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Macrobiotus pseudohufelandi Iharos as a model for cytotaxonomic study in populations of eutardigrades (Tardigrada)[1]

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Summary. The morphotype, chromosome number and Feulgen-DNA content of bisexual and unisexual populations of *Macrobiotus pseudohufelandi* were examined. Individuals of unisexual populations were triploid with ameiotic parthenogenesis. Their lowest Feulgen-DNA content is about three-fold that of sperm from a bisexual population. Egg shell shape also differs in the two types of population. However, the highest Feulgen-DNA content was the same (24 A.U.) in both diploid and triploid animals.
Key words. Cytotaxonomy; genome size; Tardigrada, *Macrobiotus*.

Cytotaxonomy allows the identification of tardigrades having similar appearance but different cytology (different chromosome number, different modality of female gametogenesis)[2-4]. The bulk of the data has been furnished by a study of oocytes, which represent good material for rapidly defining the ploidy and type of reproduction. In fact, the oocytes remain blocked at metaphase I until oviposition and are characterized by large, well-separated chromosomes[3,4]. In order to gain a deeper insight into the cytotaxonomical approach for the study of eutardigrades we considered *Macrobiotus pseudohufelandi* Iharos. In contrast to other species, clear identification is possible because of its small claw size and the presence of lunulae limited to the fourth pair of legs[5]. Moreover, this species can be readily found in coastal dune mosses. Both bisexual and unisexual populations of *M. pseudohufelandi* are known; the egg shell has a unique shape in the former[6]. Unisexual populations have 18 chromosomes in both mitoses and oocytes, that is, they are probably triploid[3,4]. The aim of the present paper was to compare information on presence or absence of males, type of reproduction, genome size, chromosome number, and taxonomy of this species.

Description of animal origin. Moss-living animals from various Italian coastal localities were used[6]. Karyotype data were obtained from two collections of a single bisexual population from Marina di Cecina (Tyrrhenian coast), and from single collections of unisexual populations from Marina Romea, Pineto (Adriatic coast), Bosco Pantano (Ionian coast), Vada, Riva degli Etruschi (three places), Tirrenia (Tyrrhenian coast), and Marina di Sorso (Sardinian coast).

Chromosome analysis. Lactic acetic orcein stain was used on whole specimens previously fixed in methanol and acetic acid (3:1). Observations were made as described below. Chromosome length was evaluated by a Leitz filar micrometer.

Genome size evaluation. Squash preparations were made of four animals from the bisexual population (Marina di Cecina) and of four from a unisexual population (Riva degli Etruschi). Specimens were fixed in formaldehyde (10%, 20 min) and the Feulgen reaction implemented as suggested by Itikawa and Ogura[7] (with hydrolysis in 5N HCl for 1 h at 23 °C and treatment with Schiff reagent for 45 min at 23 °C). To minimize variability caused by fixation and staining, all slides were processed in a single Feulgen bath. The Feulgen-DNA content was evaluated in as many nuclei as possible regardless of tissue type. Feulgen-DNA content was measured at a wavelength of 545 ± 5 nm with a Vickers M85 scanning microdensitometer directly interfaced to a P6060 Olivetti microcomputer. Readings were taken under the following instrument conditions: 10 × eyepiece; 100 × objective lens; 1.25 numerical aperture; dry condenser; 0.4 µm final diameter of the flying spot. Photometric errors due to glare and non-specific light loss were evaluated as suggested by Bedi and Goldstein[8]. Since error proved to be constant and negligible (< 3%), no instrument correction was introduced. At least 50 nuclei were

Geographic locations	Number of specimens	Chromosome number Mitosis	Oocytes	Type of reproduction
Marina di Cecina	18	—	—	
	34	12	6	Amphimixis
Marina Romea	72	18	—	—
Pineto	65	18	18	Ameiotic parthenog.
Bosco Pantano	78	18	—	—
Riva degli Etruschi I	56	18	18	Ameiotic parthenog.
Riva degli Etruschi II	66	18	18	Ameiotic parthenog.
Riva degli Etruschi III	57	18	18	Ameiotic parthenog.
Vada	15	18	18	Ameiotic parthenog.
Tirrenia	46	18	18	Ameiotic parthenog.
Marina di Sorso	22	18	18	Ameiotic parthenog.

considered for each slide (that is, per animal) considering both sperm cells and spermatids, when they were present, and any other nuclei suitable for measurement. The values obtained in arbitrary units (a.u.) were plotted as frequency distribution histograms.

