Chromosoma (Berl) (1984) 90:332–337

CHROMOSOMA

Heterochromatin in the genus *Artemia*

C. Barigozzi [1], G. Badaracco [1], P. Plevani [1], L. Baratelli [1], S. Profeta [1], E. Ginelli [2], and R. Meneveri [2]

[1] Dipartimento di Biologia, Sezione di Genetica e Microbiologia Università di Milano, Milan, Italy;
[2] Dipartimento di Biologia e Genetica per le Scienze Mediche Università di Milano, Milan, Italy

Abstract. A bisexual species of the genus *Artemia* (Crustacea, Phyllopoda), *Artemia franciscana* Barigozzi of San Francisco Bay and a parthenogenetic population of *Artemia sp.* of Tsing-Tao (China), both with 42 chromosomes, were compared with respect to the microscopic structure of the interphase larval nucleus, the microscopical structure of the prophase chromosomes and the DNA structure. – *Artemia franciscana* exhibits several chromocenters in the resting nucleus, heterochromatic blocks located at the end of the prophase chromosomes, and a large amount of repetitive DNA (Alu I 110-bp fragments). The other *Artemia sp.* lacks chromocenters, heterochromatic blocks in the chromosomes, and the Alu I DNA. The two populations thus differ by a remarkable amount of repetitive DNA.

Introduction

The chromosomes of the brine shrimp *Artemia* have been submitted to numerical analysis several times, and differences have been found in different populations and sibling species (Barigozzi 1974; Barigozzi and Baratelli Zambruni 1982). Little is known, on the other hand, of the chromosome structure. Barigozzi observed (1942) that during prophase spiralization intensity differs in the different chromosome pairs; the longer pairs spiralize more than the shorter ones with the consequence that length differences are detectable only during prophase, while at metaphase chromosome pairs are practically undistinguishable. A further contribution to the knowledge of the chromosome structure comes from Stefani (1963a, b). This author gave some evidence of the lack of a centric constriction in all chromosomes, interpreted as the lack of a differentiated centromere. The stain used, orcein, and the material, Sardinian populations, did not enable Stefani to observe any difference in eu- and heterochromatin.

Detection and analysis of the heterochromatin, both at the cytological and molecular level, was the aim of the present paper.

Material and methods

The material analyzed in this investigation was provided as dry cysts by the Laboratory of Mariculture of the University of Ghent, Belgium, which is here acknowledged. The populations were from: San Francisco Bay (U.S.A., bisexual, 42 chromosomes) and Tsing-Tao (China, parthenogenetic, 42 chromosomes). The chromosome number was determined on nearly 30 individuals. This is the first time that the Tsing-Tao population has been studied. Systematically, the brine shrimps of San Francisco Bay belong to the species *A. franciscana* Barigozzi. The other brine shrimp can presently be indicated simply as *Artemia sp.* The link between this population and *A. franciscana* is unknown. Both cells and DNA were obtained from the living nauplius larvae.

Techniques for the study of the resting nuclei and mitotic chromosomes. The mitotic stage selected for this investigation was the prophase, when the chromosomes are sufficiently thick to be individualized and the length differences are still clearly detectable.

Staining with orcein and quinacrine (4–6 nauplii on a slide): (1) treatment with hypotonic solution (tap water 0.5% or sodium citrate) for 30–40 min, (2) fixation in 1:1 methanol and acetic acid for 3 min, (3) a second exposure to 60% acetic acid (30 s) at about 40° C, (4) staining with 2% acetic orcein for 20 min or with 0.5% quinacrine for 7 min, (5) rinsing twice in 95% alcohol, transfer to absolute alcohol and mounting in Euparal.

Staining with Giemsa (C-Banding): (1) dipping the air-dried slides in 0.07 N NaOH for 40 s, (2) rinsing (three changes) in 2 × SSC (sodium chloride/sodium citrate), (3) incubation in 6 × SSC at 65° C for 3 h, (4) rinsing in ethanol first at 70° C then at 95%, (5) drying in air, (6) staining in Giemsa (10 ml stock solution improved R66 with 100 ml phosphate buffer, 0.01 M, pH 7) for 20 min, (7) rinsing in deionized running water, (8) drying in air, and (9) mounting.

