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Chromosome banding in Crustacea. I. Karyotype, Ag-NORs, C banding and treatment with EcoRI, PstI and KpnI restriction endonucleases in *Artemia franciscana*

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ABSTRACT: Karyotypic characteristics of the microcrustacean *Artemia franciscana* Kellog 1906, introduced to the salt lakes on the northeastern coast of Brazil in the 1970s, were investigated by conventional staining, C banding, restriction endonucleases (EcoRI, PstI and KpnI) and Ag-NORs. The karyotype consisted of 42 chromosomes and secondary constrictions were observed on some pairs. Large heterochromatic blocks were found distributed in the telomere portion of most of the chromosomes. Digestion with PstI and KpnI showed a similar pattern to that obtained by C banding. Preparations treated with EcoRI revealed intense action in the heterochromatic regions indicating the presence of restriction sites. Multiple Ag-NORs were shown associated to heterochromatic blocks. These data presented here no identified modifications that might have occurred after the geographic isolation of this stock and examine the evolutionary modifications in the karyotype of this group.

Keywords: chromosome banding, brine shrimp, crustacean cytogenetics.

Bandamentos cromossômicos em Crustacea. I. Cariótipo, Ag-RONs, bandamento-C e tratamento com endonucleases de restrição EcoRI, PstI e KpnI em *Artemia franciscana*

RESUMO: Características cariotípicas do microcrustáceo *Artemia franciscana* Kellog, 1906, introduzida nas salinas do litoral nordeste do Brasil, na década de 70, foram investigadas através de coloração convencional, bandamento C, endonucleases de restrição (EcoRI, PstI e KpnI) e Ag-NORs. O cariótipo consiste de 42 cromossomos, onde se individualiza sobre alguns pares a presença de constrições secundárias. Grandes blocos heterocromáticos encontram-se distribuídos nas porções teloméricas da maioria dos cromossomos. A digestão com PstI e KpnI revelou um padrão similar ao obtido pelo bandamento C. Preparações tratadas com EcoRI apresentam digestão das regiões heterocromáticas indicando a presença de sítios de restrição nestas regiões. Ag-NORs múltiplas estão associadas a blocos heterocromáticos. Os dados apresentados representam passo inicial para identificação de possíveis modificações ocorridas após o isolamento geográfico desta amostra, assim como no entendimento das modificações evolutivas ocorridas no cariótipo deste grupo.

Palavras-chave: bandamento cromossômico, camarão de água salgada, citogenética de crustáceos.

1. Introduction

Cytogenetic aspects of Crustacea have triggered less interest than in the other Arthropod groups, such as the Insecta (LAZZARETTO; LIBERTINI, 1985). This condition is partly due to technical difficulties in accessing the chromosome of species. The chromosome of crustaceans are diminutes and numerous, where visualization and complete morphological individualization is not always possible.

The *Artemia* genus is taxonomically referred to as an group of sibling species and superspecies reproductively isolated (GIL et al., 1998), consisting of five gonochoristic species *A. franciscana* Kellog, 1906 (found in the Americas), *A. persimilis* Piccinelli and Prosdocimi, 1968 (found only in Argentina), *A. salina* (Linnaeus, 1758) Leach, 1819 (found in Europe and North Africa), *A. urmiana* Günther, 1900 (in Lake Urmia, Iran) and *A. monica* Verrill, 1869 found in Mono Lake, California, USA (BOWEN et al., 1985). In the Americas, *A. franciscana* is the most intensely studied and exploited species.

This species was first introduced in Macau, Rio Grande do Norte State, Brazil (April 1977) from San Francisco Bay (California). It adapted well in the favorable climatic conditions. Since then, except for a few new introductions, the species has remained stable and isolated of its original populations (CÂMARA, 2001).

Cytogenetical analyses carried out on *A. franciscana* populations in the Americas point to an absence of numerical variability in the chromosomes of the species, both in its natural areas and in introduced populations (ABREU-GROBOIS; BEARDMORE, 1989). Cytogenetic aspects have been used as important taxonomic criteria in this group together with morphology (CAMARGO et al., 2003), genetic distance, ecological isolation and controlled crosses among the groups (GAJARDO et al., 2001).

Quantitative and qualitative knowledge of the genetics of the *Artemia* genus is increasing, ranging from molecular to chromosome aspects. However, cytogenetic analyses using higher resolution banding techniques are still incipient.

Except for *A. persimilis* (2n=44) all the gonochoric

species present the diploid number 2n=42 (ABREU-GROBOIS; BEARDMORE, 1989). The partenogenetic populations, nevertheless, show varied degrees of polyploidy (2n, 3n, 4n, and 5n) as observed in Madagascar populations that have 3n=63 (TRIANTAPHYLLIDIS et al., 1996).