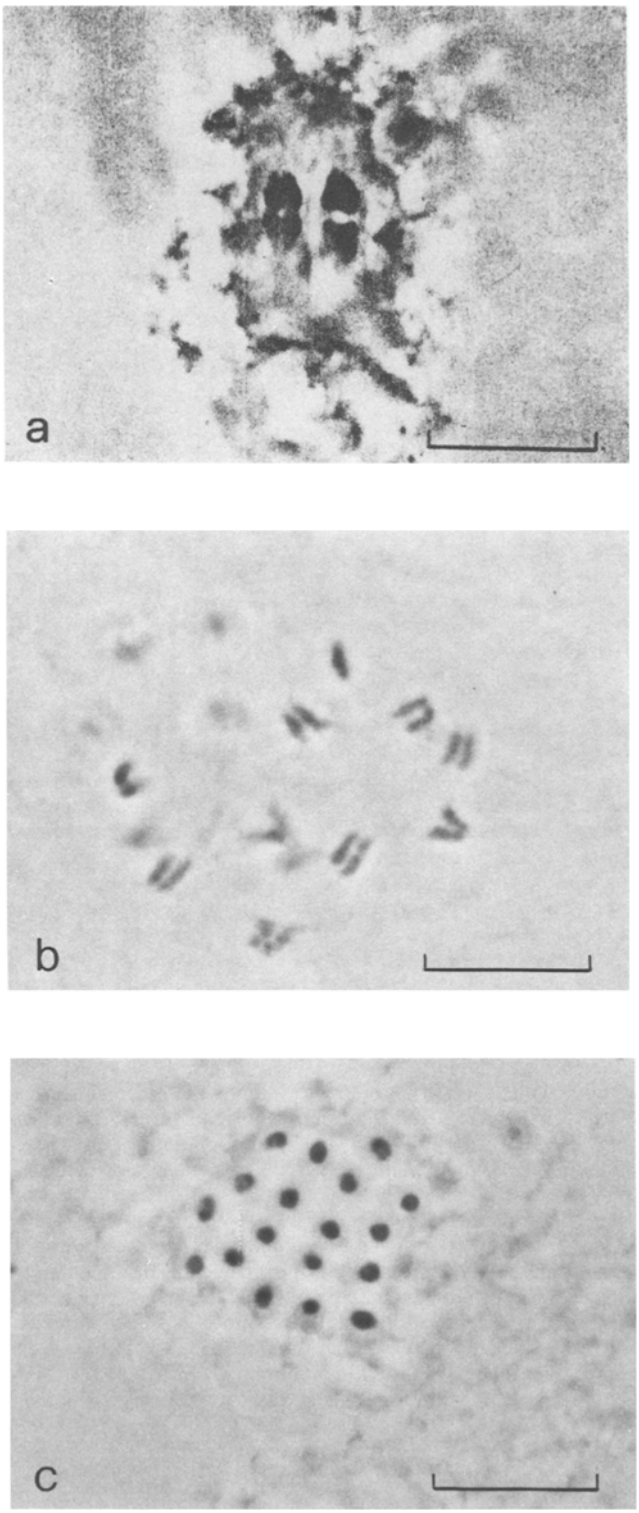

Figure 1. Orcein-stained chromosomes of *M. pseudohufelandi* (phase contrast). *a* Bivalents in a lateral view of a diploid oocyte spindle. *b* Partial view of a triploid oocyte prophase with univalents. *c* Polar view of triploid oocyte metaphase. Bar corresponds to 10 µm.

Analysis of sclerified structures. Animals were mounted in polyvinyl lactophenol for taxonomic analysis by light microscopy. Eggs were mounted in the same mounting medium or in Faure-Berlese fluid. Observations were done at maximum magnification (100 × oil-immersion objective lens) with phase contrast and Nomarski phase interference using a Leitz Dialux 20 microscope. For SEM observations, animals and eggs were fixed in boiling ethanol, washed in freon (1,1,2- trichloro trifluoro methane), transferred to a critical point dryer for dehydration and then coated with Au-Pd. A Philips SEM 500 was employed.

