAY: Finnmark east, Bugøynes	R. B. Angus, 2008	1 ♂
Subgenus *Gaurodytes* C. G. Thomson, 1859			
A. bipustulatus (Linnaeus, 1767)	ENGLAND: Surrey, Wisley Common	R. B. Angus & M. J. Clery, 2008	3♂♂
	Hampshire, Woolmer Bog	R. B. Angus, 2008	3♂♂,1 ♀
	Worcestershire, Wyre Forest	R. B. Angus & M. J. Clery, 2008	3 ♂♂
	FINLAND: Lapponia Inarensis, Inari	R. B. Angus, 2008	1 ♂
	SWEDEN: Norbotten, near Umeå	M. Drotz, 1996	1 ♂
A. bipustulatus var. *solieri* Aubé, 1837	SWITZERLAND, Valais, small lake S of Illsee	R. B. Angus, 2008	3♂♂, 1 ♀
	Valais, ditch near the Moiry glacier	R. B. Angus, 2008	2 ♂♂
	FRANCE: Hautes-Alpes, Guillestre	M. Drotz, 1998	2♂♂
A. nevadensis Håkan Lindberg, 1939	SPAIN: Granada, Sierra Nevada	M. Drotz, 1999	1 ♂, 1 ♀
A. wollastoni Sharp, 1882	MADEIRA: Pico Arieño	A. N. Nilsson, 1998	2 ♂♂, 1 ♀
A. melanarius Aubé, 1837	ENGLAND: East Sussex, Hindleap Warren	R. B. Angus & M. J. Clery, 2008	1 ♂, 1 ♀
A. biguttatus (Olivier, 1795)	EGYPT (Saleh Ahmed et al., 2000): El Noqra	R. Saleh Ahmed & R. B. Angus, 1994	1 ♂
	SARDINIA: Medio Campidano, Giara di Gesturi	R. B. Angus, 1994	1♂
A. binotatus Aubé, 1837	CORSICA: Corse-du-Sud, Col de Vizzavona.	R. B. Angus, 1993	1 ♂

Species	Locality	Collector, date	Material
A. affinis (Paykull, 1798)	ENGLAND: Hampshire, New Forest	R. B. Angus, 1987	1 ♂
A. unguicularis (Thomson, 1867)	ENGLAND: Norfolk, East Walton Common	R. B. Angus, 1987	2 ♂♂
A. ramblae Millan & Ribera, 2001	SPAIN: Huesca, Villanueva de Sigena, Barranco del Hospital	I. Ribera, G.N. Foster, D. Lott & P. Aguilera, 1995	2♂♂
	Murcia, Rambla de Majada en El Pilón	A. Millan, 1995	1 ♀
A. conspersus (Marsham, 1802)	ENGLAND: Hampshire, Keyhaven	R. B. Angus, 1993	1 ♂
A. nebulosus (Forster, 1771)	ENGLAND: East Sussex, Cuckmere Haven	R. B. Angus, 1993	1♂
	CANARY ISLANDS: Tenerife	A. N. Nilsson, 1994	1♂, 2 ♀♀
A. adpressus Aubé, 1837	NORWAY: Finnmark east, Bugøynes	R.B. Angus, 2008	1 ♂
Genus *Colymbetes* Clairville, 1806			
C. fuscus (Linnaeus, 1758)	ENGLAND: Surrey, Wisley Common	R. B. Angus, 2000	1 ♂
	FRANCE: Indre, Pinail	R. B. Angus, 2000	1 ♂
C. paykulli Erichson, 1837	SWEDEN: Ångermanland, Hörnsjö, lake Uthörnsjön	A. N. Nilsson, 2000	1 ♂
	Ångermanland, Mullsjö	A. N. Nilsson, 2000	1 ♂
C. piceus Klug, 1834	EGYPT (Saleh Ahmed et al., 2000): El Noqra	R. Saleh Ahmed & R. B. Angus, 1994	1 ♂
C. striatus (Linnaeus, 1758)	SWEDEN: Ångermanland, Hörnsjö, lake Uthörnsjön	A. N. Nilsson, 2000	1 ♂
Genus *Rhantus* Dejean, 1833			
Subgenus *Nartus* Zaitsev, 1907			
R. grapii (Gyllenhal, 1808)	ENGLAND: Dorset, Studland Heath	R. B. Angus, 1993	2♂♂
Subgenus *Rhantus* s. str.			
R. exsoletus (Forster, 1771)	ENGLAND: Dorset, Studland Heath	R. B. Angus, 1993	1 ♂
	Norfolk, Gayton Thorpe Common	R. B. Angus, 1993	1 ♂
R. frontalis (Marsham, 1802)	ENGLAND: Norfolk, Gayton Thorpe Common	R. B. Angus, 1993	1 ♂
	Norfolk, Thompson Common	R. B. Angus, 1993	1 ♂
R. suturalis (Macleay, 1825)	ENGLAND: Dorset, Studland Heath	R. B. Angus, 2000	1 ♂
	Middlesex, Staines Moor	R. B. Angus, 2000	1 ♂
	KUWAIT: Ras Az Zawr	R. B. Angus, 1996	1 ♂
R. suturellus (Harris, 1828)	FRANCE: Indre, Pinail	R. B. Angus, 2000	1 ♂
	ENGLAND: Dorset, Studland Heath	R. B. Angus, 1993, 2000	1♂, 2 ♀♀

tions came are given in the figure captions. Otherwise localities are not given apart from in the table.

Preparations were made from adult beetles, using mid-gut, testis and ovary, following the protocol given by Shaarawi and Angus (1991) and Dutton and Angus (2007). Treatment with colchicine and hypotonic KCl was for 12.5 min in each solution. C-banding was obtained using saturated $Ba(OH)_2$ at room temperature, followed by incubation in salt-sodium citrate (2 X SSC) at 60° C. Treatment times varied, and the technique evolved over the more than 25 years of the study. If a treatment has been insufficient to produce C-banding, it may be repeated. Initially Angus used to clear the stain with a short immersion in 2X SSC at 60° C, but later found this unnecessary. One set of early experiments with *Agabus congener* and *A. lapponicus* was particularly interesting: an initial treatment of 5 min in $Ba(OH)_2$ proved inadequate. A repeat treatment with 5 min in $Ba(OH)_2$ produced good centromeric C-bands, but if the second treatment was for 3 min the secondary constrictions were also stained (Fig 1 f, g, and k with the secondary constrictions, Fig. 1 j with just the centromeric C-bands).

