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High heterochromatin content in somatic chromosomes of two unrelated species of Diplopoda (Myriapoda)

R. Vitturi, M. S. Colomba, V. Caputo, I. Sparacio & R. Barbieri

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For the first time, a conventional analysis of C-banded karyotypes was carried out in two distantly related diplopod species; this revealed an impressive percentage of heterochromatin in both genomes. In *Acanthopetalum sicanum* (Order Callipodida) ($2n = 12$), heterochromatin constitutes about 60% of the total DNA in females and 56% in males, whereas in *Enologus oxypygum* (Order Julida) ($2n = 22$) it is about 67% in both sexes. Heterochromatin of the two species was found to be similar in base composition (AT rich) and heterochromatin distribution, indicating that it has accumulated in a species-specific manner. Sex-determining mechanisms of the XY type were detected in both *A. sicanum* and *E. oxypygum*. In *A. sicanum*, the Y presented the lowest heterochromatic content of all chromosomes in the karyotype, whereas the X presented the highest.

Key words: *Acanthopetalum sicanum*, chromosome banding, Diplopoda, *Enologus oxypygum*, heterochromatin

Introduction

Molecular analyses have shown that C-heterochromatin regions in plants and animals consist of short tandemly repeated sequences that are predominantly composed on non-transcribed satellite DNA (e.g. Cionini *et al.* 1985, Arnold & Shaw 1985). It has also been demonstrated that constitutive heterochromatin has a typical and non-random distribution within a given genome (e.g. John *et al.* 1985).

The idea of heterochromatin equilocality was initially based solely on the locations of C-positive regions within a complement (Heitz 1935). Later, studies on both plants (Dover & Flavell 1984, Dover 1986, Kenton 1991) and animals (John & King 1983, John *et al.* 1985), combining this technique with fluorescent DNA-binding dyes of different specificities, such as DAPI (AT specific) and CMA_3 (GC specific), led to a more refined view of heterochromatin distribution. For example, heterochromatic regions in the plant *Gibasis karwinskyana* (Commelinaceae) were found not only to be equilocally distributed in the karyotype but also to agree in base composition (AT rich) (Kenton 1991), while heterochromatins with either similar or different fluorescence behaviour were seen to be accumulated at similar sites in acridid grasshoppers (John *et al.* 1985).

Other than the above-mentioned paper of John *et al.* (1985) and other relevant papers concerning Insecta (i.e. Juan *et al.* 1990, Ugarkovic *et al.* 1994), there are few exhaustive studies of heterochromatin differentiation in other phyla of invertebrates using combined C-banding, staining with fluorochromes and chromosomal digestion with restriction endonucleases (REs). Among these few, the most important deal with the identification of six different types of heterochromatin in the mussel (Bivalvia) *Mytilus galloprovincialis* (Martínez-Lage *et al.* 1994), the existence of chromosomal markers in three different species of the genus *Mytilus* (Martínez-Lage *et al.* 1995) and supernumerary chromosomes of *Nephros norvegicus* (Crustacea, Decapoda) (Deiana *et al.* 1996).

Millipedes are invertebrates belonging to the class Diplopoda whose most characteristic feature is the presence of many legs arranged in two pairs per body segment (diplosegments). They are usually found under leaf litter or stones or just below the soil surface (Hopkin & Read 1992). Although abundant in many parts of the world, diplopods have not been fully investigated cytologically, probably because of difficulties in obtaining chromosome spreads. As far as we know, only chromosome numbers in spermatocytes from about 60 species have been determined (Akar 1984, 1986, 1987).

The present study shows that heterochromatin is not only present in impressive amounts in the genomes of the distantly related species *Acanthopetalum sicanum* and *Enologus oxypygum* but also as a species-specific mode through which it has accumulated in the two species. The heterochromatin pattern of each species was first determined by C-banding and then explored using the fluorochrome DA/DAPI in an attempt to elucidate heterochromatin composition. The heterochromatic DNA of *A. sicanum* was also analysed after digestion with the enzyme *Hae*III (5′-GGCC-3′), and nucleolus organizer regions of the two species were characterized.