Some particular chromosome characteristics hinder further detailed analyzes, such as the high number of chromosomes in some forms, reduced chromosome size, low mitotic index and non-specific associations among the chromosomes (BARIGOZZI, 1974; ABATZOPOULOS et al., 1986) that frequently makes imprecise the classification of the chromosomes types.

In this study we describe the *A. franciscana* karyotype sampled in the Northeast of Brazil, using the Ag-NORS technique to identify the number and position of the ribosomal sites, and C banding and treatment with the restriction endonucleases EcoRI and AluI to characterize the distribution and the qualitative patterns of the heterochromatic regions.

2. Material and Methods

Recently hatched *A. franciscana* nauplius from salt lakes located in Macau, Rio Grande do Norte, northeastern coast of Brazil, were used to obtained mitotic chromosomes. The specimens were immersed in 0.025% colchicine for 30 to 60 minutes. The material was later hypotonized in KCl 0.075M for one hour and fixed in methanol: acetic acid solution (3:1) for 30 minutes. To prepare the slides, the material was squashed in 50% acetic acid. Drops of the material in suspension were placed on slides pre-heated to 40°C and then aspirated. After drying, the slides were stained with Giemsa 10% solution for 20 minutes and analyzed with an optical microscope. The nucleoli organizer regions were identified by the Ag-NORs method (HOWELL; BLACK, 1980) while the C banding followed methodology proposed by Sumner (1972). The KpnI (GGTAC^C), PstI (CTGCA^G) and EcoRI (G^AATTC) restriction endonucleases were dissolved in the buffers recommended by the manufacturer (Amersham Pharmacia) to a final concentration of 1.5, 0.5, 6 U/µl, respectively. A final volume of 40µl was placed on the slide and then covered with another slide. The chromosome preparations were incubated in a moist chamber at 37°C for 10 hours. Slides were stained by 10% Giemsa. The best metaphases were photographed in Olympus BX51 epifluorescence microscope, under magnification of 1000X, with Olympus DP73 digital image capture system.

3. Results

The analyses showed a symmetrical karyotype for *A. franciscana* consisting of 42 chromosomes, visualizing the meta-submetacentric and subtelo-acrocentric types in equal proportions. The formation of chromosome chains aligned telomere to telomere (not shown) was observed in many metaphases.

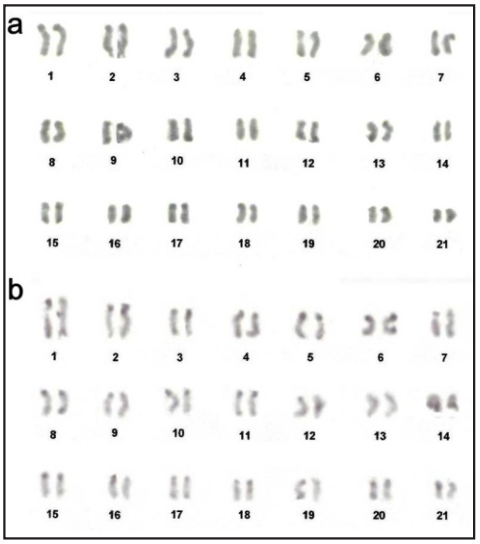

Figure 1. Karyotype of *A. franciscana* (2n=42) from Rio Grande do Norte state. Conventional staining by Giemsa (a); C-banding showing heterochromatic blocks in centromeric and telomeric regions of chromosomes (b).

Large heterochromatic segments were identified in the telomeric portion of most of the chromosome pairs (Figure 1b) even by conventional staining. The nucleoli were shown in 736 interphasic nuclei, ranging in number from one to five, of which 47% presented one large marking and 44% presented two smaller markings (Figure 2a). Supporting these data, multiple NORS were identified in the telomeric portion on three pairs of chromosomes, coinciding with the heterochromatic regions (Figure 2b). The secondary constrictions could be identified on some chromosomes under conventional staining (not shown).

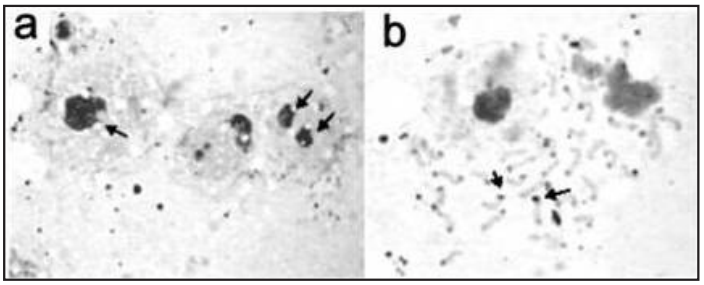

Figure 2. Ag-NORs sites in *A. franciscana*. In (a), interphase nucleus showing variations in size and number of nucleoli (arrows); in (b) ag-NORs sites localized in telomeric regions (arrows).