Chromosome measurements were made on screen and were used for calculating Relative Chromosome Length (RCL), the length of each chromosome expressed as a percentage of the total haploid autosome length in the nucleus. This compensates for differing degrees of chromosome contraction shown in different nuclei. For the *Agabus bipustulatus* group the RCL data were subjected to statistical analysis using Student's t-test, but otherwise they are given as approximate values only, to indicate the size relationships of the different pairs of autosomes. Centromere Indices (CI) are not given in detail, but are assigned to their conventional categories. Based on Sumner (2003) the categories are: metacentric–CI 46–50; submetacentric–CI 26–45; subacrocentric–CI 16–25; acrocentric–CI 3–15.

Results

Agabinae Thomson, 1867
Agabus Leach, 1817

Subgenus *Agabus* s. str.

A. serricornis (Paykull, 1799). Fig. 1 a. Published information: none. 2n = 42 + X0 (♂). The RCLs of the autosomes range from about 7.6–2.5, with sharp decreases between pairs 5 (RCL about 6.4) and 6 (RCL about 5), 15 (RCL about 4.5) and 16 (RCL about 3.4), and 20 (RCL about 3.1) and 21 (RCL about 2.5). The X chromosome (RCL about 6.4) is similar in size to pairs 4 and 5. Most of the chromosomes are metacentric to submetacentric, with pairs 8–11 subacrocentric and pairs 15 and 20 more or less acrocentric. Pair 12 has a distinct secondary constriction at the base of its short arm. The X chromosome is subacrocentric, with the centromere clearly nearer the end than in autosomes 4 and 5. No C-banded material is available.

Figure 1. *Agabus s. str.* (**a–e**) and *A.* (*Acatodes*) (**f–q**), mitotic chromosomes arranged as karyotypes. **a** *A. serricornis*, ♂, mid-gut, plain **b**, **c** *A. labiatus*, ♂, mid-gut **b** plain **c** C-banded **d**, **e** *A. labiatus*, ♀, mid-gut **d** plain, **e** C-banded **f**, **g** *A. congener*, ♂, Scotland, testis, C-banded **h** *A. congener*, ♀, Sweden, mid-gut, C-banded, with 1 B-chromosome **i–k** *A. lapponicus*, ♂, Sweden, testis **i** plain **j**, **k** C-banded **l**, **m** *A. thomsoni*, ♂, mid-gut **l** plain **m** the same nucleus C-banded **n**, **o** *A. confinis*, ♀, mid-gut, plain **n** lacking one X chromosome **o** lacking one replicate each of autosomes 1 and 2 **p** *A. sturmii*, ♂, mid-gut, plain **q** *A. infuscatus*, ♂, mid-gut, plain. Bar = 5μm.

A. labiatus (Brahm, 1791). Fig. 1 b, c (♂), Fig. 1 d, e (♀). Published information: none. 2 n = 42 + X0 (♂), 42 + XX (♀). The autosomes, all either metacentric or submetacentric, have RCLs ranging from about 7.8–2.7, with a fairly gradual decrease along the

karyotype, though this is slightly sharper between pairs 5 (RCL about 7.1) and 6 (RCL about 5.9) and 11 (RCL about 4.3) and 12 (RCL about 3.8). The X chromosome is submetacentric and the largest in the nucleus (RCL about 9). Pair 5 have secondary constrictions on the long arm and pair 13 on the short arm. C-banding (Fig. 1 c, e) shows a limited development of centromeric C-bands. These are present on autosomes 1, 3–6, 12, 14 and 17–20. The remaining autosomes, and the X chromosome, lack C-bands. Many of the C-bands are very weak, with the strongest bands present on autosomes 5 and 12.

Subgenus *Acatodes* C. G. Thomson, 1859

A. congener (Thunberg, 1794). Fig. 1 f, g (♂), Fig. 1 h (♀). Published information: none. 2 n = 42 + X0 (♂), 42 + XX (♀), 1 B-chromosome. The autosomes, all more or less metacentric, have RCLs ranging from about 7–4, with an even size decrease along the karyotype. The submetacentric X chromosome, RCL about 9, is clearly the longest in the nucleus. All the chromosomes have distinct centromeric C-bands, with some variation in strength between pairs, and autosomes 1 and 8 have secondary constrictions which may C-band, especially that on autosome 1. The C-banding reaction of the secondary constriction of autosome 8 is less pronounced, and the constriction may be apparent in only one of the replicates. The Swedish female (Fig. 1 h) has one B-chromosome, about as long as autosome 1 and appearing uniformly partly heterochromatic.

A. lapponicus (Thomson, 1867). Fig. 1 i–k (♂). Published information: none. 2n = 42 + X0 (♂). The karyotype of this species appears indistinguishable from that of *A. congener*.

A. thomsoni (J. Sahlberg, 1871). Fig. 1 l, m (♂). Published information: none. 2n = 42 + X0 (♂). The karyotype of this species is very similar to those of *A. congener* and *A. lapponicus*, but the longest autosome with a secondary constriction is placed as no. 2 as in this material it appears distinctly shorter than the longest autosome (pair 1). It is possible that additional material would show this not to be the case. As in the preceding two species, the secondary constriction on autosome 8 is more conspicuous in one of the replicates.

A. confinis (Gyllenhal, 1808). Fig. 1 n, o (♀). Published information: 2n = 40 + "XY" (sex chromosomes not identified) (Smith, 1953). 2n = 44 (♀), probably 42 + XX. The material available for study was three females, and although no intact chromosomal complement was obtained, the 43 chromosomes shown in Fig. 1 n exceed the number given by Smith. The suggestion that the X chromosome is the largest in the nucleus is based on comparison with the karyotypes of the three preceding species, all, like *A. confinis*, members of the *A. congener* group. In the interpretation given here, Fig. 1 n lacks one X chromosome while Fig. 1 o, from a different specimen, has both X chromosomes but lacks one replicate each of autosomes 1 and 2.