R. Vitturi (corresponding author), M. S. Colomba and I. Sparacio are at Dipartimento di Biologia Animale, Università di Palermo, Via Archirafi 18, 90123 Palermo, Italy. Tel: (+39) 91 6177159; Fax: (+39) 91 6172009; Email: zuvitcol@mbox.unipa.it. V. Caputo is at Istituto di Biologia e Genetica, Università di Ancona, Via Brecce Bianche, 60100 Ancona, Italy. R. Barbieri is at Dipartimento di Biologia Cellulare e dello Sviluppo 'A. Monroy', Università di Palermo, Parco D'Orleans 2, 90128 Palermo, Italy.

Materials and methods

A. sicanum and *E. oxypygum* are diplopods belonging to the orders Callipodida and Julida respectively. Details on the biology of these organisms have been compiled by Hopkin & Read (1992).

Specimens of *A. sicanum* (156 males and 194 females) and of *E. oxypygum* (130 males and 145 females) were collected from 1992 to 1995 under stones and from leaf litter in the Botanic garden of Palermo (Sicily, Italy) and processed at the Institute of Zoology, University of Palermo. In Callipodida, male specimens were identified by the presence of gonopods and in Julida by the modified first pair of legs, which form a 'crotchet-like' structure (Hopkin & Read 1992).

Somatic chromosomes were prepared by means of the air-drying technique (Vitturi *et al.* 1991) from tissues of the midgut wall, dissected from animals previously immersed in fresh-water colchicine solution (0.1%) at room temperature for 90 min. The slides were treated with various staining techniques within 1 week of their preparation.

Conventional staining of chromosomes followed current methods (Vitturi *et al.* 1991): BSG C-banding (Sumner 1972); Ag-NOR staining (Howell & Black 1980); DAPI staining (Schmid *et al.* 1983); *Hae*III restriction enzyme digestion (Mezzanotte *et al.* 1983).

Chromosomes were classified by the criteria of Levan *et al.* (1964).

Results

Acanthopetalum sicanum ($2n = 12$)

A diploid number of 12 chromosomes was determined from Giemsa-stained preparations. The very large chromosomes could be arranged into five homomorphic pairs (pairs nos 1–5) of continuously decreasing length plus a sex chromosome pair that was heteromorphic in the males (XY) (Figure 1a) and homomorphic in the females (XX) (Figure 1b). Autosomes consisted of four metacentric pairs (pairs nos 1, 2, 4 & 5) and of one pair (pair no. 3) only tentatively identified as metacentric because of the tight pairing of sister chromatids (Figure 1a). The sex pair consisted of metacentric chromosomes, of which the X was the largest in the complement and the Y the smallest (Table 1) (Figure 1a).

Specific staining with silver nitrate of either Giemsa-stained (Figure 1a) or C-banded (Figure 2a) chromosomes clearly localized nucleolar organizer regions (NORs) in a slightly subterminal position of the C-positive arm of pair no. 3 (Figures 1a & 2a, see arrows).

Analysis with barium hydroxide (C-banding) revealed that each chromosome of *A. sicanum* possessed large amounts of constitutive heterochromatin (Figure 2a). Ten high-quality C-banded metaphases of one male was selected for measurements of the haploid chromosome length and the length of the corresponding heterochromatic regions (5A + X + Y) (Table 1). An average total chromosome length of the diploid karyotype of 89.6 μm and an average heterochromatin length of 53.4 μm were found in the female (5AA + XX); an average total chromosome length of the diploid karyotype of 84 μm and an average heterochromatin length of 46.9 μm were found in the male (5AA + XY). This implies that in the females heterochromatin constitutes about 60%, while in the males 56% of total chromatin.

Whereas the X chromosome and autosomes contained large amounts of constitutive heterochromatin (Figure 2a), very little heterochromatin was located in the Y chromosome, which, besides a small and faint C-band in the centromeric region, was completely euchromatic and presented the lowest heterochromatic amount of all chromosomes in the karyotype (Figure 2b) (Table 1).

