Comparative cytogenetics in different populations of the cavernicolous diplopod *Pseudonannolene strinatii* (Diplopoda, Pseudonannolenidae)

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ABSTRACT. Different populations of *Pseudonannolene strinatii* Mauriès, 1974 collected from three caves in Iporanga, state of São Paulo, were cytogenetically compared using techniques of conventional coloration, C-banding and silver nitrate impregnation. Specimens were morphologically similar and small cytogenetic differences were observed between the populations with relation to the distribution of constitutive heterochromatin.

KEYWORDS. Millipedes, C-banding, karyotype, AgNOR, chromosomes.

RESUMO. Citogenética comparativa em diferentes populações do diplópodo cavernícola *Pseudonannolene strinatii* (Diplopoda, Pseudonannolenidae). Diferentes populações de *Pseudonannolene strinatii* Mauriès, 1974 coletadas em três cavernas em Iporanga, Estado de São Paulo, Brasil foram comparadas citogeneticamente utilizando-se técnicas de coloração convencional, bandamento C e impregnação com nitrato de prata. Indivíduos demonstraram-se morfologicamente similares e diferenças citogenéticas sutis foram observadas entre as populações com relação à distribuição da heterocromatina constitutiva.

PALAVRAS-CHAVE. Milípedes, bandamento-C, cariótipo, AgRON, cromossomos.

Despite the increasing interest in Diplopoda cytogenetic in the last years, it has still not been explored very much and consequently little is known about it (Fontanetti *et al*., 2002). White (1979) considers the Diplopoda conservative with regard to karyotype evolution in general and adds that it is necessary to know what types of chromosomic polymorphisms exist in the group and which types of chromosomic rearrangements have occurred in the evolution of its karyotypes. In a survey, it was verified that the species *Pseudonannolene strinatii* Mauriès, 1974 is widely distributed in Brazilian caves (Trajano *et al*., 2000). Carmem Silvia Fontanetti (pers. observ.) noted subtle morphologic differences between individuals from different caves, however in insufficient numbers to separate them into distinct species.

Currently the cytogenetic studies have been revealed to be important in the preparation of an efficient taxonomy of many animal groups, including diplopods (Fontanetti, 1996b), mainly in those composite species whose morphologic standard is not sufficient for its classification.

The aim for this study was to compare populations of *P. strinatii*, in order to detect possible cytogenetic differences that would assist in the taxonomy of the group.

MATERIAL AND METHODS

The analyzed specimens were collected in the Areias de Cima Cave (SP-018, 24”35’S, 48”42’W), also known as “Areias”, Iporanga, state of São Paulo in 01/2001, 04/2001 and 10/2001 by K. A. Campos and assistants; Ressurgência do Córrego das Areias de Águas Quentes Cave (SP-016, 24”35’S, 48”40’W), popularly known as “Laboratório”, Iporanga, SP in 05/2000, 07/2000, 09/2000 and 10/2000 by K. A. Campos and assistants; Jeremias Cave (SP-053, 24”38’S, 48”42’W), Iporanga, SP in 01/2001 by K. A. Campos.

The morphological comparison of the populations was based on external characteristics and the specimens were deposited in the collection of the Departamento de Biologia – UNESP, Rio Claro, SP. For the chromosomic preparations, the individuals were starved for one week and then injected with 0.08% colchicine. After approximately 16 hours (overnight), the specimens were anesthetized and dissected in physiological solution. The midgut were removed and washed in tap water for 10 minutes and after that fixated in Carnoy I (3:1 ethylic alcohol and glacial acetic acid). The slides were prepared using the method of cellular suspension, i.e., by means of centrifuging with preliminary dissociation in 45% acetic acid, followed by two washes with a fixative. The slides were stained with 3% Giemsa solution. C-banding was prepared according to Sumner (1972) and stained with silver nitrate (NOR) according to Howell & Black (1980).

The characterization of the chromosomes based on the morphology was obtained by measuring the chromosomic arms and then classified according to Guerra (1986).

RESULTS

The external morphological analysis of the animals did not demonstrate significant difference between the individuals from the three localities.