A. sturmii (Gyllenhal, 1808). Fig. 1 p (♂). Published information: 2n = 40 + Xy_p (Suortti, 1971). 2n = 42 + X0 (♂), 42 + XX (♀). The autosomes, all either metacentric or submetacentric, have RCLs ranging from about 6.8–2.7. There is a fairly even decrease in length to pair 16 (RCL about 4.8), then a more abrupt decrease to pairs 17–20 (RCL about 3.4) and a further drop to pair 21 (RCL about 2.7). The X metacentric chromosome,

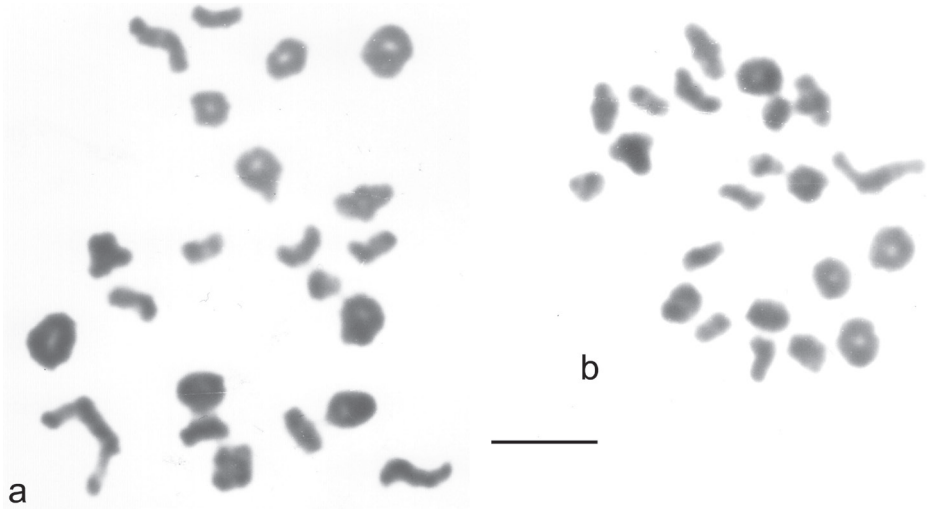

Figure 2. a, **b** *A. infuscatus* testis, first metaphase of meiosis. Bar = 5 μm.

RCL about 11.5, is by far the longest in the nucleus, almost twice as long as autosome 1. Suortti's (1971) material consists of first meiotic metaphases obtained by either sectioning or squashing, and is not clear enough to give an accurate assessment of the karyotype.

A. infuscatus Aubé, 1838. Figs 1q, 2 (♂). Published information: none. 2n = 42 + neo XY. The autosomes are nearly all either metacentric or submetacentric, but pairs 3 and 17 are subacrocentric. The RCLs of the autosomes range from about 7.9–2.9, and there is a fairly even size decrease along the karyotype, though with slightly sharper decreases between pairs 1 (RCL about 7.9) and 2 (RCL about 6.9), 11 (RCL about 4.3) and 12 (RCL about 3.6), and pairs 18 (RCL about 3.6) and 19 (RCL about 2.9). The subacrocentric X-chromosome (RCL about 7.2) has a distinct gap in its long arm and the Y chromosome, also subacrocentric, is smaller, RCL about 4.6, and matches the X chromosome minus the terminal section of its long arm. This is typical of a neo-XY system where the X chromosome fuses with an autosome to give neo-X, and the same autosome without the X fused to it becomes the neo-Y chromosome. First metaphase of meiosis (Fig. 2) shows 22 bivalents with no suggestion of a B-chromosome behaving differently from the others. Although it is not possible to identify the neo-XY the behaviour of the chromosomes is entirely consistent with a neo-XY system.

Subgenus *Gaurodytes* C. G. Thomson, 1859

The *A. bipustulatus* group

RCL data for this group are given in Table 2.

A. bipustulatus (Linnaeus, 1767). Fig. 3 a–f. Published information: 2n = 40 + Xy_p (Suortti, 1971). (See comment on Suortti's work under *A. sturmii*.) 2n = 42 + X0 (♂),