Centromere-symmetric heterochromatin could be observed in all chromosome pairs except pair no. 3. In this pair heterochromatin occupied an entire arm (the NOR-bearing one), except a small distal euchromatic region mainly detectable in decondensed chromosomes (Figure 2a, see underlined pair 3), plus a medium–large region of the other arm (Figure 2a).

After digestion with *Hae*III enzyme, chromosomal regions previously identified as heterochromatic using the C-banding appeared to be undigested and well stained (Figure 3).

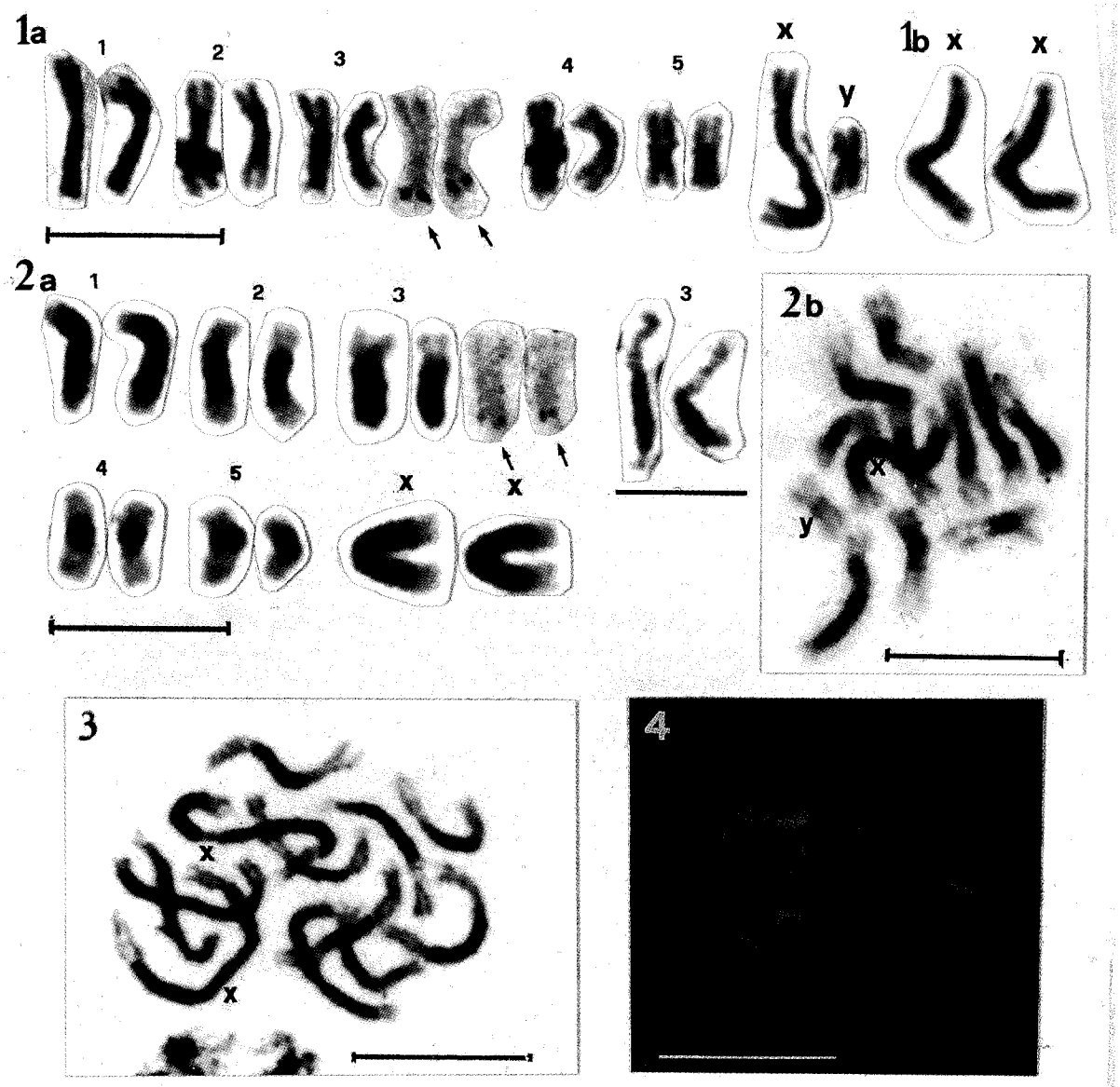
In DA/DAPI-stained metaphases, the fluorescence in all heterochromatic regions was distinctly higher than that in euchromatic chromosomal segments (Figure 4), and this intensity along the entire heterochromatic region was uniform.