Results and discussion. The table reports the data on chromosome number and type of reproduction for the various populations. Males were found in the Marina di Cecina population. The females of that population always had a seminal vesicle full of mature sperm in both collections (May and August). Unisexual populations have 18 chromosomes at mitosis and 18 univalents (fig. 1c) in the oocytes. The bisexual population has 12 chromosomes at mitosis and 6 bivalents in the oocytes (fig. 1a). In both cases mitotic chromosomes appear similar (oval shape, maximum length 1 µm). Instead, individual chromatids of univalents and bivalents have a length of 1.2 to 1.5 µm, more than mitotic chromosomes. In the Marina Romea and Bosco Pantano populations we failed to carry out the oocyte chromosomes analysis. The 18 univalents never show synapsis in prophase or metaphase (fig. 1b, c). Prophase is characterized by a gradual coiling of chromosomes. Thus it is not attributable to the second meiotic division, which moreover, as also stated

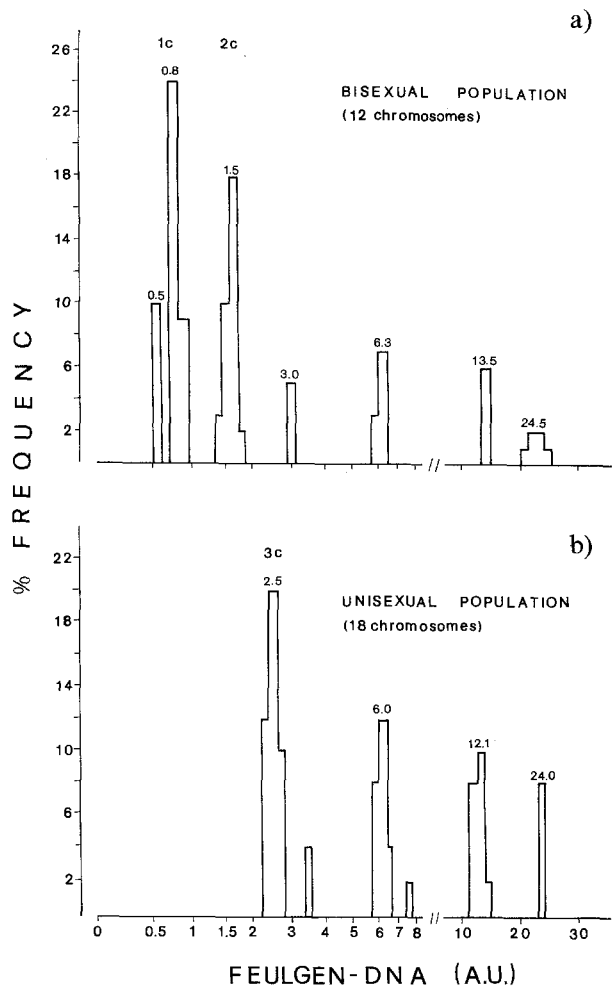

Figure 2. Percent frequency distribution of Feulgen-DNA values (cytophotometrically determined) for a bisexual *(a)* and unisexual *(b)* population of *M. pseudohufelandi*.