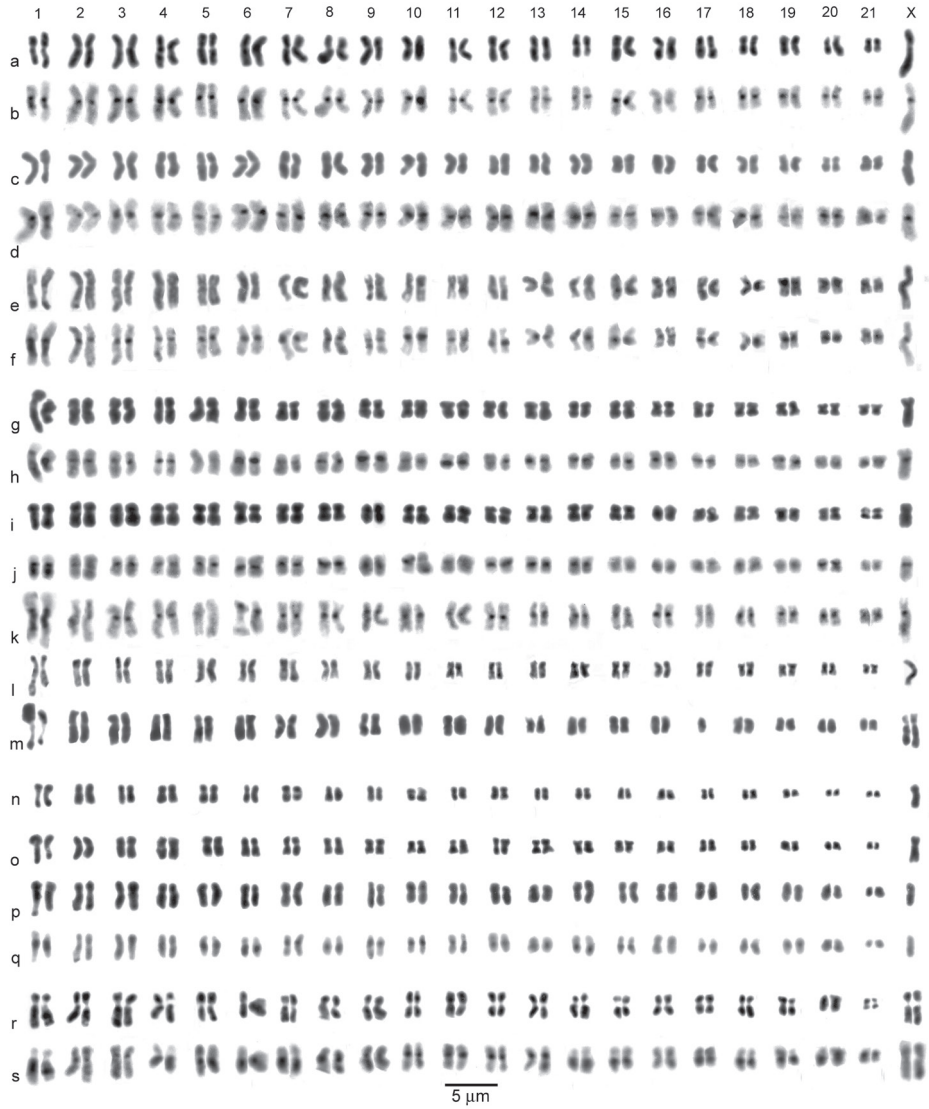

Figure 3. *Agabus* (*Gaurodytes*) part 1, the *A. bipustulatus* group, mitotic chromosomes arranged as karyotypes. **a**–**f** *A. bipustulatus*: **a–d** ♂, Inari, testis **a** plain **b** the same nucleus C-banded **c** plain **d** the same nucleus C-banded **e**, **f** ♂, Woolmer, mid-gut **e** plain **f** the same nucleus C-banded **g**–**k** *A. bipustulatus* var. *solieri*: **g, h** ♂, Moiry, testis **g** plain **h** the same nucleus C-banded **i–j, k** Illsee, ♂, testis **i** plain **j** the same nucleus C-banded **k** a different nucleus C-banded **l**, **m** *A. nevadensis*, Sierra Nevada, mid-gut, plain, **l** ♂, **m** ♀ **n**, **o** *A. wollastoni*, ♂, Madeira, testis, plain; **p–s** *A. melanarius*: **p**, **q** ♂, testis **p** plain **q** the same nucleus C-banded **r**, **s** ♀, ovary, **r** plain, **s** the same nucleus C-banded. Bar = 5 µm.

42 + XX (♀). The X chromosome is the longest in the nucleus, though its RCL value can overlap that of autosome 1 (Table 2). Autosome 1 is characterised by a secondary constriction in its long arm, frequently picked out by C-banding (Fig. 3 b, d, f). The

Table 2. *A. bipustulatus* group species, Relative Chromosome Length. Mean, 95% confidence intervals, number of chromosomes measured.

Chromosome	*A. bipustulatus*	*A. solieri*	*A. nevadensis*	*A. wollastoni*	*A. melanarius*
1	9.11 8.07–10.14 N = 14	8.86 6.69–11.03 N = 14	8.25 6.41–10.09 N = 10	8.56 6.66–10.46 N = 8	9.90 8.05–10.95 N = 4
2	9.07 8.15–9.99 N = 14	8.11 6.93–9.28 N = 14	7.20 6.32–8.08 N = 10	7.81 5.61–10.01 N = 8	8.75 7.23–10.27 N = 4
3	8.89 8.09–9.70 N = 14	8.00 6.37–9.63 N = 14	6.85 6.13–7.57 N = 10	7.25 4.99–9.51 N = 8	8.75 7.23–10.27 N = 4
4	8.29 7.33–9.24 N = 14	7.29 6.16–8.42 N = 14	6.50 5.87–7.13 N = 10	6.63 5.20–8.05 N = 8	7.87 7.11–8.64 N = 4
5	7.93 7.20–8.65 N = 14	7.00 5.83–8.17 N = 14	5.75 4.89–6.61 N = 10	6.56 4.88–8.24 N = 8	7.50 6.58–8.42 N = 4
6	7.54 6.82–8.26 N = 14	6.71 5.54–7.88 N = 14	5.40 4.59–6.21 N = 10	6.19 4.92–7.45 N = 8	6.50 5.58–7.42 N = 4
7	6.79 5.86–7.71 N = 14	6.07 4.73–7.42 N = 14	5.15 4.29–6.01 N = 10	6.00 4.14–7.86 N = 8	7.25 6.22–8.28 N = 4
8	6.54 5.63–7.44 N = 14	5.96 4.75–7.18 N = 14	4.90 4.19–5.61 N = 10	5.50 4.03–6.97 N = 8	6.50 5.58–7.42 N = 4
9	6.25 5.66–6.84 N = 14	5.68 4.64–6.71 N = 14	4.70 3.94–5.46 N = 10	5.44 3.87–7.01 N = 8	6.88 6.48–7.27 N = 4
10	6.25 5.68–6.82 N = 14	5.39 4.40–6.38 N = 14	4.35 3.60–5.11 N = 10	4.94 3.50–6.38 N = 8	6.63 5.86–7.39 N = 4
11	5.71 5.21–6.22 N = 14	5.25 4.25–6.25 N = 14	4.20 3.66–4.74 N = 10	4.63 3.35–5.90 N = 8	6.63 5.86–7.39 N = 4
12	5.75 5.23–6.27 N = 14	5.29 4.39–6.18 N = 14	3.85 3.26–4.44 N = 10	4.69 3.51–5.87 N = 8	6.38 5.18–7.57 N = 4
13	5.71 5.28–6.15 N = 14	4.93 4.05–5.81 N = 14	3.95 3.64–4.26 N = 10	4.31 3.11–5.51 N = 8	6.00 4.16–7.84
14	5.86 5.31–6.41 N = 14	4.89 4.07–5.71 N = 14	3.75 3.36–4.14	3.94 2.79–5.09 N = 8	6.38 5.37–7.38 N = 4
15	5.39 4.91–5.88 N = 14	5.07 4.18–5.96 N = 14	3.45 2.96–3.94 N = 10	3.75 2.83–4.67 N = 8	6.00 5.74–6.26 N = 4
16	5.04 4.34–5.73 N = 14	4.54 3.66–5.41 N = 14	3.55 3.09–4.01 N = 10	3.44 2.59–4.29 N = 8	5.63 4.86–6.39 N = 4

Chromosome	*A. bipustulatus*	*A. solieri*	*A. nevadensis*	*A. wollastoni*	*A. melanarius*
17	4.79 4.22–5.35 N = 14	4.29 3.46–5.11 N = 14	3.33 3.00–3.67 N = 9	3.25 2.28–4.22 N = 8	5.00 4.74–5.26 N = 4
18	4.25 3.77–4.73 N = 14	4.04 3.21–4.86 N = 14	3.05 2.62–3.48 N = 10	3.13 2.11–4.14 N = 8	5.50 4.58–6.42 N = 4
19	4.21 3.84–4.58 N = 14	3.93 3.26–4.60 N = 14	2.70 2.19–3.21 N = 10	2.63 1.89–3.36 N = 8	4.88 4.48–5.27
20	3.75 3.36–4.14 N = 14	3.36 2.71–4.00 N = 14	2.50 2.16–2.84 N = 10	2.38 1.41–2.54 N = 8	4.13 3.72–4.52 N = 4
21	3.25 2.77–3.73 N = 14	2.54 2.04–3.04 N = 14	2.05 1.62–2.48 N = 10	1.87 1.21–2.54 N = 8	2.75 1.95–3.55 N = 4
X	11.43 8.95–13.91 N = 7	8.86 5.69–12.02 N = 7	7.83 6.40–9.27 N = 5	8.25 5.75–10.75 N = 6	8.33 3.16–13.50 N = 3

expansion or contraction of this constriction can drastically alter the apparent size of the chromosome (Fig. 3 a, b). The longer chromosomes (pairs 1–10) are submetacentric, while the smaller ones are more or less metacentric. The X chromosome is submetacentric to subacrocentric. The variation in the apparent size of this chromosome in different nuclei can be striking–it is about twice as long as autosome 1 in Fig. 3 a, b, but only slightly longer that autosome 1 in Fig. 3 c, d. Since these nuclei are from the same beetle the difference must be the result of different degrees of condensation of the chromosome.