Techniques for the DNA analysis. DNA extraction. Nauplii of *Artemia* were collected, washed with 10 mM Tris-HCl, (pH 8.0), 1 mM EDTA (ethylenediaminetetraacetic acid) and lysed in the same buffer containing 1% SDS (sodium dodecyl sulfate), with three cycles of freezing and thawing. Sodium perchlorate was added to a final concentration of 1 M. After 10 min at room temperature, the lysate was deproteinized by extraction with an equal volume of phenol-chloroform followed by extraction with chloroform-isoamylic alcohol. The DNA solution was RNase treated (50 μg/ml at 37° C for 30 min), re-extracted with chloroform and finally purified by CsCl density gradient. *Artemia* DNA has an average buoyant density of 1.699 g/cm^3.

Restriction enzyme digestion and gel electrophoresis. Artemia DNA samples were digested with several restriction en-

The authors dedicate this paper to Professor Hans Bauer, on the occasion of his 80th birthday

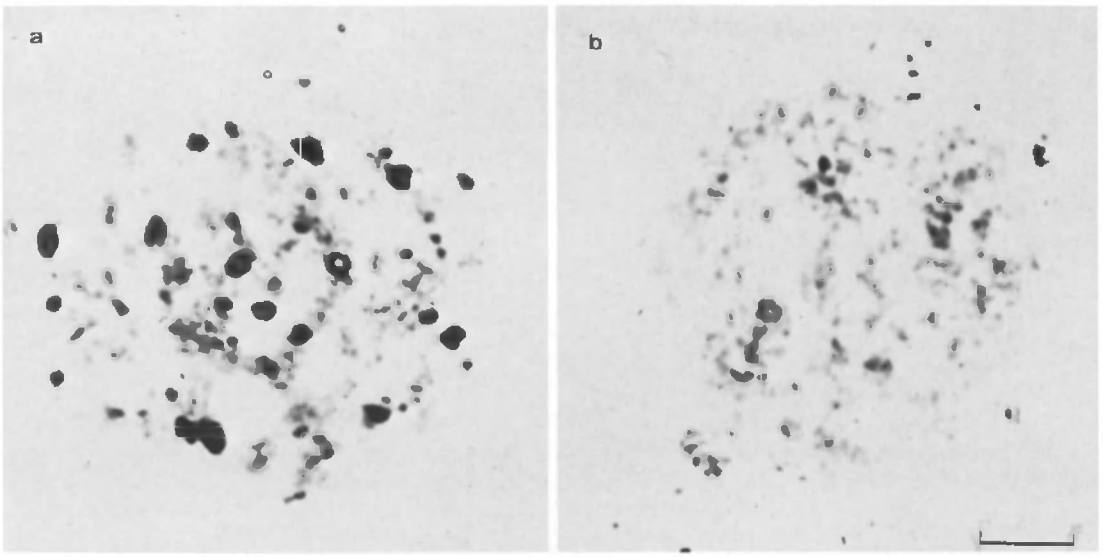

Fig. 1. Interphase nuclei (Giemsa C-banding) of *A. franciscana* (**a**) and *Artemia sp.* of Tsing-Tao (**b**). Bar represents 5 μm

zymes according to the procedure specified by the manufacturer. After digestion, DNA samples were treated with 2.5% Ficoll, 0.025% bromophenol blue and fractionated by agarose gel electrophoresis in TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA at pH 8.0). DNA bands were visualized by ethidium bromide staining and UV transillumination.

