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Immunofluorescence study of spindle microtubule arrangements during differential gonial mitosis of *Acricotopus lucidus* (Diptera, Chironomidae)

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Abstract. The changes in microtubule arrangements correlated with the behavior of the germ line limited and soma chromosomes were studied during the last unequal gonial mitosis, the so-called differential mitosis, of the chironomid *Acricotopus lucidus* by indirect immunofluorescence using a monoclonal anti-β-tubulin antibody, and by simultaneous staining with the DNA-specific fluorescence dye DAPI. An impressive difference in microtubule density between both half spindles was determined in metaphase and during the monopolar anaphasic migration of the germ line limited chromosomes. In the following normal separation of the soma chromosomes, a similar microtubule density in both half spindles occurred. In each of the half spindles, chromosome movement and spindle elongation occurred independently, and in one half spindle two anaphasic chromosome movements ran off one after another, the second without a simultaneous spindle elongation.

Key words: Antitubulin antibody/differential mitosis/germ line limited chromosomes/spindle microtubules

Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; EGTA, ethylene glycol-bis(β-aminoethyl ether)-tetraacetic acid; FITC, fluorescein isothiocyanate; Ks, germ line limited chromosomes (K= "Keimbahn"); MT, microtubule; PBS (-), calcium- and magnesium free phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid); Ss, soma chromosomes.

Introduction

In the dipteran *Acricotopus lucidus* (Chironomidae, Orthocladiinae) the last gonial mitosis proceeds in an unusual manner. During this so-called differential mitosis the germ line limited chromosomes (=Ks, K derived from "Keimbahn", 1) migrate undivided towards only one cell pole, while the soma-chromosomes (Ss) separate equally, but only after the arrival of the Ks at the pole.

Differential mitosis, which takes place in 4th larval stage, compensates for the elimination of about half of the Ks in the first gonial mitoses in newly hatched first instar larvae (=germ line elimination, 2). The cells with both the S- and the K-sets develop to meiotic cells, while the cells with only the Ss develop to aberrant spermatocytes in males and to nurse cells in females.

The aim of the present investigation was to study, by simultaneous fluorescent staining of microtubules (MTs) and chromosomes, the sequence of changes in spindle architecture correlated with the behavior of chromosomes during differential mitosis in *A. lucidus*.

Materials and methods

Animals

Animals of a laboratory stock of *Acricotopus lucidus* (Diptera, Chironomidae) were used. Rearing conditions and development of the larvae were described earlier (16).

Indirect immunofluorescence

Testes from young 4th instar larvae of *A. lucidus* were prepared in microtubule-stabilizing buffer (100 mM PIPES, pH6.8, l mM MgSO4, l mM EGTA) (17), and were transferred to a bored slide on which a coverslip had been mounted with paraffin on the bottom side (10). Subsequently the testes were minced with fine tweezers and the cells were centrifuged onto the coverslips in a special slide rotor (2000 rpm, 5 min, Labofuge GL, Heraeus Christ, Germany).

The cells were fixed in 4% formaldehyde (freshly prepared before use from paraformaldehyde) in PBS, pH 6.9, for 10 min, rinsed in PBS and treated with 1% Triton X-100 in PBS for 5-10 min. Subsequently the slides were rinsed again in PBS and were stored in 100% Methanol (-20°C) for about 1-2 h.

After three washes in PBS for 4 min each, the cells were covered with a monoclonal anti-β-tubulin antibody (Sigma, T4026, St. Louis, USA), diluted 1 : 50 in PBS, for 1 h at room temperature. Subsequently the cells were washed three times for 4 min with PBS and were then incubated for 45 min at room temperature with fluorescein isothiocyanate-(FITC)-conjugated goat anti-mouse IgGl (Serva, 80254, Heidelberg, Germany), diluted 1 : 50 in PBS. After three 4 min washes in PBS the preparations were stained in 1 µg/ml 4',6-diamidino-2- phenylindole (DAPI) in PBS for 30 s. They were then rinsed in PBS and the paraffin-mounted coverslips were separated from the slides. For fluorescence observations, the preparations were embedded in a 1 : 10 (v/v) mixture of PBS (pH 8.2): glycerol and were then stored at 5°C.