A. bipustulatus var. *solieri* Aubé, 1837. Fig. 3 g–k. Published information: none. 2n = 42 + X0 (♂), 42 + XX (♀). All the preparations illustrated are from the Swiss Alps, and are chosen because good plain and C-banded preparations were obtained from the same nuclei. The nuclei shown in Fig. 3 g–j are more condensed than the typical *A. bipustulatus* shown, but the one in Fig. 3 k shows a comparable degree of condensation. These karyotypes show no obvious difference from those of typical *A. bipustulatus*. The dark area at the end of the X chromosome in Fig. 3 k is where it overlapped one of the autosomes in the preparation. The extreme size difference between the two replicates of autosome 1 in Fig. 3 g, h is very striking, but C-banding (Fig. 3 h) shows that this size difference is entirely due to the degree of expansion of the secondary constriction.

A. nevadensis Håkan Lindberg, 1939. Fig. 3 l, m. Published information: none. 2n = 42 + X0 (♂), 42 + XX (♀) The preparations are from old material in R. B. Angus' archive, and no C-banding is available. The heavy short arm of one replicate of autosome 1 in Fig. 3 m is the result of its lying on top of dark material. The sizes and shapes of these chromosomes show no detectable differences from those of *A. bipustulatus* and *A. bipustulatus* var. *solieri*.

A. wollastoni Sharp, 1882. Fig. 1 n, o. Published information: none. 2n = 42 + X0 (♂). As with *A. nevadensis*, this is archive material and no C-banding is available. Only two karyotypes could be obtained, both from rather condensed nuclei, but the general arrangement of the chromosomes is very similar to, if not identical with, those of the species already discussed.

A. melanarius Aubé, 1837. Fig. 3 p–s. Published information: none. 2n = 42 + X0 (♂), 42 + XX (♀). The general layout of the karyotype is very similar to those of the *A. bipustulatus* complex described above, but there appear to be more secondary constrictions. Thus in the female (Fig. 3 s), where the C-banding is better displayed, secondary C-bands are clear in autosomes 1, 3, 6, 7 and 14, and even in the male (Fig. 3 q) the secondary C-bands are clear in autosomes 1, 6 and 14.

Other *Gaurodytes* species

A. biguttatus (Olivier, 1795). Fig. 4 a, b. Published information: 2n = 42 + X0 (♂), 22 + XX (♀) (Saleh Ahmed et al., 2000). The present material, from both Egypt and Sardinia, confirms the data of Saleh Ahmed et al. We have altered the position of the long chromosome with the secondary constriction from pair No. 3 to pair No. 1 as this matches the Sardinian specimen better, and there is sufficient variation in the RCL of this chromosome, due to opening of the secondary constriction to justify this move. The autosomes are all either metacentric or submetacentric with an even size decrease along the karyotype from RCL about 6 to about 3. The X chromosome has RCL about 6 and is more distinctly submetacentric than the larger autosomes, except of autosome 1 which has the secondary constriction. No C-banded preparation is available.

A. binotatus Aubé, 1837. Fig. 4 c. Published information: none. 2n = 42 + X0 (♂). The karyotype of this species appears very similar to that of *A. biguttatus*, with a similar spread of RCLs. However, autosomes 14–21 are clearly less metacentric than in *A. biguttatus*, in some cases approaching subacrocentric. The X chromosome, RCL about 8.5, is clearly the largest in the nucleus, thus distinctly larger than in *A. biguttatus*.

A. affinis (Paykull, 1798). Fig. 4 d. Published information: none. 2n = 42 + X0 (♂). The RCLs of the autosomes range from about 8–2.7, with an abrupt size decrease between pair 4 (RCL about 7.4) and pair 5 (RCL about 5.4), but otherwise with a gradual decrease. Most to the autosomes are either metacentric or submetacentric, but autosomes 12, 17, 20 and 21 are subacrocentric. The X chromosome is submetacentric, RCL about 6. No C-banded material is available.

A. unguicularis (Thomson, 1867). Fig. 4 e. Published information: none. 2n = 42 + X0 (♂). The RCLs of the autosomes range from about 10–2.4. There is an abrupt size decrease between pairs 2 and 3 (RCLs about 9.4 and 7.6) and pairs 3 and 4 (RCL of pair 4 about 6.5), but apart from that the size decrease is fairly even. Most of the autosomes are metacentric or almost so, but a few are clearly submetacentric. The X chromosome, RCL about 6.5, is similar in size to autosome pair 4, but much more clearly submetacentric. No C-banded material is available.

Figure 4. *Agabus* (*Gaurodytes*) part 2, mitotic chromosomes arranged as karyotypes. **a**, **b** *A. biguttatus*, ♂, mid-gut, plain: **a** El Noqra **b** Giara di Gesturi; **c** *A. binotatus*, ♂, mid-gut, plain **d** *A. affinis*, ♂, mid-gut, plain **e** *A. unguicularis*, ♂, mid-gut, plain **f** *A. ramblae*, ♂, Murcia, testis, plain **g** *A. conspersus*, ♂, mid-gut, plain **h**, **i** *A. nebulosus*, ♂, mid-gut, plain **h** Cuckmere **i** Tenerife **j–l** *A. adpressus*, ♂, mid-gut **j** plain **k, l** the same nucleus **k** plain **l** C-banded. Bar = 5 μm.

A. ramblae Millan et Ribera, 2001. Fig. 4 f. Published information: none. 2n = 42 + X0 (♂), 42 + XX (♀).The RCLs of the autosomes range from about 7–2.9, with a fairly even decrease in length along the karyotype. The autosomes are a mixture of metacentrics and submetacentrics (some at the extreme end of the range), with autosomes 10–12, 15, 16 and 20 subacrocentric. The X chromosome is about the same size as autosome 1, but more clearly submetacentric. No C-banded material is available.