Cloning. The Alu I 110-bp (base pairs) *Artemia franciscana* fragments were isolated essentially as described by Taback and Flavell (1978). A 100-μg sample of total *Artemia* DNA was digested to completion with Alu I and then subjected to two-dimensional 2.5% agarose gel electrophoresis in TAE buffer (0.04 M Tris-acetate, 0.002 M EDTA at pH 8.0) and the 110-bp fragments were recovered by hydroxylapatite absorption in the second dimension. The DNA was eluted from hydroxylapatite with 0.6 M phosphate buffer at pH 7.2 and desalted on a Sephadex G-50 column. The Alu I fragments were cloned in the Sma I site of the pUC8 cloning vector (Vieira and Messing 1982). The plasmid was dephosphorylated and ligated to the Alu I fragments by standard recombinant DNA procedures (Maniatis et al. 1982). The ligated plasmids were used to transform the JM-103 *Escherichia coli* bacterial strain (Messing et al. 1981). Recombinant bacterial clones were identified as blue colonies by plating on ampicillin in the presence of X-gal and IPTG (Messing et al. 1981). Twenty independent recombinant bacterial clones were picked up and tested for the presence of a foreign DNA insert (Holmes and Quigly 1981). All the clones tested contained foreign DNA, and plasmids from ten separate clones were purified on large scale by ethidium bromide-CsCl density gradient (Radloff et al. 1967).

Nick translation and hybridization. A 1-μg sample of one of the previously characterized plasmids, identified as pUA41, was labeled with α-[^{32}P]dCTP by a nick-translation reaction as decribed by Rigby et al. (1977) and hybridized to Alu I-digested *Artemia* DNA blotted onto nitrocellulose filters (Southern 1975). The filters were prehybridized for 6–8 h at 65° C in 10% dextran sulfate, 1% SDS, 10 × Denhardt's solution, 50 mM Tris-HCl at pH 7.0, 1 M NaCl, 0.01% sodium pyrophosphate, and 100 μg/ml low molecular weight denatured herring sperm DNA. At the end of prehybridization, 0.1 μg of the denatured radioactive plasmid probe (10^8 cpm/μg) was added and the incubation continued for an additional 12–14 h. Filter washings were carried out in 2 × SSC, 1% SDS at 65° C and lastly, in 0.1 × SSC at room temperature. Autoradiography was performed on preflashed Fuji RX films at −70° C with an intensifying screen.

In situ hybridization. Unstained slides to be hybridized were prepared as described above. The pUA41 plasmid DNA was tritium-labeled by nick translation with [^{3}H]TTP and [^{3}H]dATP as the labeled substrates to a specific activity of 4×10^6 cpm/μg DNA. The slides were RNase treated (50 μg/ml at 37° C for 10 min), washed at 70° C in 2 × SSC and dehydrated with ethanol. Chromosomal DNA was denatured by heating at 65° C for 150 min in 90% formamide and hybridized with 25 ng of the labeled plasmid DNA (10^5 total cpm) in 2 × SET (0.15 M NaCl, 0.02 M Tris-HCl at pH 7.8), 2 × Denhardt's solution and 10% dextran sulfate for 36 h at 65° C. After incubation, the slides were washed with 0.1 × SET at 4° C for 10 min, 1 × SET at 37° C for 30 min and finally 1 × SET at 65° C for 30 min. After ethanol drying the slides were autoradiographed with Kodak NTB-2 emulsion for 12 days.

Results

The interphase nucleus

The interphase nucleus was studied both in the nauplius (undifferentiated cells) and in the intestinal epithelium of the adults. The same structure was found in both cases. The structure varies in different populations owing to the presence or absence of chromocenters, which stain with orcein, Giemsa (C-banding), and quinacrine.

The different nuclear types of the nauplius are illustrated in Figure 1 a, b. The species from San Francisco Bay, *A. franciscana*, had 14.8 ± 1.8 chromocenters and the population from Tsing-Tao, *Artemia sp.*, had no chromocenters and one to two only very small chromatin masses.

