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The genome of *Epiperipatus biolleyi* (Peripatidae), a Costa Rican onychophoran

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Abstract: Cytologic studies of *Epiperipatus biolleyi*, a Costa Rican onychophoran, reveal the presence of a large genome (C-value=4.3 pg/C) with many chromosomes, and a bimodal karyotype. Analysis of male meiosis indicates that all chromosomes are rod-shaped, without distinct primary constrictions. Two large bivalents are consistently associated with nucleoli. Meiosis proceeds through the pachytene stage, with a few thick bivalents that break up into many small and a few large chromosomes at the diplotene stage. At metaphase the rod-shaped bivalents seem to be pulled from the telomeres into opposite poles of the spindle, lacking well defined kinetochores. DNA isolated from *E. biolleyi* presents a complex profile with very low GC-content in isopycnic cesium gradients. Digestion with restriction enzymes produces a homogenous spread of molecules, except with Hae III, Hind III and Bgl II, which generate distinct bands of fluorescence. A Bgl II repeat of 1.9 Kb, has been cloned for evolutionary studies. Based on these results, many repetitive DNA families should be found in this genome.

Key words: Onychophorans, cytogenetics, evolution, C-value, repeated DNA families.

Cytogenetic studies have provided useful insights into the evolution of diverse groups of animals, since chromosome morphology and DNA content are relatively conserved traits within most animal species (Makino 1953; White 1977; Achar 1987; Alegria and León, 1991).Very little research has been done on onychophoran genomes, probably because these invertebrates are rare, secretive animals. The only modern study on onychophoran cytogenetics, so far, was recently reported on Australian onychophorans from the family Peripatopsidae (Rowell *et al.* 1995). Peripatopsids present a range of chromosome numbers 2N=18 to 42. Early work by Montgomery (1900) with tissue sectioning techniques of spermatocytes from peripatopsids provide the only information on chromosomes from African species. Montgomery reported a karyotype with N=14 chromosomes, but tissue sections are notoriously unreliable sources of chromosome information.

Here we present the first genomic study of a New World onychophoran, including the first observations of male meiosis in this species and the first C-value determination in the phylum. In *E. biolleyi* we find a large genome, very A+T rich, with a complex karyotype. Much more work is needed on onychophorans in general to establish trends in chromosome evolution, and with American species, in particular, to determine if the karyotype of *E. biolleyi* is representative of the whole genus and similar to other genera in the New World.

MATERIAL AND METHODS

Animals: During the last ten years we have been studying a population of *E. biolleyi* from Las Nubes de Cascajal, near San José, Costa Rica, found almost exclusively on moss embankments along rural roads. The animals

can be kept in humid moss terraria for long periods of time, feeding on small insects. Further details on the habitat of this population and on the species are offered in Monge-Nájera *et al.* (1993) and Monge-Nájera and Brenes (1994).

Chromosome preparations: Meiotic chromosomes were observed in testicular squashes, with material fixed in 3:1 (ethanol:acetic acid) and treated as described elsewhere (León and Kezer 1978, Macgregor and Varley 1983). A 2 min hypotonic treatment with distilled water was attempted without useful results. Preparations were stained with Giemsa and photographed with Panatomic-X film using a Zeiss microscope (MC63A).

Determination of DNA content: Feulgen reagent was used to stain endolymph smears fixed in methanol-acetic acid as described by Macgregor and Varley (1983). Human lymphocytes included in the same slides were used as standard. One hundred cells of each species were measured in a Reickert integrating microdensitometer and the average value was used to estimate C-value (DNA content/haploid nucleus), assuming 3,4 pg for the human C-value.

Isolation of DNA: DNA was isolated from carcasses frozen in liquid nitrogen, as described in detail elsewhere (Mora 1991). Briefly, powdered carcasses were resuspended in STE (0.01 M Tris [pH=8.5]; 0.1 M NaCl,; 0.02 M EDTA); sodium dodecyl sulfate (to a final concentration of 1%) and proteinase K (100 ug/ml, final concentration) were added. After overnight digestion at 53° C, the solution was extracted with phenol and chloroform as usual (Sambrook *et al.* 1989), and the DNA in the aqueous phase was precipitated with two volumes of ethanol and washed twice with 70% ethanol.

Isopycnic gradients: DNA was dissolved in TE buffer (0.01M Tris-Cl [pH=7.4], 1 mM EDTA), mixed with concentrated solutions of CsCl to a final concentration of 1.7 g/ml, determined by measuring the refractive index with a Bausch and Lomb refractometer. Centrifugation and fractionation of the gradients was done as described elsewhere (León and Gámez 1981). Gabriel Macaya kindly analyzed this DNA preparation in an analytical ultracentrifuge at the University of Paris VII, to obtain an independent determination of the buoyant density. Standard used was DNA from phage C2 (1.742 g/cm^3) .