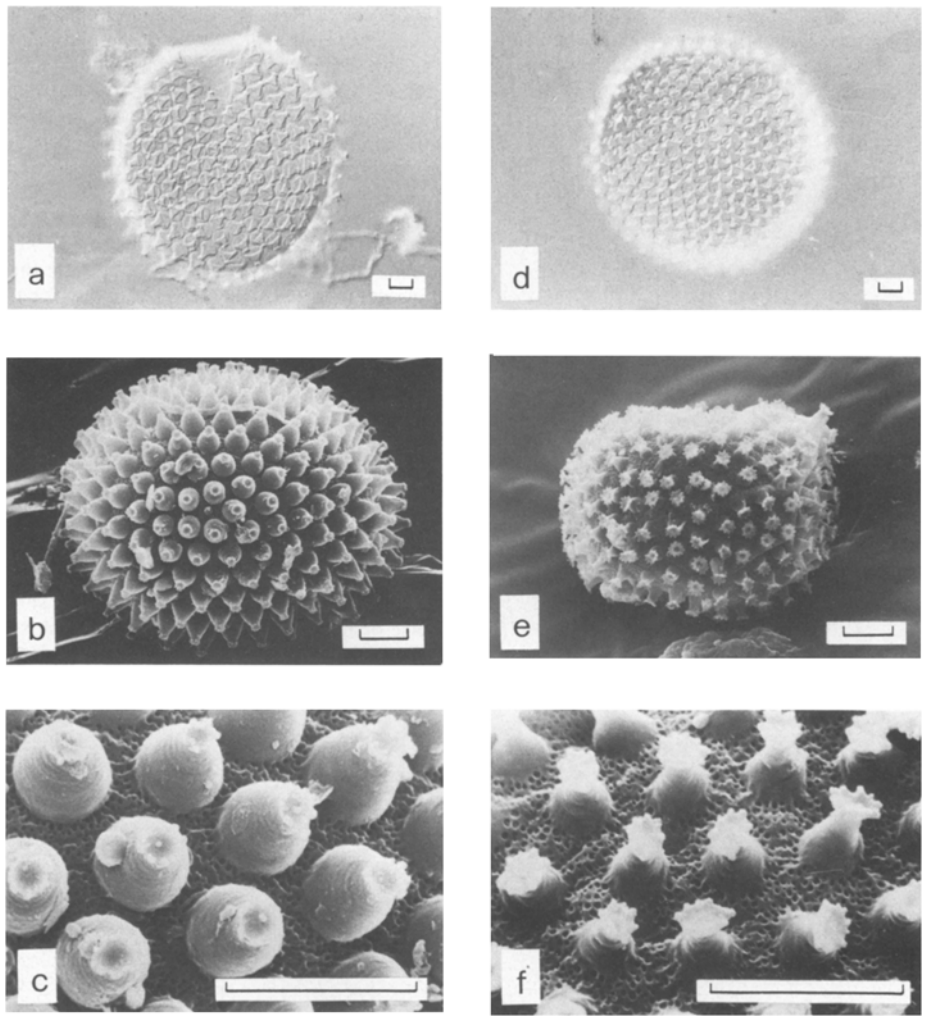

Figure 3. Egg shell shape in *M. pseudohufelandi*. *a* Egg of bisexual population (Nomarski interference contrast microscopy). *b* SEM appearance of the same egg as in *a*. *c* Detail of processes of egg from bisexual population. *d* Egg of unisexual population (Nomarski); note the smaller and more numerous egg processes. *e* SEM appearance of the same type of egg as in *d*. *f* Detail of egg processes (SEM) from unisexual population. Bar corresponds to 10 µm.

above, does not occur until after oviposition. Figures 2a and 2b show the histogram distribution of the nuclear Feulgen-DNA content determined cytophotometrically in the two populations. In the bisexual population the distribution ranges from a minimum Feulgen-DNA content of 0.5 and 0.8 a.u. (referrable to sperm values) and reaches a maximum of 24.5 a.u., corresponding to 8.7 pg, on the basis of a calibration curve that considers both chicken sperm and erythrocytes (1.36 and 2.52 pg) and mouse sperm (3.4 pg). The other Feulgen-DNA values have a lower frequency. This is probably due to the preparation of the samples by the squash method: cells with larger nuclei probably remain in the areas of the preparations which cannot be measured. The highest DNA-Feulgen content is about 24 a.u. in both populations. Haploid values range from 0.5 to 0.8 a.u. In the triploid population the lowest DNA contents can be attributed to the 3c class (as seen for the values of the 1c and 2c class of the diploid population). The absence of values attributable to classes below 3c is correlated with the type of parthenogenetic reproduction (ameiotic).

Figure 3 shows that the eggs from a bisexual population have fewer shell processes which are more developed in height and have wider bases. In contrast, light microscope and SEM studies failed to show differences in the sclerified animal parts.

Within the *M. pseudohufelandi* populations, Feulgen-DNA contents are strictly correlated with the triploid and diploid state. The minimum DNA content changes between the two cytotypes in strict correlation with the variation of the chromosome number from 0.8 a.u., pertaining to the haploid set, to 2.5 a.u., referring to a triploid set of organisms which do not pair their chromosomes and do not reduce the number. In other words this means that the mean chromosome DNA content is maintained between the two cytotypes. Within the different value classes found in the two population types the highest class of Feulgen-DNA content reaches the same value (24.5 a.u.). The existence of a maximum value not exceeded even with diversified mechanisms of reproduction suggests that in tardigrades the maximum DNA content may constitute a critical value having a regulative significance. We noted that haploid values of the same cell population range from 0.5 to 0.8 a.u. This range is probably due to different Feulgen reactivity of spermatids at various developmental stages of cytodifferentiation. A similar pattern has been reported for the development of the male germ cell in other animal groups[9,10].

Cytological analysis, as already described, demonstrates the existence of ameiotic parthenogenesis in *M. pseudohufelandi* based on the absence of synapsis in the oocyte prophase. This type of egg maturation is the same as that of many other eutardigrades[3,4]. In this species animals belonging to the two different cytotypes (diploid and triploid) do not present morphological differences of the sclerified parts, whereas differences exist in the

appearance of the egg shell. This finding can be used to recognize populations easily, without having recourse to more specific cytological analyses. The two populations of *M. pseudohufelandi*, however, have very similar sclerified structures, as is found in all the other known eutardigrades, and mean chromosome DNA content. These similarities suggest that the present parthenogenetic populations (in which the genetic flow is interrupted) must have differentiated from the bisexual strain in a not very remote period.