Fluorescence microscopy

A Zeiss-Standard microscope equipped with an epifluorescence illumination (mercury lamp, HBO50 W, Osram, Germany), with the filter combinations BP 450-490, FT 510, LP 520 (FITC) and BP 365, FT 395, LP 397 (DAPI), and with a Neofluar 100/1,3 objective were used for observation and photography. Microphotographs were made within 1-2 days on Kodak T-MAX 400 film processed in T-MAX developer to 3200 ASA.

Silverstaining

Testes were fixed in ethanol acetic acid (3 : 1, v/v) and squashed in 45% acetic acid. The coverslips were then removed after freezing on dry ice. Air-dried preparations were silver-stained using a modified method of Howell and Black (6) as described in (15).

Vital observations

Freshly explanted testes were transferred in a small drop of Firling medium (4) onto a slide and were covered with a coverslip. The weight of the coverslip induces a slight squashing of the gonial cells. Examination and photography were done in phase contrast using a photomicroscope II (Zeiss) equipped with a Planapo 63/1,4 objective.

Results

In *A. lucidus* during differential mitosis all Ks move undivided to only one cell pole, while the Ss still remain somatically paired in the equatorial plate. Only after arrival of the Ks at the pole, the Ss separate equally as in normal mitosis. That pole to which all Ks and the Ss move, is named the K/S-pole in the following text, while the opposite pole to which only the Ss move is named the S-pole.

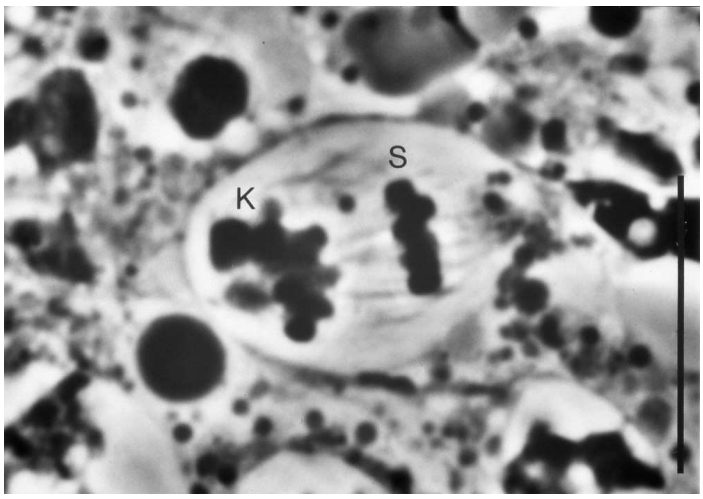

Fig. 1. Living-cell phase contrast micrograph of a male differential gonial mitosis of *Acricotopus lucidus*. All K-chromosomes (K) are moving as unseparated sister chromatids to the left spindle pole, while the S-chromosomes (S) still lie in the equatorial plate. Some spindle fibers extending from the poles to the Ss are visible. Bar represents 10 µm.

In the living cell micrograph of a male differential mitosis in Fig. 1, the Ks are in anaphasic migration just before reaching the K/S-pole (left). Spindle fibers are visible in the two half spindles between Ss and S-pole and in the interzone between Ss and Ks. The pole to pole distance is about 11 µm.