A. conspersus (Marsham, 1802). Fig. 4 g. Published information: 2n = 38 + XY (Yadav et al., 1984). 2n = 42 + X0 (♂). The RCLs of the autosomes range from about 6.1–3.6, with an even decrease in chromosome size along the karyotype. The autosomes are all either metacentric or submetacentric, and autosome 3 has a prominent secondary constriction in its long arm and autosome 15 has what appears to be a terminal NOR at the end of its short arm. The X chromosome, RCL about 5.6, is submetacentric and similar in size to autosomes 4–6. No C-banded material is available. This karyotype is clearly very different from that reported by Yadav et al. (1984). They report a number of nuclei supporting their conclusions, so the most likely explanation is that they were working with a different species. It may be noted that Marsham (1802) described *A.*

conspersus from England so the material here may be regarded as true *A. conspersus*. Yadav et al. worked with Indian material.

A. nebulosus (Forster, 1771). Fig. 4 h, i. Published information: none. 2n = 42 + X0 (♂), 42 + XX (♀). The general layout of the karyotype in terms of RCLs of the autosomes is very similar to that of *A. conspersus*. Autosome 3 has a similar secondary constriction in its long arm, but the small chromosome with the terminal apparent NOR is relatively larger than in *A. conspersus*, and is placed as pair 12 as against 15. The X chromosome, RCL about 7.3, appears relatively larger than that of *A. conspersus*, and is metacentric. The Tenerife specimen whose chromosomes are shown in Fig. 4 i is of a form whose dark pronotal spots are absent or scarcely apparent, but the chromosomes clearly associate it with the British well-spotted *A. nebulosus* rather than *A. conspersus* which lacks the pronotal spots.

A. adpressus Aubé, 1837. Fig. 4 j–l. Published information: none. 2n = 42 + XY (♂). The autosomes are all either metacentric or submetacentric, with RCLs ranging from about 7.2–3.1 and with an even decrease in size along the karyotype. Autosome 2 has a secondary constriction in its long arm and autosome 8 has one in its short arm. The X chromosome is submetacentric (almost metacentric), about as long as autosome 1. The Y chromosome, RCL about 5, looks like the X chromosome with most of one arm missing. C-banding (Fig. 4 l) shows considerable variation in the centromeric C-bands of the autosomes. Autosome 1 lacks any C-band, 2 and 3 have strong C-bands and 4 has a weak one. Autosome 5 lacks a C-band and that on autosome 6 is very weak. Autosomes 7–9 have strong centromeric C-bands and 10–13 have weaker ones. Pair 14 has very weak bands. Pairs 15–21 have strong C-bands. The secondary constriction of autosome 2 shows as a C-band, but that of autosome 8 appears to be merged with the strong centromeric C-band. The sex chromosomes both have very large strong centromeric C-bands, which is a powerful piece of evidence that this is a neo-XY system rather than an X0 system and a B-chromosome. Unfortunately no meiotic preparation is available.

Colymbetinae Erichson, 1837

Colymbetes Clairville, 1806

C. fuscus (Linnaeus, 1758). Fig. 5 a, b. Published information: 2n = 35–37 (♀) (Günthert, 1910). 2n = 40 + X0 (♂). The RCLs of the autosomes range from about 7.8–2.1, with an even decrease in chromosome size along the karyotype. Autosomes 2, 9, 11, 12, 14 and 15 are subacrocentric, while the remainder are more or less metacentric. The X chromosome, RCL about 5.7, is metacentric, similar in size to autosome 8. All the chromosomes have distinct centromeric C-bands and autosome 4 has a fainter band, possibly a secondary constriction, in its short arm.

C. paykulli Erichson, 1837. Fig. 5 c, d. Published information: 18 pairs including Xy_p? (Suortti, 1971). 2n = 40 + X0 (♂). The RCLs of the autosomes range from