Digestion with restriction enzymes: Digestions were done using the conditions described by distributors of the restriction enzymes Bgl II, Alu I, Bam HI, Eco RI, Hae III, Hind III, Pst I and Sma I, all obtained from Bethesda Research Labs., Bethesda, MA. USA. After overnight digestion with a 10-fold excess of enzyme (10 units/ug), DNA samples were analyzed in 1-2% agarose gels, stained with ethidium bromide and photographed with Polaroid film 667 with an ultraviolet transilluminator (Sambrook *et al.* 1989).

Cloning repetitive sequences: Bands were excised from gels with a razor blade and frozen. To obtain clonable sequences the frozen bands were squeezed and the DNA solution was clarified by centrifugation to eliminate small pieces of agarose (Sambrook *et al.* 1989). A 1.9 Kb sequence produced by Bgl II digestion was cloned into pUC13 previously linearized with Bam HI, as described by Sambrook *et al* (1989). Twenty recombinant colonies (Vieira and Messing 1982) were tested for inserts of the appropriate size, after growing overnight in 10 ml LB-broth cultures containing ampicillin or carbenicillin (60 ug/ml). T4 DNA ligase was used for the ligation reactions at 15°C. Host cells used were JM103, a strain capable of α-complementation (Sambrook et al. 1989).

RESULTS

Genomic DNA studies: Genomic DNA from *E. biolleyi* is very A+T rich (70% A+T), with a complex profile containing several distinct components on the light and heavy side of the main band in the isopycnic gradients (Fig. 1). The main band peak is close to 30% G+C, based on the refractive index of the peak fractions, and on the analytical ultracentrifugation results. There is at least one very large satellite component on the heavy side of the gradient (Fig. 1). Digestion of DNA with a 10-fold excess of restriction enzyme usually produces diffuse fluorescence along gel lanes, after staining with ethidium bromide. However, three enzymes- Bgl II, Hae III, and Hind III- out of eight

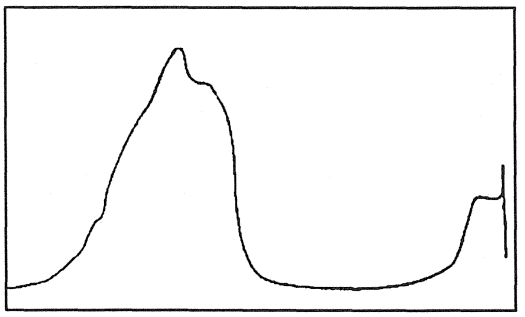

Fig. 1. DNA from *E. biolleyi* centrifuged to equilibrium in CsCl. The top of the gradient is to the right. The peak of absorbance has a GC-content of 30%.

that were tested were found to generate repetitious sequences and produce distinctly visible bands over the diffuse background. We have cloned the 1.9 Kb. Bgl II digestion product, into the pUC13 vector. Of twenty white colonies tested, two had 1.9 Kb inserts. These were designated pEb1 and pEb4. According to preliminary tests with restriction enzymes and sequencing studies (M. Mora, unpublished), both clones have identical sequences. The sequence is AT-rich and contains restriction sites for Eco RI, Hind III and Bam HI (Mora 1991).

Cytologic and cytophotometric studies: The average fluorescence of 100 Feulgen-stained endolymph cells measured with a microspectrophotometer, was used to estimate DNA content, using 100 human white blood cells on the same slide as reference. *E. biolleyi* cells consistently had higher UV absorbance values. We estimated that *E. biolleyi* diploid nuclei contain about 8.6 pg of DNA, consequently the C-value is about 4.3 pg (compared to 3.4 pg in haploid human cells).

Consistent with this high C-value, *E. biolleyi* presents many chromosomes in metaphase cells, with a wide range of sizes, from very small members that could be considered microchromosomes, to intermediate and large size members of the set (Fig. 2A). A striking observation is the absence of primary constrictions at metaphase or during other stages of meiosis in these chromosomes. In testicular squashes, pachytene stage cells are abundant, containing a few thick "bivalents" (Fig. 2B and C). These structures break up into individual chromosomes as diplotene stage begins. It appears that several different chromosomes are packed into each of these individual structures, consequently, these are not pachytene bivalents in the traditional sense (White 1977, Kezer and Macgregor 1973). Nucleoli are often observed attached to two large chromosomes in pachytene cells (Fig. 2B, arrows). We have counted the number of chromosomes in 10 metaphase plates, with number ranging in diplotene and metaphase cells between N= 28 to N=32. The discrepancies are due to variation in the number of small chromosomes. We cannot offer a definitive karyotype for this species, without primary constrictions to define arm ratios and without well defined G-bands.