The analyses did not indicate the presence of numerical or structural chromosome heteromorphisms nor alocyclic behavior in the complement of this species, suggesting absence of differentiated sex chromosomes.

The banding patterns induced by selective DNA extraction by the KpnI, PstI and *EcoRI* (Figure 3b-d) restriction endonucleases were peculiar indicating different responses in the heterochromatic regions of the chromosome pairs. Patterns obtained by KpnI and PstI digestion were coincident with those shown by C banding, revealing strong markings in the centromeric and telomeric regions of the different pairs.

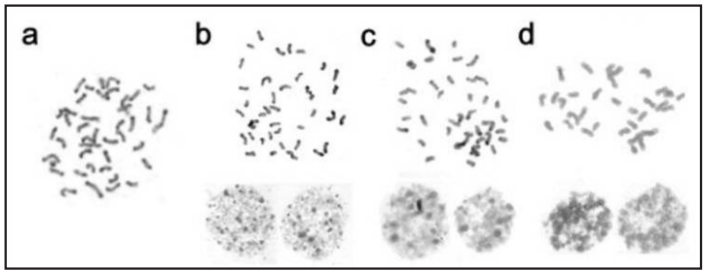

Figure 3. Somatic metaphases of *A. franciscana* and interphasic nuclei. C-banding (a); digestions with KpnI (b); PstI (c); EcoRI (d) endonucleases.

The interphasic nuclei were reticulated permitting easy identification of the chromocenters. Digestion with EcoRI acted intensely on the heterochromatic portions, removing large quantities of DNA from these regions. This fact was particularly evident in the interphasic nuclei that were homogeneous, without defined chromocenters or showing gaps regions (removal of heterochromatic sequences).

4. Discussion

Characteristics unfavorable to obtaining mitotic chromosomes are common among the different Crustacea groups (LAZZARETTO; LIBERTINI, 1986, LIBERTINI; LAVARETTO, 1993). However, an increasing number of cytogenetic data have been gathered for this group of organisms.

The cytogenetic analyses of the *A. franciscana* species showed a diploid number of 2n=42, similar to the previous descriptions (ABREU-GROBOIS; BEARDMORE, 1989), and heteromorphic chromosomes were not observed although heterogametic sex was indicated in females (BROWNE et al., 1991). However, although the complete definition of the chromosome types was not possible, some chromosome types were defined, such as metacentrics, submetacentrics and acrocentrics, previously observed (GIL et al., 1998 e COLIHUEQUE; GAJARDO, 1996).

The high incidence of telomeric heterochromatic blocks masked the identification of Ag-NORS signs in many cases. The nucleolar marking in interphasic nuclei (1-5 signs) with predominance of two markings corroborated the idea of multiple NORs, located in telomeric position on three chromosome pairs.

There is little information on ribosomal sites in Crustacea, especially multiple sites, such as those observed here in *A. franciscana* or in the copepod *Tigriopus brevicornis*, where they were located on three chromosome pairs, visualized by the presence of secondary constrictions or by the association of chromosomes in the nucleus, at the end of the meiotic prophase (AR-RUSHDI, 1963; LAZZARETTO; LIBERTINI, 1986).

Although the NORs are efficient cytotaxonomic markers for detection of inter or intra populational variation, and in understanding the karyotypic evolution of a group, the Ag-NORs technique is not always executable in crustaceans. This fact was observed in *Dactylopodia* sp. where no visible marking was shown (LIBERTINI; LAZZARETTO, 1993). This may be related to heterochromatic characteristics associated to these regions or to the organization and size of the ribosomal sites. The 5S rRNA subunit of Anostraca *Artemia salina* that are beginning to be understood are linked in tandem repeats of the genes in the histone (ANDREWS et al., 1987).

The chromocenters visualized during the interphase were correlated with the heterochromatic regions abundantly distributed on the telomeres in most of the *A. franciscana* chromosome pairs. Polymorphisms were observed in these regions in a population in Argentina (PAPESCHI, 2000). The mean number of chromocenters in the *Artemia* genus has been shown to be valid cytotaxonomic data and useful in characterizing species and lines (BARGIGOZZI; BARATELLI-ZAMBRUNI, 1981; GAJARDO et al., 2001a).