Enologus oxypygum ($2n = 22$)

The diploid chromosomal number was found to be $2n = 22$. As in *A. sicanum*, in *E. oxypygum*, the chromosomes were large so that they often overlapped. The morphology of these chromosomes was not easy to determine because sister chromatids were tightly paired. Hence, the chromosomes of *E. oxypygum* could be tentatively arranged into ten homomorphic pairs (autosomes), which were identified as metacentric or submetacentric from analysis of several spreads, and one small-sized pair that was heteromorphic in the males (XY) (Figure 5a) and homomorphic in the females (XX) (Figure 5b). The X chromosomes was metacentric and slightly larger than the Y, which was submetacentric.

Analysis with silver nitrate of a few Giemsa-stained metaphases showed positive Ag signals on five chromosomes (Figure 6), of which only two were identified as homologues because of their resemblance in morphology and size. NORs were terminally located in four chromosomes (Figure 6, see arrows) and interstitially located in one chromosome. Additional Ag signals seem to occur in two chromosomes. Because the silver pattern of this species mainly includes unpaired chromosomes, NOR polymorphism may occur in *E. oxypygum*.

A characteristic pattern of very large C-positive blocks could be observed (Figure 7a & b). These constantly occupied one entire arm of all chromosomes and, except for a few pairs, small pericentromerical regions of the other arm. As chromosomes of pairs from 5 to 10 and the X chromosome could hardly be identified, only average total chromosome length of



Figures 1–4. Mitotic chromosomes of *Acanthopetalum sicanum*. Giemsa-stained male karyotype (**Figure 1a**, arrows indicate silver-stained chromosomes of pair 3). Giemsa-stained female sex chromosome pair (**Figure 1b**). C-banded female karyotype (**Figure 2a**, arrows indicate silver-stained chromosomes of pair No. 3; decondensed chromosomes of pair 3 showing C-positive arm with distal euchromatic regions are underlined). C-banded male metaphase (**Figure 2b**). Metaphase after *Hae*III enzyme digestion (**Figure 3**). DAPI stained metaphase (**Figure 4**) (Bar = 10 μm).

80.8 μm and average length of 54.2 μm of the total heterochromatin from ten C-banded metaphases of one male was measured. Thus, heterochromatin was about 67% of the total genome size in the males (10AA + XY), whereas the heterochromatin percentage in female specimens was almost equal that in male specimens because the sex pair was composed of chromosomes very similar in size and with almost identical heterochromatin amounts (Figure 7c).

As observed in *A. sicanum*, in *E. oxypygum* also, DAPI staining showed heterochromatin with a uniform fluorescence intensity distinctly higher than that of the euchromatic regions (Figure 8).