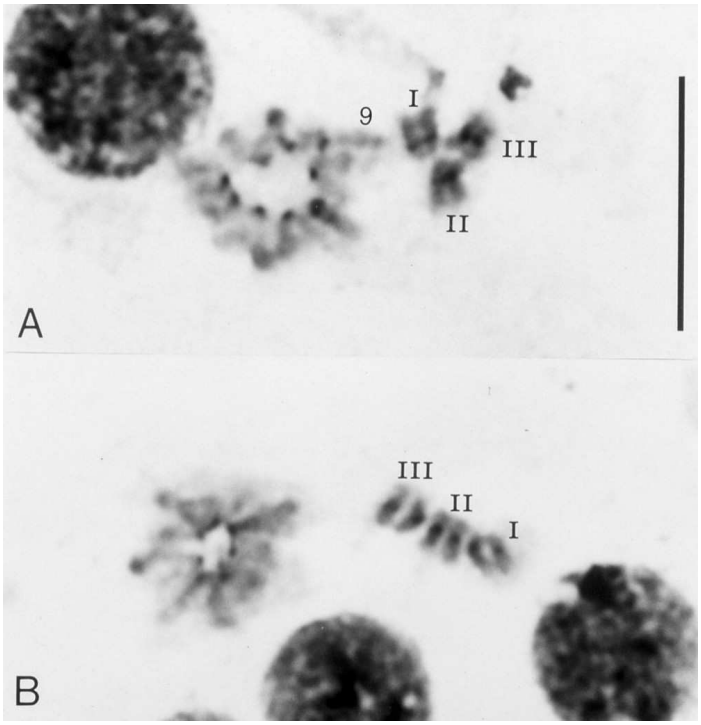

Fig. 2A, B. Silver staining of the centromeric heterochromatin of the Ss and the paracentromeric heterochromatin of the Ks on male differential mitoses of *Acricotopus lucidus*. The pole-migrating Ks show ring-like arrangements with pole orientation of the centromeres. The somatically paired Ss are grouped at the equatorial plates. In **A** the K chromosome K9 shows a delayed migration to the pole. I, II, III = S chromosome no. I, II, III. 9 = K-chromosome K9. Bar represents 10 µm.

In the differential mitoses in Figs. 2A, B the centromeric heterochromatin of the Ss and the paracentromeric heterochromatin-bands of the Ks are specifically stained with silver. The anaphasic Ks are arranged ring-like around the K/S-pole, and the centromeres and the surrounding silver-positive heterochromatin are clearly oriented polewards.

Anti-β-tubulin fluorescence and MT-distribution in spindles of a normal gonial mitosis and of differential gonial mitoses are presented in Fig. 3A and in Figs. 4AF. The corresponding DAPI-stained chromosomes are to be seen in Fig. 3B and in Figs. 4A-F'.

Figs. 3A, B show a lateral view of metaphase of a normal gonial mitosis, where Ss and Ks are grouped in the equatorial plate. The fluorescence distribution is equal in the two half spindles. The pole to equatorial plate distance is about 4 µm. In comparison with neighboring areas, tubulin fluorescence is strongly reduced in the equatorial zone. Only faint fluorescent fibers are to be seen between the chromosomes arranged in the equatorial plate.

In differential mitosis an increasing anti-β-tubulin fluorescence appears at the K/S-pole during late prophase and prometaphase (Fig. 4A, A'), indicating an increasing MT-density at this pole. Figs. 4B, B' show a lateral view of a differential mitosis metaphase. A conspicuous higher MT-

density is present in the K/S-half spindle as compared with the S-half spindle. With the pole-migration of the Ks (Figs. 4C, C), i.e., with the reduction of the kinetochore to pole distance (=anaphase A), an increase in the spindle length (=anaphase B) of the K/S-pole half-spindle occurs. The distance between the Ss and the S-pole remains unchanged during this process. From the equatorial plate to the K/S-pole the Ks migrate over a distance of about 6 µm (Fig. 4D, D'). At the time, when the Ks group around the K/S-pole, the relation of the lengths of the two half spindles is about 2:1.

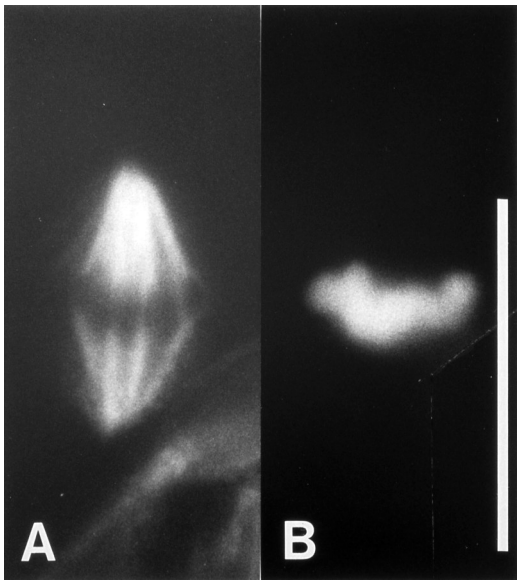

Fig. 3. Metaphase of a normal gonial mitosis of *Acricotopus lucidus*. **A**. Indirect FITC-immuno-fluorescent staining of microtubules with a monoclonal anti-β-tubulin antibody showing a symmetric spindle. **B**. Complementary presentation of Ss and Ks by DAPI-staining. Bar represents 10 µm.