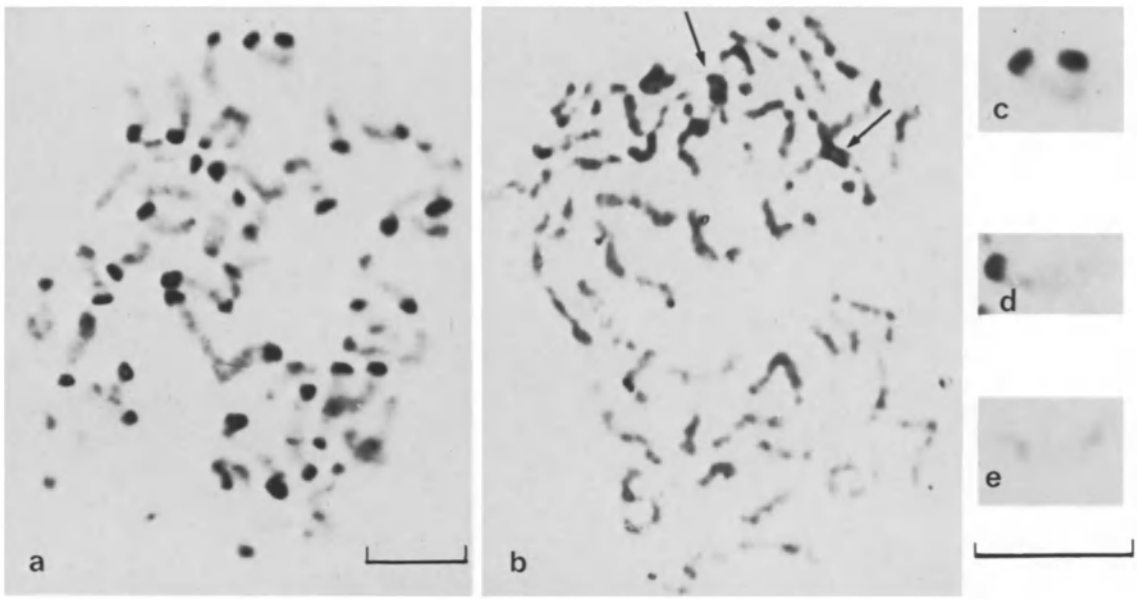

Fig. 2. Prophases (Giemsa C-banding) of *A. franciscana* (**a**) and *Artemia sp.* of Tsing-Tao (**b**); Notice two small heterochromatic masses (*arrowed* in **b**). Isolated prophase chromosomes with heterochromatic blocks (**c, d**) and without heterochromatic blocks (**e**). Bars represent 5 μm

Chromocenter counting was difficult in the epithelial cells, but the structure of their nuclei reflected the structure described in the nauplius.

The distribution of the heterochromatin in mitotic chromosomes

This section does not give the karyograms, which would have required a special investigation, but only points out the differences between the populations of *Artemia.*

Stained with Giemsa C banding, nearly all chromosomes *A. franciscana* exhibit chromatin condensed masses at one or both ends of the chromosome. The frequency is difficult to ascertain because well-spread prophases are very rare. It can be estimated that approximately 34 of 42 chromosomes carry Giemsa-positive masses, which can be considered as heterochromatin. A minority of heterochromatin-carrying chromosomes (8–10) show also a restricted number of thin and unclear bands.

The frequency of chromosomes carrying a single mass is higher than that of chromosomes carrying two masses. An estimate is difficult because a perfect spreading of the 42 chromosomes is rarely found. A small group of chromosomes (about 8) is devoid of heterochromatic masses.

In two prophases that were particularly unambiguous we found: 8 chromosomes with no heterochromatic mass, 24 with one heterochromatic mass, and 10 with two heterochromatic masses.

In no chromosome did we find anything similar to a constriction (Fig. 2a, b).

In the *Artemia sp.* population of Tsing-Tao (China) the prophase chromosomes fail to show any compact mass of heterochromatin. The general aspect of a prophase is, thus, Giemsa-negative (Fig. 2b).