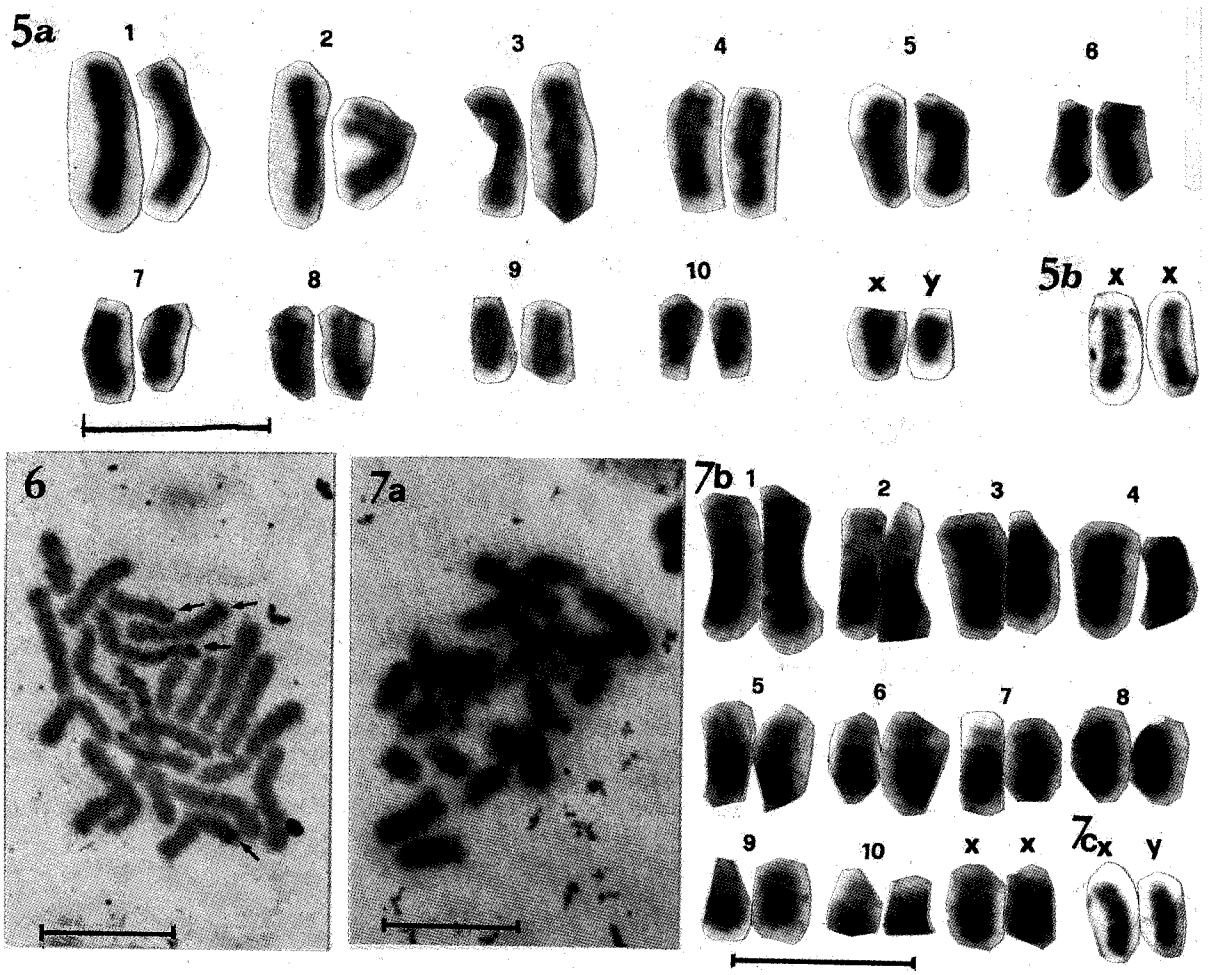
Discussion

The unrelated diplopod species *A. sicanum* and *E. oxypygum* are extremely attractive for cytogenetic studies both because they possess long chromosomes and because their heterochromatin is abundant. In fact, heterochromatin in *A. sicanum* constitutes about 60% and 56% of the total female and male genome length,

Table 1. Eu- and heterochromatin absolute lengths, arm ratio and centromere position of *Acanthopetalum sicanum**

Chromosome	Length (μm)			Arm ratio	Centromere position
	Euchromatin	Heterochromatin	Total ± SD		
1	1.6	6.6	8.2 ± 2.3	1.23	M
2	3.5	3.7	7.3 ± 1.1	1.30	M
3	2.5	4.3	6.8 ± 2	1.16	M
4	3.8	2.1	5.9 ± 1.9	1.03	M
5	3.5	2.4	5.9 ± 1.9	1	M
X	3.2	7.4	10.6 ± 2.4	1.23	M
Y	4.5	0.5	5 ± 1.9	1.05	M

*The chromosomes of 10 high-quality C-banded metaphases from the midgut wall of one male animal were used for the measurements.