The frequency of interphasic chromocenters identified in *A. franciscana* from Macau corroborated previous cytogenetic data in the same population (ABREU-GROBOIS; BEARDMORE, 1989) revealing a mean value of 16.9 chromocenters. These results are similar to those observed in natural North American populations, source of the samples introduced to Brazil, but greater than those identified in *A. franciscana* from Yavorus, in Mexico (3.90) and *A. persimilis* (3.03) from Argentina. This parameter was distinctive among gonocorist species from the New and Old Worlds and among Caribbean and North American populations (*A. franciscana* and *A. monica*) (ABREU-GROBOIS; BEARDMORE, 1989). Apparently the frequency of heterochromatic segments is closely related to the evolutionary process, where heterochromatin gain or loss can lead to the establishment of populational differences or interspecific barriers. The current cytogenetic data still do not permit identification of the plesiomorphic condition of this trait. The existence of differential heterochomatic contents among the species indicates deviation of the speciation process bearing in mind the relative absence of gene flow during the geographic isolation (CAMARGO et al., 2003) and evolution of the group.

The heterochromatic telomeric regions in *A. franciscana* are formed by highly repetitive DNA, such as the sequence called satellite I, composed of 113pb, organized in tandem, containing sites for *AluI*, MboII and TaqI. Such sequences are present only in the American populations (BARIGOZZI et al., 1984).

The digestion patterns identified by *KpnI and PstI* indicated a weak presence of restriction sites in the heterochromatic portions, causing a typical C banding pattern. On the other hand, the response to tratament by EcoRI showed large loss of material in the

heterochromatic portion of all the chromosome pairs. Because DNA content extracted by an enzyme is proportional to the restriction site frequency existing for endonuclease data (VIÑAS et al., 1998), it is evident that the similar responses among the heterochromatic segments for digestion with *KpnI and PstI* or with *EcoRI* showed the high degree of conservatism among the heterochromatic sequences through the genome of the species. This result indicated that possible dispersion mechanisms occurred in the karyotypic evolution. The telomeric disposition of the heterochromatic sequences in *A. franciscana* are intrinsically related to the formation of pro-metaphasic chromosome chains and frequently metaphasic chains, could ease the dissemination of these repetitive sequences of the *A. franciscana* genome. This reinforces a possible constitutive/functional similarity of the regions involved.

The absence of G-like bands strongly suggests the non existence of compartmentalization of the *A. franciscana* genome thar may be a characteristic of the Crustacea in general.

Knowledge is increasing of the karyotypic evolution of the *A. franciscana* genus. The conserved karyotypic numbers (2n=42) observed in the bisexual species suggest some degree of chromosome conservatism. However, structural aspects still deserve greater investigation. A varied degree of ploidy (2n, 3n, 4n, and 5n) has been observed among the partenogenetic forms (2n, 3n, 4n, and 5n) (BARIGOZZI, 1974).

The presence of Bs chromosomes has not been observed in *A. franciscana*, as verified in *Hyperiella dilatata* (LIBERTINI; LAZZARETTO, 1993), a widely spread condition in Amphipods (LOP, 1989). The modulation mechanisms of the DNA contents is a condition found in Crustacea, better described for copepods (WYNGAARD; GREGORY, 2001), above all the elimination during cleavage of pre-somatic cells during embryonic development. This mechanism has not been studied in *A. franciscana* but if it exists, it might explain the occurrence of karyotypes with much or little heterochromatin in the genus.

The presence of many metacentric chromosomes is a characteristic observed in both copepods (LAZZARETTO; LIBERTINI, 1986) and decapods of the *Penaeidae* family. In *A. franciscana* there is apparently an equal proportion of chromosomes of one or two arms. Karyotypic surveys carried out on Copepods have included three of its seven orders. The chromosome evolution in the *Tisbe* (LAZZARETTO, 1983) and *Tigriopus* genera (LAZZARETTO; LIBERTINI, 1985) seemed to have occurred more frequently by structural rearrangements and only in a few species by reduction in the chromosome number. Due to the difficulties in the exact definition of chromosome types in *A. franciscana*, tendencies cannot yet be established in its chromosome evolution. *A. franciscana* populations from Argentina, studied through base specific fluorochromes (DAPI, CMA_3), revealed the presence of an intermediary number of heterochromatic segments (PAPESCHI, 2000).

Studies in progress involving the location of repetitive sequences and ribosomal sites by *in situ* hybridization in addition to the use of base specific fluorochromes will provide a better understanding of the chromosome structure of this group.

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