Repetitive DNA sequences in Artemia DNA

Restriction pattern of *Artemia* DNA. The difference in heterochromatin amount in *A. franciscana* and in the Tsing-Tao *Artemia sp.* induced us to undertake a DNA analysis. We compared the two DNA patterns after digestion with several restriction enzymes. Complete digestion of *A. franciscana* DNA with Alu I clearly demonstrated the presence of two prominent bands of 110 bp and 220 bp that are undetectable after digestion of *Artemia sp.* DNA with the same enzyme (Fig. 3). A comparison of totally with partially Alu I-digested *A. franciscana* DNA showed that the

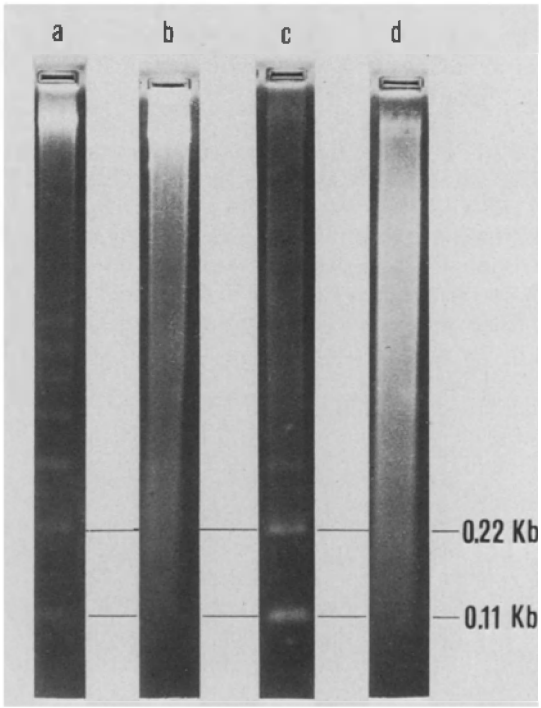

Fig. 3. Partial (**a, b**) and complete Alu I digestion (**c, d**) of DNA from *A. franciscana* (**a, c**) and *Artemia sp.* of Tsing-Tao (**b, d**) by incubating at 37° C 10 μg DNA with 10 units of enzyme 5 min and 3 h, respectively. 1 Kb = 1,000 bp

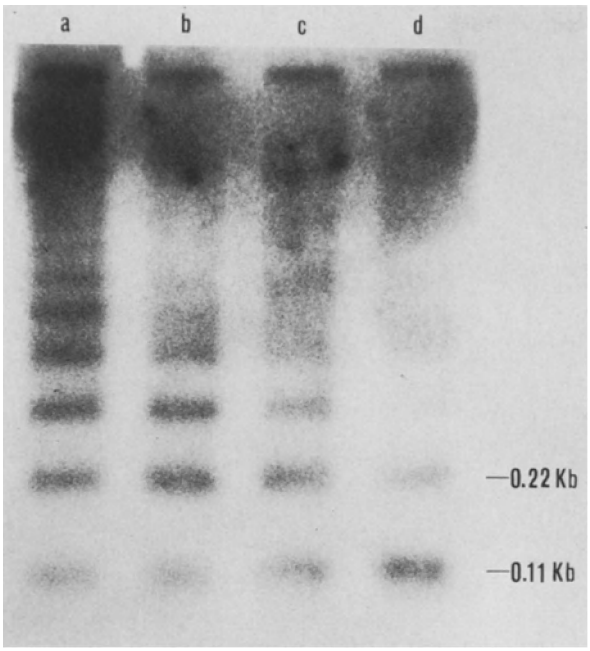

Fig. 4. Autoradiogram of hybridization of nick-translated pUA41 to Southern blots of Alu I partial and complete digests of *A. franciscana* DNA. A 10-μg sample of DNA was digested with 10 units of enzyme for 5 min (**a**), 15 min (**b**), 30 min (**c**) and 120 min (**d**), subjected to electrophoresis on a 2% agarose gel, and blotted onto a nitrocellulose filter. 1 Kb = 1,000 bp

110-bp band accumulated in totally restricted DNA (Fig. 3a, c). These results suggest that a family of clustered repetitive sequences is present in *A. franciscana* and absent in the *Artemia sp.* of Tsing-Tao. These observations parallel completely those obtained with cytological techniques on interphase and mitotic chromosomes, and indicate that Alu I repetitive DNA may correspond to the heterochromatin blocks.