After arrival of the Ks at the K/S-pole, the S-chromatids start to separate as in normal mitosis (Fig. 4E, E). During anaphasic migration of the Ks, a higher MTdensity is not only present between the K/S-pole and the moving Ks, but also in the interzone between the Ks and the equatorial plate.

In the course of the migration of the S-chromatids an elongation of the S-pole half spindle occurs (Fig. 4F, F') - to about the same length (about 6 µm) as the previous K/S-half spindle. The length of the latter remains unchanged during that process. In Fig. 4F' the migrating S-chromatids arrive at the opposite poles. The similar anti-tubulin fluorescence intensities between the equatorial plate and both poles in Fig. 4F indicate a similar distribution of MTs.

In Figs. 4A-F' - with the exception of K9, which in contrast to the other Ks frequently showed a delayed migration to the K/S pole or even a partial pairing with SI (arrow in Fig. 4D', see also Fig. 2A) - no individual chromosomes can be identified.

In Fig. 4F' the great difference in DAPI fluorescence at the opposite poles shows impressively the very different DNA content of the daughter cells resulting from a differential mitosis.

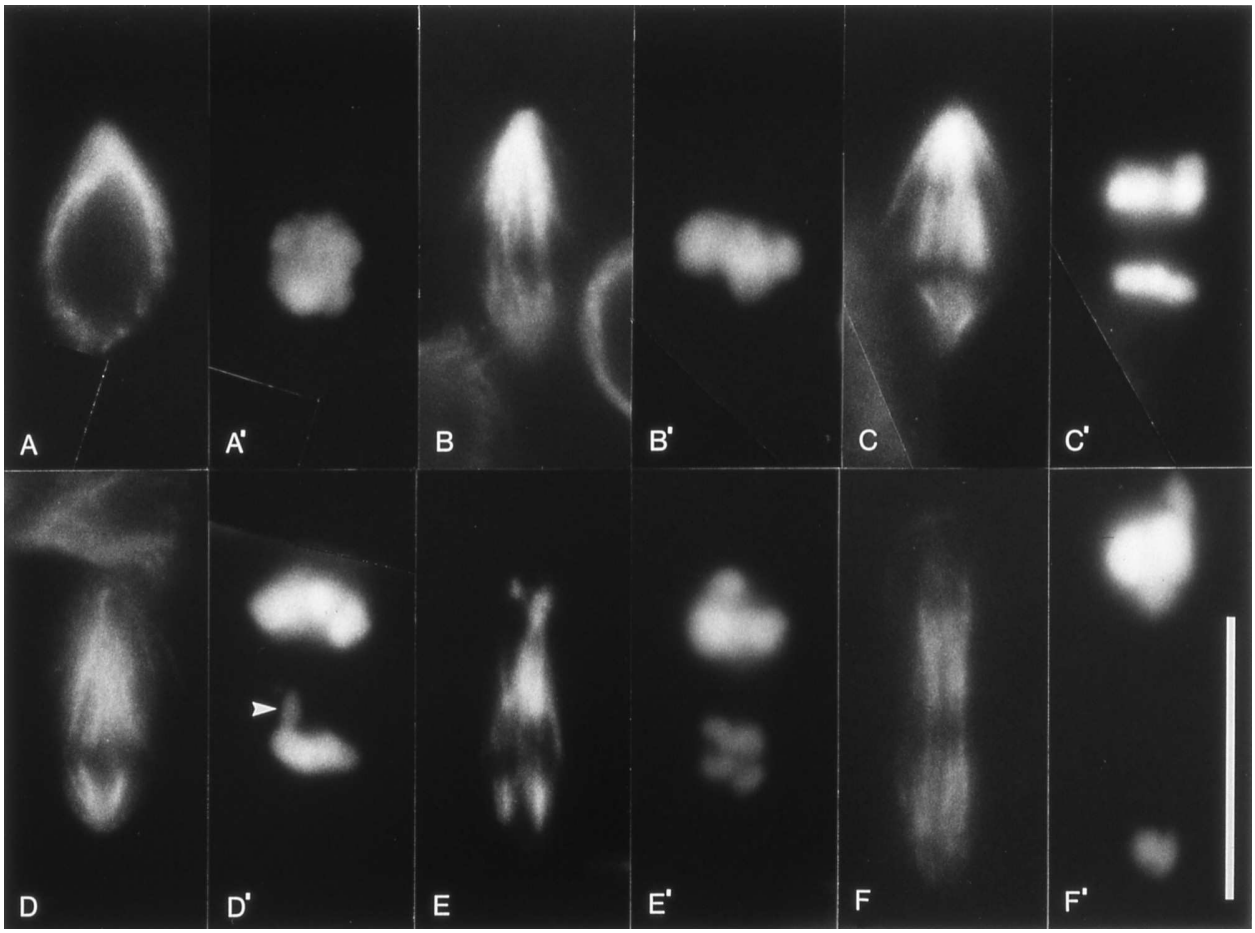

Fig. 4. Male differential (=last) gonial mitoses of *Acricotopus lucidus*. **A-F**. Anti-β-tubulin immunofluorescence. **A'-F'**. DAPI-staining of Ss and Ks. **A-D**. Formation of asymmetric spindles and **B'-D'**. migration of all Ks to the upper cell pole. **E'-F'**. Segregation of the sister chromatids of the Ss and migration to the opposite poles. The lower cells with the Ss only develop to aberrant spermatocytes and the upper cell with Ss and Ks enter in meiosis after the next replication cycle. The arrow in **F'** indicates K chromosome K9. Bar represents 10 µm.

Discussion

The simultaneous fluorescent staining of MTs with FITC and of chromosomes with DAPI, as seen in Figs. 4A, A' to 4F, F', enables study of the sequence of changes in spindle architecture in combination with the movement of chromosomes during cell-divisions.

The result of a differential mitosis is the appearance of two cells with very different DNA-content. In *A. lucidus* from 6 to 16 Ks can move as unseparated chromatids to the K/S-pole (Table 1 in 14). This can explain the exceptionally high MT-densities observed in the K/S half spindles as compared with the S-half spindles. The future meiotic cells (2S- and 2K-sets) have a multiple of the DNA-content as do the future aberrant spermatocytes (2S).