Within the animal model studies here, a strict correlation appears between different types of data: mechanisms of reproduction, presence or absence of males, number of chromosomes, and shape of the egg shell.

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Residual influences on fecundity in drosophilid species

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Summary. A residual influence of males and females on oviposition has been examined in 7 drosophilids. There was evidence for oviposition deterrence in *Drosophila funebris*, with males as well as females producing the inhibitory effect. In contrast, male residues stimulated oviposition in *Zaprionus tuberculatus*. Male residues also stimulated oviposition and appeared to serve as an aggregation cue in *D. melanogaster*.
Key words. Drosophila; reproductive behavior; oviposition; pheromones.

There is evidence for a non-contact, residual influence on fecundity in *Drosophila*. Mainardi[1,2] reported that fly medium previously exposed to *Drosophila melanogaster* males received more eggs than unexposed medium. In a recent study[3] we suggested that Mainardi's results may have been due to the transmission of microbes onto medium. The growth of microorganisms on food could attract inseminated females and stimulate oviposition. However, we found evidence for a male effect on fecundity in *D. melanogaster* by confining males on non-nutritive agar. Residues from these males increased oviposition on an adjoining medium surface. These experiments were conducted without live yeast on the surface of the agar or medium and consequently fecundity was low.

The goal of the present study is to explore non-contact effects of residues on fecundity in other drosophilids. We have conducted a survey of male and female residual effects in species of *Drosophila* and one species of a related genus, *Zaprionus*. Within the constraints of a limited study it was not feasible to examine many species or multiple lines from a species. Tests were conducted on several species closely related to the previously studied *Drosophila melanogaster*[3], and several more distantly related taxa. We have also retested *Drosophila melanogaster* for residual male influence when yeast is added to the agar surface, a treatment which results in more eggs laid than in a previous experiment[3].

Materials and methods. The experiments employed the testing method of Hoffmann and Harshman[3]. Briefly, the open end of a 30-ml glass scintillation bottle was inserted into a 40-ml glass vial. The bottle contained 10 ml of 1% agar and the vial had 12 ml of fly medium (6.2% cornmeal, 3.1% semolina, 3.6% sucrose, 7.1% dextrose, 1.1% agar, 1.5% dead yeast, 0.5% propionic acid). In order to provide a smooth surface for oviposition, 2 ml of molten medium was added to an initial aliquot of 10 ml of medium in each vial. The scintillation bottles were used to collect adult residues. Twenty males or females were held in a bottle for 24 h before discarding the flies. A new set of flies was used to expose bottles for the next day. Unexposed bottles served as controls. Three inseminated females of the same species were aspirated into the assembled vials and bottles.

Species survey. There are inherent difficulties in using a variety of species with different rates of sexual maturation. In order to standardize the experiments and ensure that females were inseminated the following procedure was employed. Individuals that had emerged within the previous 16 h were placed, 50 of each sex, in a bottle with live yeast on the surface of the medium. When larval activity was observed the males were removed using CO_2 anesthesia and the females used in the testing devices. They were placed horizontally on shaded shelves with a light intensity of approximately 30 lx and a dark period of 7–10 h. Experiments were conducted at room temperature (22–24 °C). After 24 h females were transferred to a fresh bottle-vial and the number of eggs in vials from the previous day were counted. Typically, few or no eggs were laid in bottles on the agar surface. The experiments were run for 7 days which is an arbitrary period with respect to variation in reproductive characteristics of the species tested. Nevertheless, these experiments consistently evaluate the early phase of reproduction of each species.

Table 1 shows the drosophilids employed, where they were collected and approximately how long they have been in laboratory culture. All flies were maintained at Davis by mass transfer for at least 6 months before being tested. Residues were obtained from males and females that had been separated 2–7 days earlier under CO_2 anesthesia. For each species there were 20 replicates per treatment. Repeats with 30 replicates were carried out for those species which had statistically significant results in the first experiments. The data were analyzed by single classification analysis of variance on the total number of eggs, the number laid from days 1–3 and days 4–7, and the time taken for half the eggs to be deposited ($T_{½}$).

Added yeast. This part of the study used the same stock of *D. melanogaster* previously employed[3], 2½ years after it was initiated from the field. Male residues were introduced to the bottles with agar as previously described[3]. After males were removed one or two grains of live yeast were added to control bottles and bottles with male residues. The yeast granules rapidly absorb moisture, becoming soft and swollen. As a result, females oviposit in the yeast and on the agar as well as in the vial with fly medium. Fly transfers and egg counts were made every