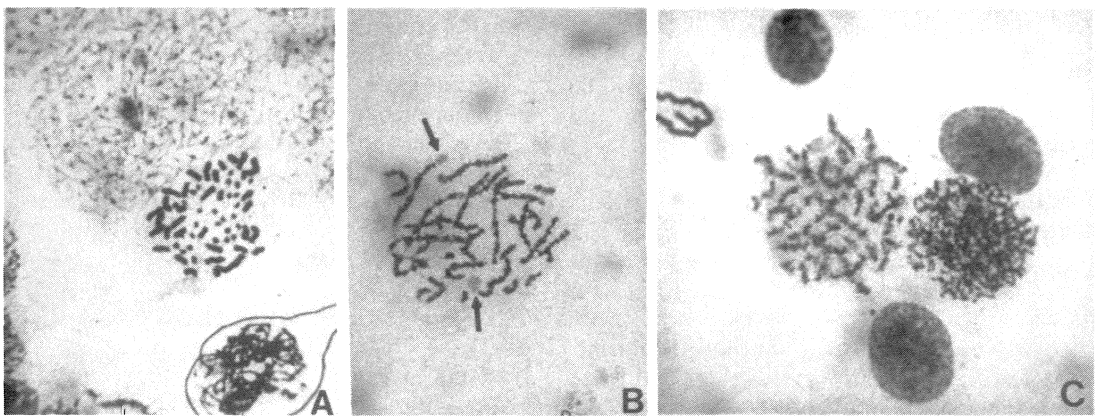

Fig. 2. Spermatocyte squashes of *E. biolleyi* stained with Giemsa and photographed under the 40x objective lens for a final magnification of x800. A metaphase I cell is shown in (A) with 2N=60. In (B) a late pachytene cell is shown, displaying two nucleoli attached to the ends of two large bivalents (arrows). An early pachytene is observed in the middle of (C), with a leptotene cell to the right of it.

DISCUSSION

A vast literature is available on the chromosomes of insects, myriapods and annelids (White 1977, Achar and Chowdaiah 1980, Achar 1986, 1987, Colmagro *et al.* 1986, Singhal *et al.* 1986, Fischer 1987). Comparisons of these chromosomes with the *E. biolleyi* karyotype described here, reveal morphological similarities with chilopods, in which many species lack primary constrictions and have similar bimodal karyotypes. For instance, the primitive chilopods in the genus *Lithobius* (Achar 1984, 1986; Colmagro et al. 1986) have many chromosomes with small, medium and large members in the set, similar to *E. biolleyi.* The more derived diplopods have fewer, more homogenous chromosomes (2N=12 to 2N=30). The few pauropods that have been studied have chromosomes that vary in number from 2N=12 to 2N=28. Annelid chromosomes, however, are also variable in number, particularly in polychaetes (Vitturi *et al* 1984), although they show little resemblance, otherwise, with the *E. biolleyi* chromosomes. Altogether, the greatest resemblance that we have encountered is with the Chilopoda. Clearly, a single karyotype is of limited value for phylogenetic comparisons, until more is known about karyologic trends in the family Peripatidae and in the whole phylum Onychophora. It is noteworthy that chilopods and onychophorans also present similar embryologic properties (Anderson, 1973) and other evolutionary similarities (Ballard *et al*, 1992; Emerson and Schram, 1990; Tiegs and Manton, 1958;).

Peripatopsids present a range of chromosome numbers 2N=18 to 42 (Rowell *et al.* 1992). No morphological data were presented and the position of centromeres was not reported. Montgomery's (1900) initial report described a karyotype with N=14 chromosomes, but as already indicated, tissue sections are unreliable sources of chromosome information. His *camera lucida* drawings, however, show chromosomes without well defined primary constrictions, as we have found.

A shotgun approach to the study of repetitive families has been previously applied to genomic studies in vertebrate (Alegría and León 1991). This approach consists of digesting total DNA with a battery of restriction endonucleases, and searching for distinct bands after electrophoresis of the digested products and cloning in small vectors. If repetitive families are present and have a particular restriction site, bands can be easily observed over the diffuse fluorescent background in each lane of the gel. Digestion of *E. biolleyi* DNA with restriction enzymes has again disclosed the existence of repetitive DNA families that we have cloned for phylogenetic studies.

RESUMEN

Estudios citológicos del onicóforo costarricense *Epiperipatus biolleyi* demostraron la presencia de un genoma grande (valor C= 4.3 pg/C) con muchos cromosomas en un cariotipo bimodal. El análisis de la meiosis en machos indica que todos los cromosomas tienen forma homogénea, sin constricciones primarias, aunque dos de estos presentan constricciones secundarias asociadas a nucleolos. Durante el paquitene de la meiosis aparecen todos los bivalentes asociados entre sí en cilindros gruesos que al entrar en diplotene se desintegran para dar origen a muchos cromosomas pequeños y algunos cromosomas grandes. Durante la metafase meiótica los cromosomas aparentemente son separados a polos opuestos, sin que pueda observarse un cinetocoro bien definido. El ADN de esta especie presenta un complejo perfil con un alto contenido A+T, en gradientes isopícnicos de cloruro de cesio. La digestión con enzimas de restricción produce una distribución homogénea de moléculas, excepto cuando se utilizan las enzimas Hae III, Hind III y Bgl II, las cuales generan bandas bien definidas, por la presencia de secuencias repetidas. Una de estas secuencias de 1.98 Kb producida con la enzima Bgl II fue clonada en un plásmido bacteriano para estudios evolutivos. Con base en estos resultados esperamos encontrar muchas familias de ADN repetitivo en este organismo.

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