During migration of the Ks to the K/S-spindle pole (anaphase A) an elongation of the K/S-half spindle occurs (anaphase B), while the S-half spindle remains unchanged. Then, during the equal separation and the migration of the Ss (now in the S-half spindle) anaphase A and anaphase B occur, while in the K/S-half spindle only anaphase A takes place. These observations indicate

that, in both half spindles of the same cell, anaphase A and anaphase B can occur independently of each other, and that in the same half spindle two A anaphases can run off one after another, the second anaphase A without a simultaneous spindle elongation.

Forces developed by spindle microtubules, acting on both sister kinetochores and oriented to the opposite poles, are necessary to arrange and stabilize the Ss as also the Ks in the equatorial plate (12). Unknown is what triggers the release device and what is the mechanism of the movement of the Ks to only one pole.

Because all Ks exhibit two paracentromeric heterochromatin bands and the Ss only one centromeric heterochromatin band (15), it was supposed that the paracentromeric K-heterochromatin bands may serve for the recognition of the Ks as such, and/or may influence the interaction of the Ks with the spindle fibers during such processes as the germ line elimination or the soma elimination of the Ks, or as the monopolar migration of the Ks during differential mitosis.

Different mechanisms responsible for the monopolar movement of the Ks are possible. For example, the forces of the S-pole oriented MTs may fail by inactivation of the S-pole oriented kinetochore, or the S-pole oriented kinetochore may change to interact with MTs coming from the K/S-pole as on its sister kinetochore, or the balance of the forces may change by insertion of more MT on the K/S-pole oriented kinetochore than on the opposite one. Further studies are necessary to decide this, for example, the electron microscopic examination of sequential ultrathin sections of centromeric regions of Ks during anaphasic movement.

Chromosomes limited to the germ line were found in various groups of animals, such as in crustaceans-copepods (3), in insects-cecidomyids, chironomids and sciarids (2, 11, 18), and in vertebrates-Japanese hag fish species (7, 9). A further case of monopolar movement of germ line limited chromosomes is known from meiosis of Sciarids. In the first meiotic division of sciarid spermatogenesis a monopolar spindle occurs on which the maternal set of ordinary chromosomes and all germ line limited chromosomesare collected at the single pole and retained by the spermatocyte. The paternal set of ordinary chromosomes "moves backwards" from the pole, being excluded in a bud of cytoplasm and discarded (8, 11, for review 5).