Pseudonannolene strinatii presents 2n=16 and the mechanism for determination of the sex is of XY/XX type. In the three populations, chromosomic pairs 1 and 2 are metacentric, pairs 3, 7 and chromosome X are

submetacentric and pair 6 is acrocentric (Figs 1, 4, 7). In the population of the Laboratório and Areias Caves, pair 4 is acrocentric and chromosome Y is submetacentric (Figs 1, 4); in the population of the Laboratório Cave, pair 5 presents one of the bigger elements as submetacentric with the other smaller one as metacentric (Fig. 1, Tab. I). The individuals of the Areias Caves have pair 5 as submetacentric; meanwhile the population of Jeremias Cave presents pairs 4 and 5 submetacentrics and acrocentric Y (Fig. 7). The respective indexes as centromeric, arm ratio and chromosome morphology can be seen in tables I to III.

C-banding revealed the constitutive heterochromatin distributed in interstitial, telomeric and centromeric bands, each chromosomic pair having a specific marking (Figs 2, 5, 8). Pairs 1, 2, 3 and 5 were almost heterochromatic, pair 4 and the X and Y chromosomes showed the short arm as completely heterochromatic, having a telomeric marking in the long arm of the X; pairs 6 and 7 presented pericentromeric markings. Some heteromorphisms related to C-banding were observed in the populations of the Laboratório and Jeremias Caves. In the population of the Laboratório Cave, the elements of pairs 5 and 6 presented C+ blocks of different sizes in all cells that, consequently, reflected in the differences of size and morphology between the homologous ones (Fig. 2, Tab. I); the individuals of the population of the Jeremias Cave presented, in the same direction, heteromorphisms in pairs 4 and 5 (Fig. 8, Tab. III).

The NORs were subterminally highlighted in pair 4 (Figs 3, 6, 9); this region was also stained by C-banding (Figs 2, 5, 8). The animals in the Jeremias Cave, like in the Areias Cave, presented duplicate marking in one of the pair elements (Figs 6, 9). Based on the results obtained, the different populations of *P. strinatii* could be compared to one another. Furthermore, for better visualization of the data obtained, ideograms of these populations were constructed (Figs 10-12).

DISCUSSION

The cytogenetic analysis of the individuals from the studied populations revealed the chromosomic 1, 2, 3, 5, 6, 7 pairs and X chromosome with the same morphology in the three populations; however, morphologic differences between the 4 pair and Y chromosome are observed. Such chromosomes are visually similar, being the morphologic difference between the populations, observed by the results of the calculation of the centromeric index and arm ratio; these differences can be a result of the condensation degree of each chromosome or even due chromosomal rearrangements (Tabs I-III).

The standard distribution of C-bands in the chromosomes of the individuals of the three populations is very similar. These present high amounts of the constitutive heterochromatin, about 65% of the diploid genoma of the species, a fact already observed in this and other species of other orders (Vitturi *et al*., 1997; Fontanetti *et al*. 2002; Campos & Fontanetti, 2004; 2005; Souza *et al*., 2005; Godoy *et al*., 2008). Pairs 5 and 6 of the individuals of the Laboratório Cave and pairs 4 and 5 of Jeremias Cave are heteromorphic based on C-banding. Studying Anura amphibians, King (1991) observed the same phenomenon in chromosomic pairs 9, 10 and 12 of *Litoria chloris*, *L. coplandi* and *L. meriani* respectively, commenting that these pairs are heteromorphic because of an addition of heterocromatin in one of the pair elements. Deletion and duplication could also be involved in the heteromorphism of these pairs. It is known that these mechanisms tend to occur in multi-gene families or non-codifier sequences, mainly in

Tab. I. Chromosomal centromeric index (CI), arm ratio (R) and morphology (Mo) of Laboratório Cave individuals, Iporanga, state of São Paulo, Brazil (m, metacentric; sm, submetacentric; a, acrocentric).