Cloning of the 110-bp Alu I fragments and molecular hybridization

Total DNA of *A. franciscana* was digested with Alu I and the 110-bp fragments isolated from agarose gel and cloned in the Sma I site of the cloning vector pUC8. Four hundred independent recombinant bacterial clones were isolated and ten clones were chosen randomly to be tested for the presence of the foreign Alu I 110-bp fragment. This can be detected by digestion of plasmid DNA with Eco RI and Bam HI, whose recognition sites flank the Sma I site in the polylinker region of pUC8 (Vieira and Messing 1982). The DNA of the recombinant plasmid pUA41 was labeled by nick translation and hybridized to *A. franciscana* DNA that was digested with Alu I for different times and blotted onto a nitrocellulose filter. As it is shown in Figure 4 only the ladder of bands that can be shown after ethidium bromide staining of the Alu I-digested DNA, positively hybridized with the radioactive plasmid DNA probe.

This result demonstrates that the cloned *Artemia* DNA sequence is representative of the Alu I repetitive DNA family. The radioactive pUA41 probe was also hybridized to the *Artemia sp.* of Tsing-Tao DNA totally and partially restricted with Alu I. As it is shown in Figure 5 no hybridization to the Alu I repetitive DNA family was observed in the *Artemia sp.* DNA samples (Fig. 5b, d), while strong

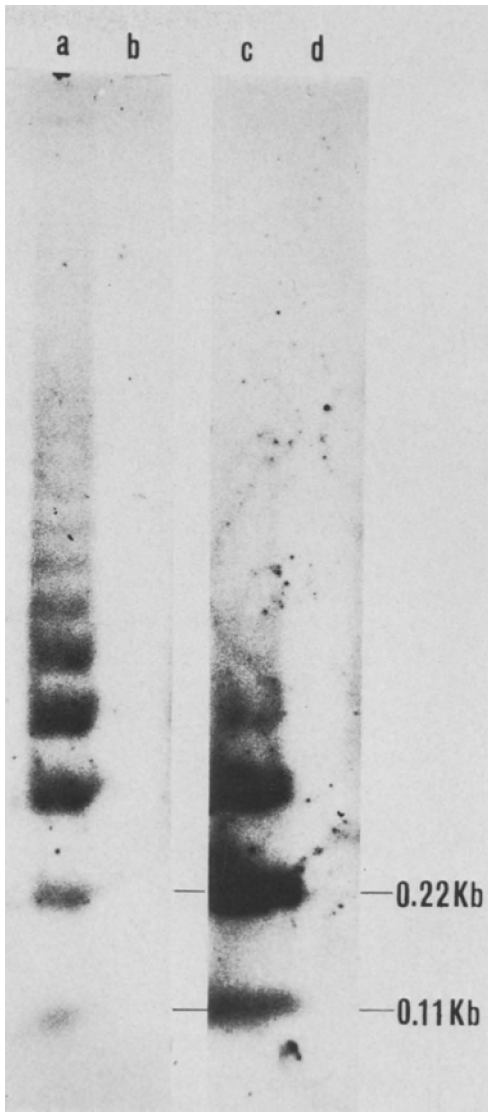

Fig. 5. Autoradiogram of hybridization of nick-translated pUA41 plasmid to Southern blots of Alu I partial (**a, b**) and complete (**c, d**) digests of the DNA of *A. franciscana* (**a, c**) and the *Artemia sp.* of Tsing-Tao (**b, d**). A 10-μg sample of DNA was digested with 10 units of enzyme for 5 min (**a, b**) and 120 min (**c, d**), subjected to electrophoresis on a 2% agarose gel, and blotted onto a nitrocellulose filter. 1 Kb = 1,000 bp

positive signals to Alu I DNA were clearly detected in the control *A. franciscana* DNA samples (Fig. 5a, c). These results substantiate the assumption that the heterochromatic blocks observed by cytological investigations in *A. franciscana* and not in *Artemia sp.* may be rich in the repetitive Alu I DNA family.