Figures 5–7. Mitotic chromosomes of *Enologus oxypygum*. Giemsa-stained male karyotype (**Figure 5a**). Giemsa-stained female sex chromosome pair (**Figure 5b**). Silver-stained metaphase (**Figure 6**, arrows indicate terminal NORs). C-banded female metaphase (**Figure 7a**). C-banded female karyotype (**Figure 7b**). C-banded male sex chromosome pair (**Figure 7c**). (Bar = 10 μm).

respectively, and in *E. oxypygum* about 67% of the total genome in both sexes. In the tree frog *Agalychnis callidryas* (Schmid *et al.* 1995) and in the mealworm beetle *Tenebrio molitor* (Petitpierre *et al.* 1988), considered as exceptional, heterochromatin is only about 40% and 50% of the whole genome respectively.

This study suggests that heterochromatin distribution reflects two different species-specific modes through

Figure 8. DAPI-stained mitotic metaphase of *E. oxypygum* (Bar = 10 μm).

which heterochromatin has accumulated in both diplopod species. In *A. sicanum*, heterochromatin is typically distributed symmetrically with respect to the centromere in the X chromosome and all autosomes except pair no. 3 and is centromerical in the Y (Figure 9A). In *E. oxypygum* on the other hand, heterochromatin is accumulated asymmetrically with respect to the centromere in all chromosomes, including the sex pair (Figure 9B).

On the whole, these different modes of heterochromatin accumulation in the diplopod species here investigated, irrespective of heterochromatin functions, may be the expression of a general karyoevolutionary trend towards an increase in karyotype symmetry (Stebbins 1950).

Evidence from this study strongly indicates that the karyoevolutionary tendency towards an increase of the heterochromatin amount, although expressed to a different degree in the two species, also involved those pairs that have been identified as the sex chromosome pairs. Whether the heteromorphism of the sex chromosomes is the result of a loss of amplified heterochromatin, as reported in the marsupial frogs *G. walkeri* and *G. ovifera* (Schmid *et al.* 1988), or of a lack of heterochromatin amplification cannot be evaluated from the available data. However, whatever the process operating in the constitution of the heteromorphic pair, it certainly involves the Y chromosome in both species.

It is worth remarking that, like *G. walkeri* and *G. ovifera* (Schmid *et al.* 1988), *A. sicanum* and *E. oxypygum* also present examples of Y chromosomes with less heterochromatin than the X chromosomes. This contrasts with most amphibian Y chromosomes and the Y or W chromosomes of most other vertebrates with only a few exceptions in fish (e.g. Phillips & Ihssen 1985, Lloyd & Thorgaard 1988) and reptiles (Bull 1978, Sites *et al.* 1979). As in *G. walkeri* and *G. ovifera* (Schmid *et al.* 1988), in *A. sicanum* the Y contains the smallest amount of heterochromatin of all the chromosomes. Moreover, as the X contains the highest amount of heterochromatin, then total diploid heterochromatin in males is 5% lower than in females (Table 1).

The uniform fluorescence intensity, which was distinctly higher than that of euchromatin detected with DAPI in every heterochromatin region of *A. sicanum* and *E. oxypygum*, would suggest that the repetitive DNA of these species is not only peculiarly distributed but also agrees in base composition (AT rich). Responses to DAPI staining similar to those reported here have been obtained in the chromosomes of the mealworm beetle *Tenebrio molitor* (Juan *et al.* 1990), the mouse *Mus musculus* (i.e. Mezzanotte & Ferrucci 1984) and the plant *Gybasis karwinskiana* (Kenton 1991).