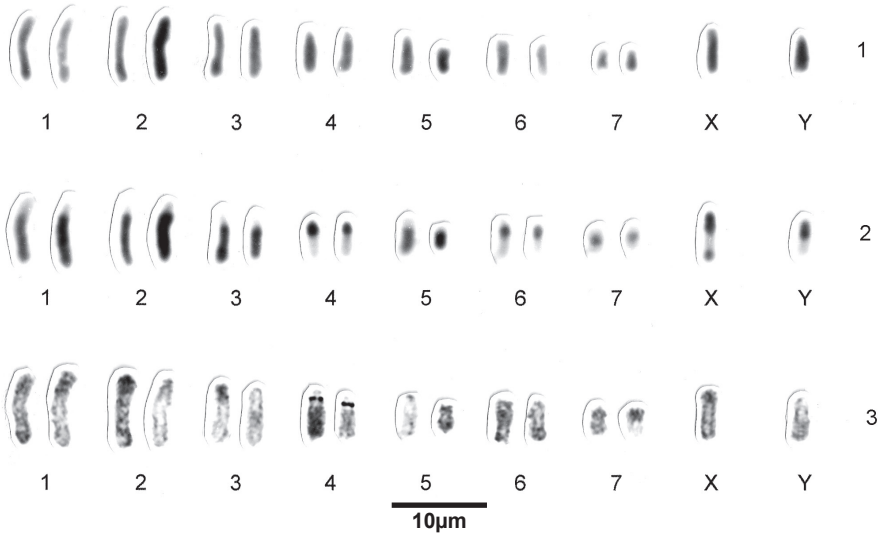
Chromosomal pair	R	CI	Mo
1	1.1	47.8	m
2	1.1	47.4	m
3	1.7	36.7	sm
4	5	17	a
5a	2	33	sm
5b	1.3	44	m
6a	5.3	16	a
6b	5	17	a
7	2	33	sm
X	2.9	26	sm
Y	2	33	sm

Tab. II. Chromosomal centromeric index (CI), arm ratio (R) and morphology (Mo) of Areias Cave individuals, Iporanga, state of São Paulo, Brazil (m, metacentric; sm, submetacentric; a, acrocentric).

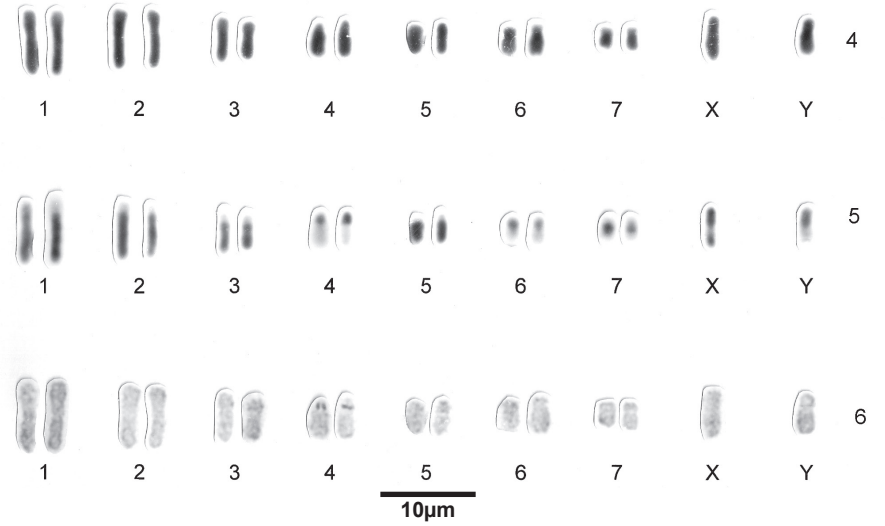
Chromosomal pair	R	CI	Mo
1	1.1	48.5	m
2	1	47.5	m
3	2.4	29	sm
4	3	25	a
5	2	33.7	sm
6	3.7	21	a
7	1.7	37	sm
X	2.6	28	sm
Y	3	25	a

Tab. III. Chromosomal centromeric index (CI), arm ratio (R) and morphology (Mo) of Jeremias Cave individuals, Iporanga, state of São Paulo, Brazil (m, metacentric; sm, submetacentric; a, acrocentric).