Hybridization in situ

Here both interphase nuclei and prophases were considered. Interphase nuclei (Fig. 6a, b) differ greatly in *A. franciscana* and *Artemia sp.* the former being strongly rich in grana, and the latter showing very few or no grana. Parallel observations on prophases show (Fig. 6c, d) no grana on the Tsing-Tao chromosomes and a strong accumulation of grana on those of *A. franciscana.*

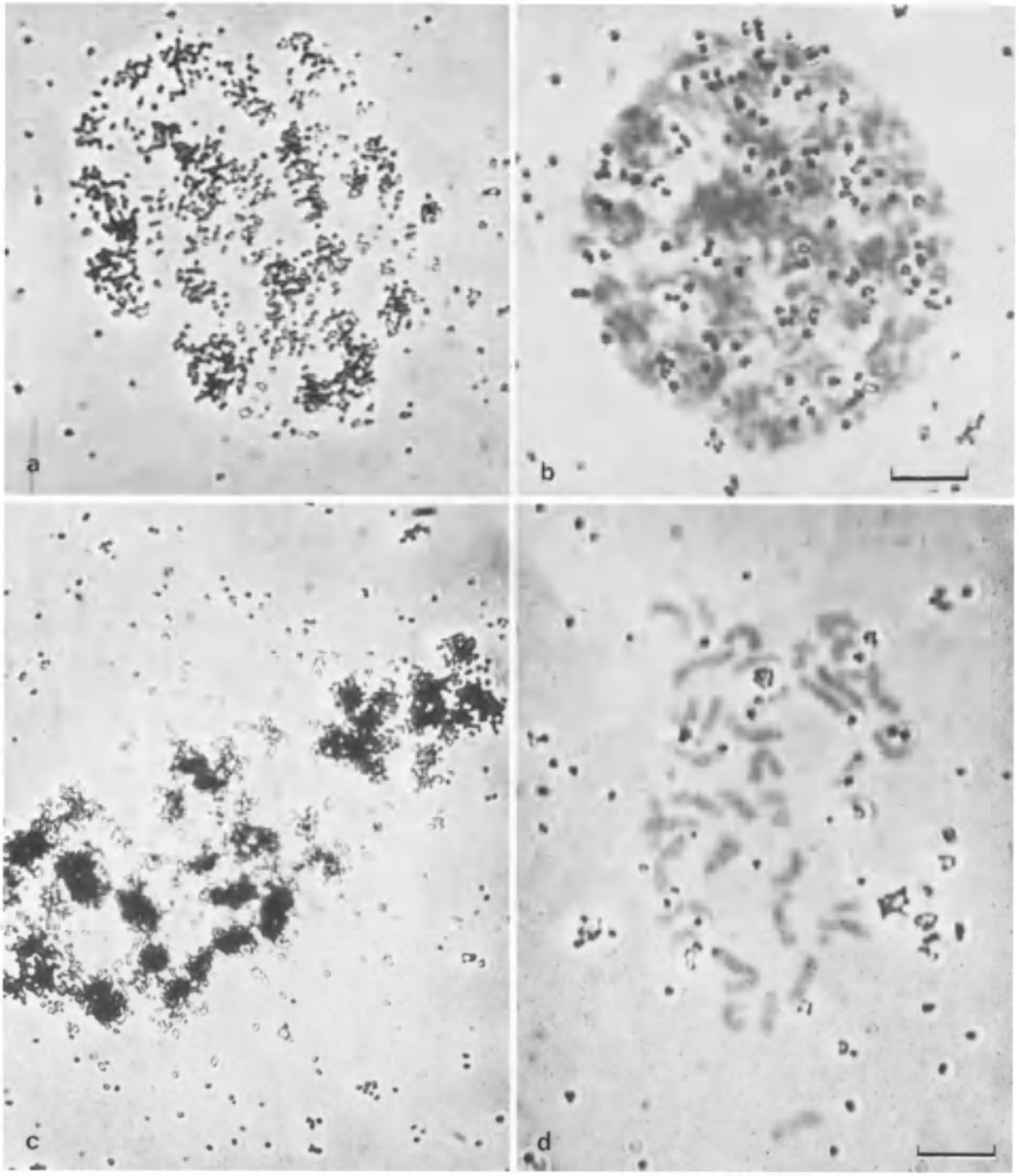

Fig. 6. Interphase (**a, b**) and prophase (**c, d**) nuclei of *A. franciscana* (**a, c**) and *Artemia sp.* of Tsing-Tao (**b, d**) after in situ hybridization. Bars represent 5 μm

Clearly, all the data confirm the presence of a considerable amount of repetitive DNA localized in heterochromatin in *A. franciscana* and an undetectable amount of repetitive DNA in the *Artemia sp.* of Tsing-Tao.