In conclusion, the present results add to the findings of former investigations on chromosomal behavior of Ss and Ks (2), the facts that in differential mitoses, before and during the monopolar migration of the Ks, there is a very high MT density to the K/S-spindle pole, and a conspicuous difference in MT density between both half spindles, and that chromosome movement and spindle elongation occur independently in each of the half spindles.

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References

1. Bauer, H. 1970. Rearrangements between germ-line limited and somatic chromosomes in *Smittia parthenogenetica* (Chironomidae, Diptera). Chromosoma, **32**: 1-10.
2. Bauer, H. and Beermann, W. 1952. Der Chromosomencyclus der Orthocladiinen (Nematocera, Diptera). Z. Naturforsch., **7b**: 557-563.
3. Beermann, S. 1959. Chromatin-Diminution bei Copepoden. Chromosoma, **10**: 504-514.
4. Firling, C.E. and Kobilka, B.K. 1979. A medium for the maintenance of *Chironomus tentans* salivary glands in vitro. Insect Physiol., **25**: 93-103.
5. Gerbi, S.A. 1986. Unusual chromosome movements in sciarid flies. In Results and Problems in Cell Differentiation, vol. 13, Germ Line-Soma Differentiation (W. Hennig, ed.). Springer, Berlin Heidelberg New York Tokyo, pp. 71-104.
6. Howell, W.M. and Black, D.A. 1980. Controlled silverstaining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia, **36**: 1014-1015.
7. Kohno, S., Nakai, Y., Satoh, S., Yoshida, M., and Kobayashi, H. 1986. Chromosome elimination in the Japanes hag fish, *Eptatretus burgeri* (Agnatha, Cyclostomata). Cytogenet. Cell Genet., **41**: 209-214.
8. Kubai, D.F. 1982. Meiosis in Sciara coprophila: Structure of the spindle and chromosome behavior during the first meiotic division. J. Cell Biol., **93**: 655-669.
9. Kubota, S., Nakai, Y., Kuro-o, M., and Kohno, S. 1992. Germline-restricted supernumerary (B) chromosomes in *Eptatretus okinoseanus*. Cytogenet. Cell Genet., **60**: 224-228
10. MacGregor, H. and Varley, J. 1983. Working with animal chromosomes. John Wiley & Sons, p. 134.
11. Metz, C.W. 1938. Chromosome behavior, inheritance and sex determination in *Sciara*. Am. Nat., **72**: 485-520.
12. Nicklas, R.B. 1985. Mitosis in eukaryotic cells: an overview of chromosome distribution. In Aneuploidy. Etiology and Mechanisms (V.L. Dellarco, P.E. Voytek, and A. Hollaender, eds.). Plenum, New York, pp. 183-195.
13. Staiber, W. 1987. Unusual germ line limited chromosomes in *Acricotopus lucidus* (Diptera, Chironomidae). Genome, 29: 702-705.
14. Staiber, W. 1988. G-banding of germ line limited chromosomes in *Acricotopus lucidus* (Diptera, Chironomidae). Chromosoma, **97**: 231-234.
15. Staiber, W. 1991. Characterization of heterochromatin of germ line limited and soma chromosomes in *Acricotopus lucidus* (Diptera, Chironomidae) by differential banding methods. Hereditas, **114**: 91-96.
16. Staiber, W. and Behnke, E. 1985. Developmental puffing activity in the salivary gland and Malpighian tubule chromosomes of *Acricotopus lucidus* (Diptera, Chironomidae). Chromosoma, **93**: 1-16.

17. Wolf, K.W. and Bastmeyer, M. 1991. Cytology of Lepidoptera. V. The microtubule cytoskeleton in eupyrene spermatocytes of *Ephestia kuehniella* (Pyralidae), *Inachis io* (Nymphalidae), and *Orgyia antiqua* (Lymantriidae). Eur. J. Cell Biol., **55**: 225-237.
18. White, M.J.D. 1973. Animal cytology and evolution, 3rd ed. Cambridge Univ. Press, Cambridge. 961 pp.