Finally, although the *in situ* digestion using only one restriction enzyme is not sufficient to provide exhaustive information on the relative composition of heterochromatic DNA, the enzyme *Hae*III (GG/CC) digestion of mitotic chromosomes in *A. sicanum* seems to support results obtained with fluorochrome DAPI, i.e. that the heterochromatic DNA of this species is poor in GC base pairs. In fact, *Hae*III digestion produces a C-band-like

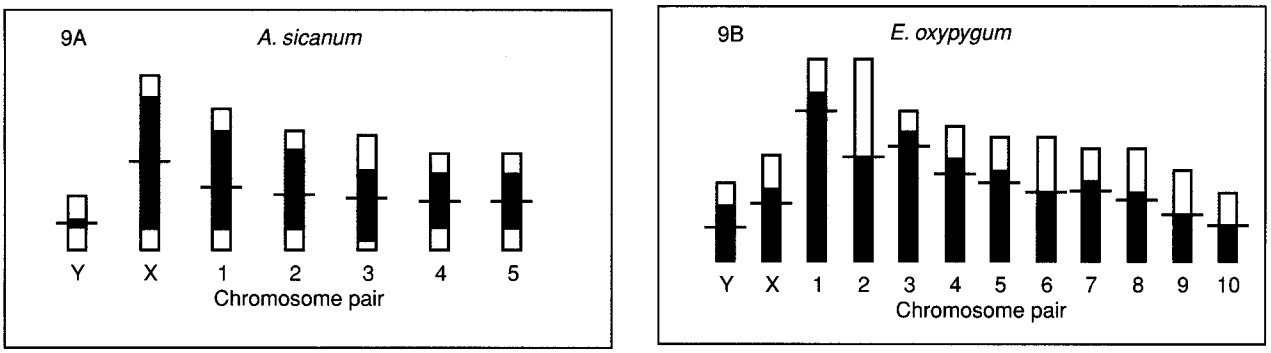


Figure 9. Diagrammatic representation of karyotypes and C-bands determined in somatic metaphase chromosomes of males of *A. sicanum* (**A**) and *E. oxypygum* (**B**). White sectors, euchromatin; dark sectors, constitutive heterochromatin.

band pattern, the heterochromatin of this species appearing undigested and well stained.

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References

Akar KP (1984) Analysis of male meiosis in five species of indian Diplopoda (Myriapoda). *Caryologia* **37**: 373–386.

Akar KP (1986) Analysis of male meiosis in seven species of indian pill-millipedes (Diplopoda: Myriapoda). *Caryologia* **39**: 89–101.

Akar KP (1987) Chromosomal evolution in Diplopoda (Myriapoda: Arthropoda). *Caryologia* **40**: 145–155.

Arnold ML, Shaw DD (1985) The heterochromatin of grasshoppers from the *Caledia captiva* species complex. II. Cytological organization of tandemly repeated DNA sequences. *Chromosoma* **93**: 183–190.

Bull J (1978) Sex chromosome differentiation: an intermediate stage in a lizard. *Can J Genet Cytol* **20**: 205–209.

Cionini PG, Bassi P, Cremonini R, Cavallini A (1985) Cytological localization of fast renaturing and satellite DNA sequences in *Vicia faba*. *Protoplasma* **124**: 106–111.

Deiana AM, Coluccia E, Milia A, Salvadori S (1996) Supernumerary chromosomes in *Nephrops norvegicus* L. (Crustacea, Decapoda). *Heredity* **76**: 92–99.

Dover GA (1986) Molecular drive in multigene families: how biological novelties arise, spread and are assimilated. *Trends Genet* **2**: 159–165.

Dover GA, Flavell RB (1984) Molecular co-evolution: DNA divergence and the maintenance of function. *Cell* **38**: 662–623.

Heitz ER (1935) Die Herkunft Der Chromozentren. *Planta* **18**: 571–635.

Hopkin SP, Read HJ (1992) *The Biology of Millipedes*. Oxford University Press.