Discussion

The present investigation offers for the first time the possibility of discussing for the brine shrimp *Artemia*, the microscopic structure and DNA composition of its heterochromatin.

Two contrasting cases were found, one corresponding to a well-defined bisexual species (*A. franciscana* Barigozzi), the other to a less systematically defined parthenogenetic entity, indicated as *Artemia sp.* Both forms are diploid with 42 chromosomes. *Artemia franciscana* chromosomes are rich in Giemsa- and quinacrine-positive blocks, detectable also in interphase as chromocenters. Much of their DNA

is a repetitive Alu I monomer of 110 bp. This type of chromatin exhibits all the microscopic and chemical structural characteristics for being considered heterochromatin. Heterochromatic blocks are lacking in the *Artemia sp.* of Tsing-Tao, where only one or two small masses were observed during prophase.

This contrasting condition within a single genus raises questions on the possible links between the two forms, which are undistinguishable by means of morphological characters and cannot be submitted to any genetic investigation because the cross between a parthenogenetic female and a male of a bisexual strain or species is infertile.

The presence or absence of a large amount of repetitive DNA distributed in nearly all chromosomes suggests two models for its explanation. If it is assumed that the form lacking heterochromatic blocks is the primitive one; duplication and amplification processes working on its small amount of heterochromatin (the rare and thin bands) might have led to the condition found in *A. franciscana.* If, on the other hand, it is assumed that the primitive condition is *A. franciscana,* the postulated mechanism would be that of repeated deletions.

Obviously the speciation mechanisms must be applied to two bisexual forms, the parthenogenetic form being derived from a bisexual genotype.

Our research supplies no arguments in favor of one or the other mechanism. But we can quote the hypothesis formulated by Abreu-Grobbois and Beardmore (1982) that the American forms are derived from those living in Europe and Asia. This hypothesis is based also on geological grounds.

In any case the genus *Artemia* exhibits a rare example of speciation steps consisting mainly in changes of a repetitive DNA sequence. A final remark regards the lack of a primary constriction. This condition may support the view (Stefani 1965a, b) that the chromosomes of *Artemia* are devoid of a localized centromere.

References

Abreu-Grobbois FA, Beardmore A (1982) Genetic differentiation of the brine shrimp Artemia. In: Mechanisms of speciation. Liss, New York, 347–375

Barigozzi C (1974) Artemia: A survey of its significance in genetic problems. Evol Biol 7:221–252

Holmes DS, Quigley M (1981) A rapid boiling method for the preparation of bacterial plasmids. Anal Biochem 114:193–197

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory

Messing J, Crea R, Seeburg PH (1981) A system for shotgun DNA sequencing. Nucleic Acids Res 9:309–321

Radloff R, Bauer W, Vinograd (1967) A dye buoyant density method for the detection and isolation of closed circular duplex DNA: the closed circular DNA in HeLa cells. Proc Natl Acad Sci 57:1514–1518

Rigby PWJ, Dieckmann H, Rhodes C, Berg P (1977) Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J Mol Biol 113:237–251

Southern EM (1975) Detection of specific DNA sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–527

Stefani R (1963a) La digametia femminile in Artemia salina Leach e la constituzione del corredo cromosomico nei biotipi anfigonico e partenogenetico. Caryologia, 16:625–636

Stefani R (1963b) Il centromero non localizzato in *Artemia salina* Leach. Acc Naz Lincei Rend Cl Sci Fis Mat Nat 35:375–378

Taback HF, Flavell RA (1978) A method for the recovery of DNA from agarose gels. Nucleic Acids Res 5:2321–2332

Vieira J, Messing J (1982) The pUC plasmids, and M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19:259–268

Received April 26, 1984
Accepted by W. Beermann