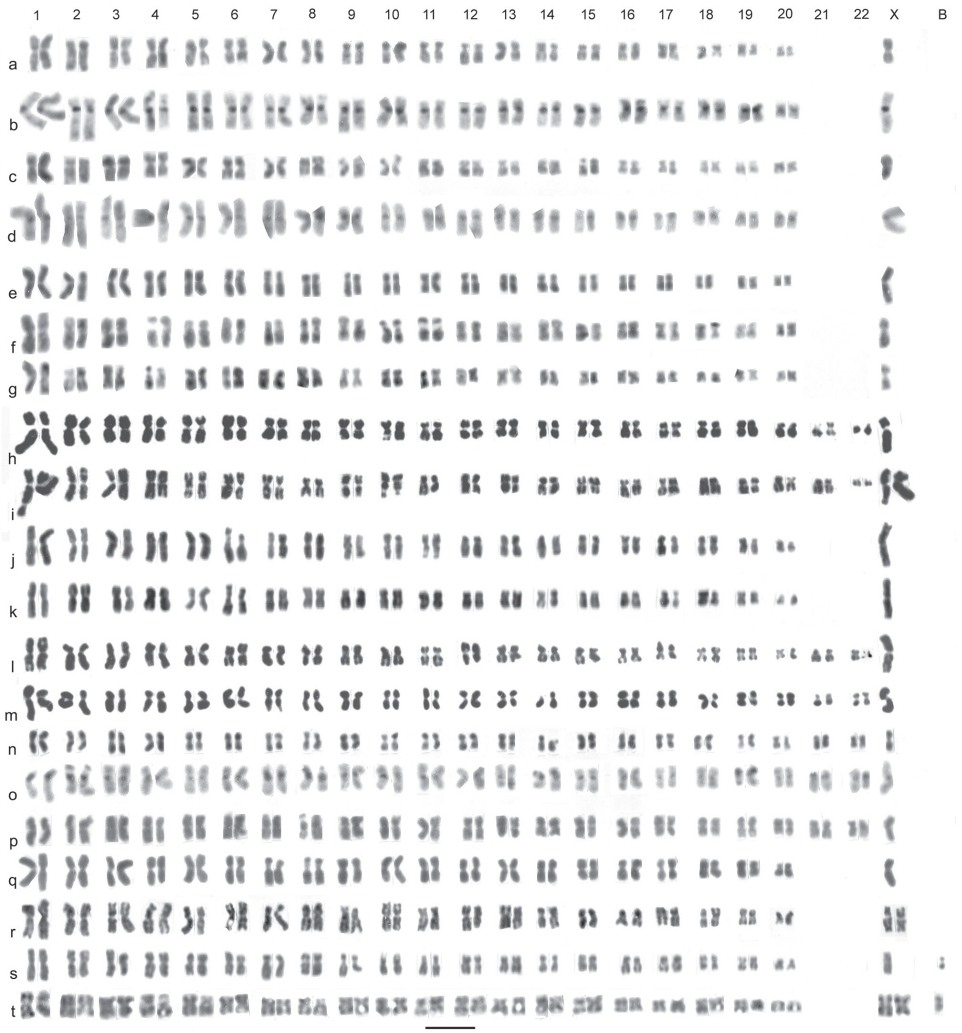

Figure 5. *Colymbetes* (**a–g**) and *Rhantus* (**h–t**), mitotic chromosomes arranged as karyotypes. **a**, **b** *C. fuscus*, ♂, mid-gut **a** Pinail, plain **b** Wisley, C-banded **c**, **d** *C. paykulli*, ♂, midgut, Mullsjö **c** plain **d** C-banded **e** *C. piceus*, ♂, Egypt, mid-gut, plain **f**, **g** *C. striatus,* ♂, mid-gut, plain **h**, **i** *R. grapii*, ♂, mid-gut, plain **j**, **k** *R. exsoletus*, ♂, mid-gut, plain **j** Studland Heath **k** Gayton Thorpe Common **l**, **m** *R. frontalis*, ♂, plain **l** mid-gut, Gayton Thorpe Common **m** testis, Thompson Common **n–p** *R. suturalis*, ♂ **n**, **o** mid-gut, Staines Moor **n** plain **o** C-banded **p** testis, Ras Az Zawr, Kuwait, plain **q–t** *R. suturellus*, plain **q** ♂, testis, Pinail **r–t** Studland Heath **r** ♀ mid-gut **s** ♂ mid-gut with 1 Bchromosome **t** ♀ mid-gut with 1 B-chromosome. Bar = 5 μm.

about 8.4–3.2, with an even decrease in chromosome size along the karyotype. The X chromosome, RCL about 6.8, is metacentric. Autosomes 2 and 12–16 are borderline submetacentric-subacrocentric, while autosome 17 is more clearly submetacentric. The general arrangement appears very similar to that of *C. fuscus* but the centromeric C-

bands appear less bold (perhaps a preparation artefact). The unreliability of Suortti's data has been mentioned under *Agabus sturmii* and *A. bipustulatus*.

C. piceus Klug, 1834. Fig. 5 e. Published information: 2n = 40 + X0 (♂), 40 + XX (♀) (Saleh Ahmed et al., 2000). The karyotype shown in Fig. 5 e is the one published by Saleh Ahmed et al. and is included for comparison with the other species. The RCLs of the autosomes range from about 8.2–1.9, with a fairly even decrease in chromosome size along the karyotype. Autosomes 2, 8, 12, 14 -17 and 19 are submetacentric, pair 9 is subacrocentric, and the remainder are metacentric. Autosomes 6 and 7 have secondary constrictions in their short arms. The X-chromosome, RCL about 8.2, is similar in size to autosome 1, but is less evenly metacentric. No C-banded material is available.

C. striatus (Linnaeus, 1758). Fig. 5 f, g. Published information: 19 - 21 pairs + Xy_p? (Suortti, 1971). 2n = 40 + X0 (♂). The RCLs of the autosomes range from about 8–2.7, with a more noticeable decrease in length between autosomes 1 and 2 (RCL about 6.1) than in the other species, but otherwise with a fairly even decrease in chromosome length along the karyotype. Autosomes 2, 3, 5, 7 and 9 are submetacentric, but the others are more or less metacentric. Autosomes 4, 8 and 9 have secondary constriction in their short arms. The X chromosome, RCL about 9, is similar in length to autosomes 2–4, more nearly metacentric than pairs 2 and 3, but less so than pair 4. No C-banded material is available. For Suortti's data, see comment under *C. paykulli*.

Rhantus Dejean, 1833

Subgenus *Nartus* Zaitsev, 1907

R. grapii (Gyllenhal, 1808). Fig 5. h, i. Published information: none. 2n = 44 + X0 (♂), 44 + XX (♀). The RCLs of the autosomes range from about 9.6–2.5, with an even decrease in chromosome length along the karyotype, apart from sharp decreases in size between pairs 1 and 2 (RCL about 7) and between pair 21 (RCL about 5.5) and pair 22 (RCL about 1.2). Most of the autosomes are more or less metacentric, but pairs 4, 5, 7, 10–12, 14, 16–18 and 20 are clearly submetacentric and pairs 21 and 22 are subacrocentric. Autosome 9 has a secondary constriction towards the end of its long arm. The X chromosome, RCL about 7.7, is the second to longest in the nucleus and is submetacentric. No C-banded material is available.

Subgenus *Rhantus* s. str.

R. exsoletus (Forster, 1771). Fig. 5 j, k. Published information: 20 pairs + Xy_p (Suortti, 1971). 2n = 40 + X0 (♂). The RCLs of the autosomes range from about 8–2.2, with a fairly even decrease in chromosome length along the karyotype. Autosome pairs 4, 7, 8, 10, 11, 15–17, 19 and 20 are clearly submetacentric, with pairs 9, 12 and 14 more or less subacrocentric. The remaining seven pairs are more or less metacentric. Pair 6

has a secondary constriction on its short arm. The X chromosome, RCL about 9, is the longest in the nucleus. No C-banded material is available.

R. frontalis (Marsham, 1802). Fig. 5 l, m. Published information (as *R. notatus* F.): 22 pairs including Xy_p (Suortti, 1971). 2n = 44 + X0 (♂). The RCLs of the autosomes range from about 8.3–2.7 with a sharper decrease in length between pair 1 and pair 2 (RCL about 6.4) but otherwise an even decrease in chromosome length along the karyotype. Autosomes 2–5, 7, 9–11, 14, 15 and 21 are submetacentric while the rest are more or less metacentric. Autosomes 2, 3 and 17 have secondary constrictions in their short arms. The X chromosome, RCL about 9.1, is the longest in the nucleus. No C-banded material is available.

R. suturalis (Macleay, 1825). Fig. 5 n–p. Published information: none. 2n = 44 + X0 (♂). The RCLs of the autosomes range from about 6.1–3.5. The rate of decrease along the karyotype is very even with many of the adjacent pairs appearing more or less the same size. Most of the autosomes are more or less metacentric but pairs 13, 14 and 22 are clearly submetacentric and pair 20 is subacrocentric. C-banding (Fig. 5 o) shows all the chromosomes with centromeric C-bands, of varying strengths. Pairs 1, 8 and 14 have secondary constrictions on their short arms. The X chromosome, RCL about 5.8, is metacentric with a rather weak centromeric C-band. The Kuwaiti material (Fig. 5 p) shows no differences from the British.