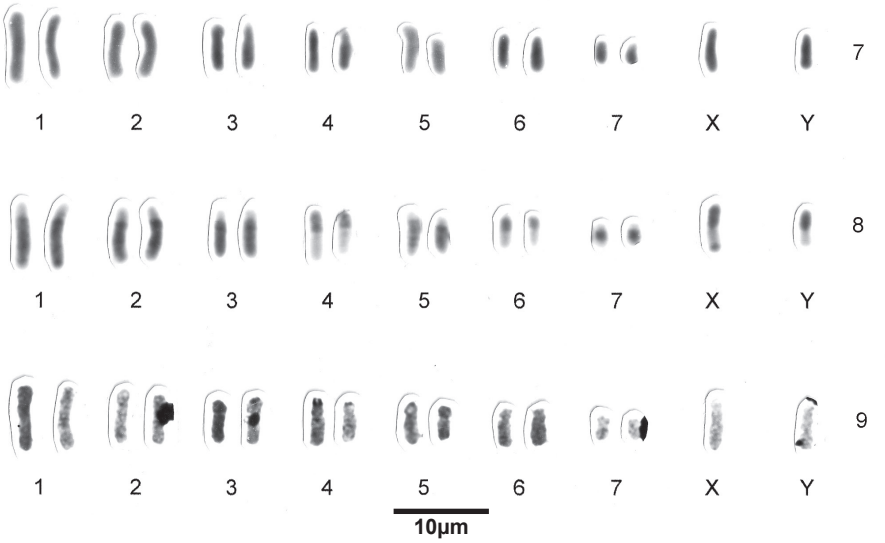
Chromosomal pair	R	CI	Mo
1	1.1	48.5	m
2	1.1	48	m
3	2	33	sm
4a	2.8	26	sm
4b	2	31	sm
5a	2.5	29	sm
5b	1.7	36.5	sm
6	4	19	a
7	2	34	sm
X	2.7	28	sm
Y	3.5	22	a



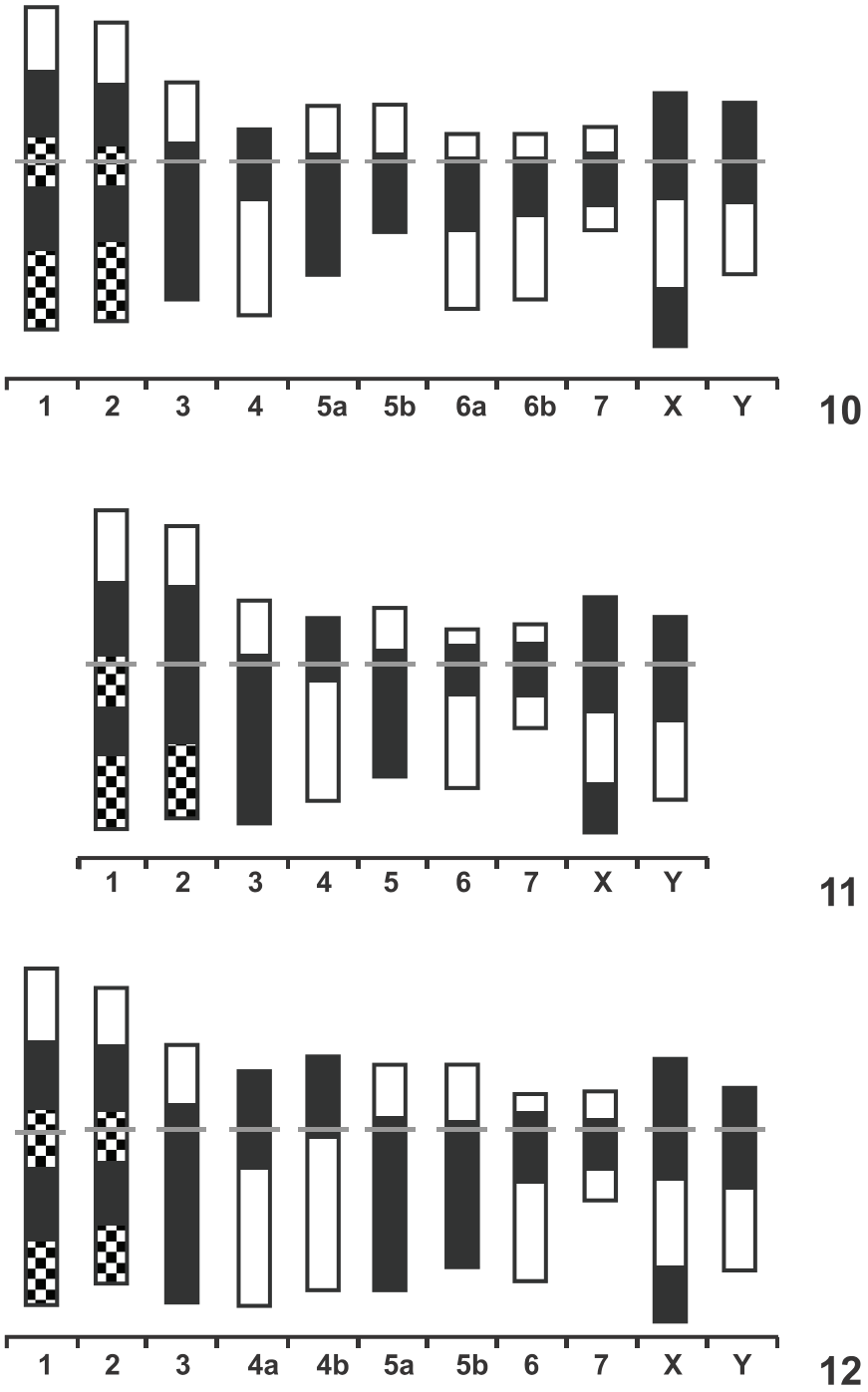
Figs 1-3. Karyotype of individuals of *Pseudonannolene strinatii* Mauriès, 1974 from Laboratório Cave, Iporanga, state of São Paulo, Brazil obtained with: 1, conventional staining with Giemsa; 2, C-banding technique and 3, Ag-NOR technique.