Howell WM, Black DA (1980) Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. *Experientia* **36**: 1014–1015.

John B, King M (1983) Populational cytogenetics of *Atractomorpha similis* I. C-band variation. *Chromosoma* **88**: 57–68.

John B, King M, Schweitzer D, Mendelak M (1985) Equilocality of heterochromatin distribution and heterochromatin heterogeneity in acridid grasshoppers. *Chromosoma* **91**: 185–200.

Juan C, Gosálvez J, Petitpierre E (1990) Improving beetle karyotype analysis: restriction endonuclease banding of *Tenebrio molitor* chromosomes. *Heredity* **65**: 157–162.

Kenton A (1991) Heterochromatin accumulation, disposition and diversity in *Gibasis karwinskyana* (Commelinaceae). *Chromosoma* **100**: 467–478.

Levan A, Fredga K, Sandberg AA (1964) Nomenclature for centromeric position of chromosomes. *Hereditas* **52**: 201–220.

Lloyd MA, Thorgaard GH (1988) Restriction endonuclease banding of rainbow trout chromosomes. *Chromosoma* **96**: 171–177.

Martínez-Lage A, González-Tizón A, Méndez J (1994) Characterization of different chromatin types in *Mytilus galloprovincialis* and restriction endonuclease treatments. *Heredity* **72**: 242–249.

Martínez-Lage A, González-Tizón A, Méndez J (1995) Chromosomal markers in three species of the genus *Mytilus* (Mollusca: Bivalvia). *Heredity* **74**: 369–375.

Mezzanotte R, Ferrucci L (1984) Alterations induced in mouse chromosomes by restriction endonucleases. *Genetica* **64**: 123–128.

Mezzanotte R, Bianchi U, Vanni R, Ferrucci L (1983) Chromatin organization and restriction endonuclease activity on human metaphase chromosomes. *Cytogenet Cell Genet* **36**: 562–566.

Petitpierre E, Gatewood J, Schmid CW (1988) Satellite DNA from the mealworm beetle. *Tenebrio molitor*. *Experientia* **44**: 498–499.

Phillips RB, Ihsenn PE (1985) Identification of sex chromosomes in lake trout (*Salvelinus namaycush*). *Cytogenet Cell Genet* **39**: 14–18.

Schmid M, Haaf T, Geile B, Sims S (1983) Chromosome banding in Amphibia. VII. An unusual XY/XX sex chromosome system in *Gastrotheca riobambae* (Anura, Hylidae). *Chromosoma* **88**: 69–82.

Schmid M, Steinlein C, Feichtinger W, De Almeida CG, Duellman WE (1988) Chromosome banding in Amphibia. XIII. Sex chromosomes, heterochromatin and meiosis in marsupial frogs (Anura, Hylidae). *Chromosoma* **97**: 33–42.

Schmid M, Feichtiger W, Weimer R, Mais C, Bolaños F, León P (1995) Chromosome banding in Amphibia. XXI. Inversion polymorphism and multiple nucleolus organizer regions in *Agalychnis callidryas* (Anura, Hylidae). *Cytogenet Cell Genet* **69**: 18–26.

Sites JW, Bickham JW, Haiduk MW (1979) Derived X chromosomes in the turtle genus *Staurotypus*. *Science* **206**: 1410–1412.

Stebbins GL (1950) *Variation and Evolution in Plants*. Columbia University Press, New York.

Sumner AT (1972) A simple technique for demonstrating centromeric heterochromatin. *Exp Cell Res* **75**: 304–306.

Ugarkovic D, Plohl M, Petitpierre E, Lucijanic V, Juan C (1994). *Tenebrio obscurus* satellite DNA is resistant to cleavage by restriction endonuclease in situ. *Chrom Res* **2**: 217–223.

Vitturi R, Colombera D, Catalano E, Amico FP (1991) Karyotype analysis, nucleolus organizer regions and C-banding pattern of *Eisenia foetida* (Oligochaeta, Lumbricidae). *Genetica* **83**: 159–165.