R. suturellus (Harris, 1828). Fig. 5 q–t. Published information: none. 2n = 40 + X0 (♂), 40 + XX (♀), sometimes with 1 B-chromosome. The RCLs of the autosomes range from about 7–2.7, with a fairly even decrease in chromosome length along the karyotype. Autosomes 5, 7, 9, 11, 12, 15 -17, 19 and 20 are clearly submetacentric, with the remainder more or less metacentric. Pairs 4, 8 and 9 have secondary constrictions in their short arms. The X chromosome, RCL about 5.3, is metacentric and similar to chromosomes 4–8. No C-banded material is available. This karyotype is unusual in having a B-chromosome, a small metacentric, RCL about 3, which has so far been found in Studland Heath material. The first Studland Heath material, in 1993, comprised a male with a B-chromosome and a female without one, giving the impression that this species had an XY sex chromosome system. However, the 2000 material, a mail from Pinail lacking the B-chromosome and a female from Studland Heath with the B-chromosome, revealed the true nature of the situation.

Discussion

In considering the data presented here, two aspects are of particular note: the extent to which the different genera have characteristic karyotypes and details of any deviations from generic karyotypes; and the extent to which the karyotypes of related species show clear differences.

In *Agabus* 18 of the 20 species reported have a karyotype involving 21 pairs of autosomes and sex chromosomes which are X0 (♂) and XX (♀), but the remaining 2, *A. infuscatus* and *A. adpressus*, have 21 pairs of autosomes and sex chromosomes which are

XY (♂) and XX (♀). These two species are not closely related (they are placed in different subgenera), but appear to have evolved similar neo-XY sex chromosomes. What makes this particularly surprising is that, since the development of a neo-XY system involves fusion of the original X chromosome with an autosome, there should be an initial reduction by one in the number of pairs of autosomes. However, both the species involved here show no such reduction, so have presumably undergone fission of one autosome to give two and hence restore the original number. It may be noted that Yadav et al. (1984) describe their "*Agabus conspersus*" as having 38 autosomes (19 pairs) and XY sex chromosomes. Assuming their chromosome data are correct and they are working with an *Agabus* species, this one has a reduced number of autosomes as well as an XY system.

Among the *Agabus* species reported here, there are two groups of particularly close relatives, *A. congener*, *lapponicus* and *thomsoni*, and the *A. bipustulatus* group. *A. congener* and *lapponicus* show no interspecific chromosomal differences despite a good number of high-quality preparations. *A. thomsoni* may show a slight difference in the RCL of the longest secondary constriction-bearing autosome, but more material would be needed to confirm this.

The *A. bipustulatus* group comprises *A. melanarius* and the *A. bipustulatus* complex within which the overriding impression from the present investigation is the extreme similarity between the karyotypes of the species. In the case of *A. bipustulatus* and *A. bipustulatus* var *solieri* this is not surprising as these are regarded as conspecific. The case of *A. nevadensis* is perhaps more interesting as this is currently regarded as a distinct species in spite of the lack of clear morphological characters to distinguish it from *A. bipustulatus*. The karyotype of *A. wollastoni* also shows no obvious difference from those of the other species, but in this case the species does have a very clear morphological character to distinguish it from *A. bipustulatus*–the inner anterior tarsal claw of the male is simple, not expanded to give the "scooped-out" appearance characteristic of *A. bipustulatus*, *solieri* and *nevadensis*. Only *A. melanarius*, not really a member of the *A. bipustulatus* complex, shows some karyotype differences, most clearly in the more extensive development of heterochromatic (C-banding) regions on the chromosomes. These findings may be considered in the light of the phylogenetic trees obtained by Drotz et al. (2010) from their studies of mitochondrial DNA of these beetles. Drotz et al. place the *A. bipustulatus* group as a complex within a slightly larger *A. tristis* Aubé group. Their Fig. 5 shows a strict consensus phylogenetic tree of the group. This figure is particularly interesting: *A. melanarius* is shown to be among the most isolated of the *A. tristis* group species, with it plus *A. tristis* placed as a sister taxon to all the rest combined. The remaining species, including *A. wollastoni*, comprise the *A. bipustulatus* complex, within which *A. wollastoni* is the first to come out, being placed as sister to all the others. It is at once apparent that the karyotypes of all these *A. bipustulatus* complex species are the ones showing no difference from one another. *A. melanarius* does show chromosomal differences, and it would be very interesting to know whether this is also true of *A. tristis*. However, this is a Nearctic and east Palaearctic species, not available for study here.

Examination of the material of *A. bipustulatus*, *solieri* and *nevadensis* included in their study shows how they came to their conclusions as to their taxonomic status. They are concerned with forms in which the primary reticulation (the fine meshes inside the larger elongate secondary meshes) is progressively reduced. These forms are referred to the varieties *dolomitanus* Scholz, 1935, *falcozi* Guignot, 1932, *kiesenwetteri* Seidlitz 1887 and *pyrenaeus* Fresneda and Hernando, 1989. The most striking thing is that these various *solieri* forms come out in a number of different places, often with ordinary *bipustulatus* from neighbouring areas. *A. nevadensis*, with its very restricted distribution, almost inevitably comes out in only one place, but very closely associated with a population of *solieri* (*kiesenwetteri*) from France. The claim of *A. nevadensis* to species status appears weak. The mitochondrial DNA separation is very slight, the karyotype appears identical with those of other *A. bipustulatus* forms, and the morphological characteristics are less clear than those of *solieri*.

The case of *A. wollastoni* is interesting. This species is isolated on Madeira and has had time to diverge from other *A. bipustulatus*, both in its mitochondrial DNA and also in its morphology–simple inner anterior tarsal claws of males, and generally larger size. Only the chromosomes show no difference.

The four species of *Colymbetes* share the same basic karyotype with 2n = 40 + X0 (♂), with the X chromosome a large more or less metacentric. There are minor differences in the RCL sequences between the species, which may or may not stand up to more detailed analysis if more material becomes available. Autosome 1 of *C. striatus* appears larger than in the other species.

The karyotypes of the *Rhantus* species are interesting in showing two different numbers, with 2n = 40 + X0 (♂) in *R. exsoletus* and *R. suturellus,* but 2n = 44 + X0 (♂) in the other species studied. Interestingly, this number difference does not reflect the subgeneric classification. The B-chromosome of *R. suturellus* is interesting in that it could be confused with a neo-XY sex chromosome system comparable with that of *Agabus infuscatus* and *A. adpressus*.

The Kuwaiti material of *R. suturalis* is interesting as it shows no differences from British material. Balke et al. (2009) demonstrated that this "supertramp" species almost certainly originated in the highlands of New Guinea from where it extended its range in two separate lineages, one southern going into Australia and New Zealand, and the other northern, going into Asia and Europe. Clearly Kuwaiti and European material belong to this northern lineage, but it is good to see the absence of chromosomal differences between specimens from these areas supporting the integrity of this species.

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