Figs. 4-6. Karyotype of individuals of *Pseudonannolene strinatii* Mauriès, 1974 from Areias Cave, Iporanga, state of São Paulo, Brazil obtained with: 4, conventional staining with Giemsa; 5, C-banding technique and 6, Ag-NOR technique.



Figs. 7-9. Karyotype of individuals of *Pseudonannolene strinatii* Mauriès, 1974 from Jeremias Cave, Iporanga, state of São Paulo, Brazil obtained with: 7, conventional staining with Giemsa; 8, C-banding technique and 9, Ag-NOR technique.



Figs. 10-12. Ideograms representing the morphology and pattern of chromosomal C-banding of *Pseudonannolene strinatii* Mauriès, 1974 from Iporanga, state of São Paulo, Brazil: 10, Laboratório Cave; 11, Areias Cave; 12, Jeremias Cave.

the heterocromatin (Guerra, 1988; Macgregor, 1993). It is clear, therefore, that the small existing cytogenetic differences between the populations of *P. strinatii* studied are relative to the constitutive heterochromatin. Heteromorphism of the heterocromatin frequently occur, not only between species, but also between populations of the same species without, however, any phenotypic effect (John, 1988). The role of the heterochromatin in the evolution and speciation still remains obscure. Imai (1991) studied ants, and suggested that heterochromatin has a reconstitution role in telomere stability, being added after centric fission, in the torn extremity of the chromosome. It had been considered that the presence of the heterochromatin facilitates the occurrence of structural rearrangements, leading to reproductive isolation (John, 1988). Sumner (1990), however, affirms that chromosomic variants, such as variations in the heterocromatin, are kept in populations as a heteromorphism, not necessarily causing reduction of fertility and in this way not becoming reproductive barriers, and thus not leading to speciation.

As expected, the nucleolus organizer regions are

observed in pair 4 in all populations and correspond to heterochromatic portions (CAMPOS & FONTANETTI, 2004), as described by various authors for different groups animals, including others diplopods (SOUZA *et al*., 2005; GODOY *et al*., 2008). The silver impregnation revealed heteromorphisms for the size of the NOR in the chromosomes of the individuals of Jeremias and Areias de Cima Cave. These heteromorphisms can be the result of the distinguishing genic activity of the rDNA segments, duplication of the rDNA content or significant incidence of non-reciprocal exchanges that can be occurring between the homologous individuals (MACGREGOR *et al*., 1977; SILVA *et al*., 1999; KING *et al*., 1990). CAMPOS & FONTANETTI (2004) comment that none of these events can be eliminated, since cytogenetic studies in Diplopoda are scarce, which prejudice the comparison inside the group and the attainment of a conclusive hypothesis.

The cytogenetic analysis could, therefore, point to the beginning of the differentiation of these populations, since the C-banding disclosed small differences between them. However, as previously seen, the role in the speciation process of the heterochromatin is not known, if there is any. Anyway, such interpopulational differences are small and only joint studies on biology, ecology and the cytogenetics of the species could supply conclusive data to evaluate the degree of isolation of these populations.

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