sperm entry into the egg[3,14]. The reason why such a variety of physiological roles of acrosin have been proposed may be explained by the fact that trypsin inhibitors which are not so specific for acrosin have been used in the studies of mammalian fertilization. In this connection, it is noteworthy that we used the potent cognate inhibitors specific for acrosin, spermosin, and the chymotrypsin-like enzyme of spermatozoa in the present study. By employing three specific inhibitors, we demonstrated for the first time the timing of action of sperm proteases in fertilization in the ascidian. Further studies on morphological events that occur within 4 min after insemination, and on the subcellular localization of the proteases in the spermatozoon passing through the vitelline coat, will be necessary to establish the definite timing of action and the precise role of sperm proteases in fertilization.

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Feulgen-DNA amounts and karyotype lengths of three planarian species of the genus *Dugesia*

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Summary. Genome sizes of the planarians *D. lugubris* (2n = 8), *D. polychroa* (2n = 8) and *D. benazzii* (2n = 16) were evaluated on metaphase plates by measuring both the Feulgen-DNA contents and the karyotype lengths. In the three species, genome sizes are significantly different; this finding rules out the possibility of a karyotype evolution through simple chromosome rearrangements between *D. lugubris* and *D. polychroa*. A different Feulgen-DNA content per unit length of karyotype in the three species studied was also found, which suggests that DNA could be differently packed along metaphase chromosomes.
Key words. Cytotaxonomy; genome size; karyotype length; nuclear DNA amount; planarians.

In the planarian genus *Dugesia*, the '*D. lugubris-polychroa* group' and *D. gonocephala* s.l. are made up of various sibling species, which are reproductively isolated within each group, although they show few morphological differences. The karyological studies and genetic experiments involving breeding, carried out by Benazzi and coworkers over many years, have made it possible to clarify some karyotype relationships among the species belonging to the two groups and to put forward some interesting evolutionary hypotheses (for a review see Benazzi[1,2]).
In order to gain a deeper insight into the karyotype differences in the two groups of the genus, we evaluated the genome sizes of some species. In fact, genome size, as a species specific constant, is a very useful cytological parameter in connection with evolutionary processes[3].
Measurements of genome size have been obtained for several animal groups by means of the microdensitometric and/or microfluorometric evaluation of the DNA content on either interphase nuclei or metaphase plates after cytochemical reactions specific for DNA[4-6]. By means of this methodological approach, we recently demonstrated the genome size constancy of E and F biotypes of *D. lugubris*[7], and supported the hypothesis, for these biotypes, of karyotype evolution through a Robertsonian mechanism, as suggested by Benazzi and Puccinelli[8].
Estimates of genome size were also obtained by linear measurement of the whole karyotype length on metaphase chromosomes[9].

In the present paper, the possible genome size differences among *D. polychroa* (A biotype), *D. lugubris* (E biotype), and *D. benazzii* (diploid biotype) were studied; both the microdensitometric and karyometric methods were applied for the analysis of metaphase plates from regenerative blastemas.
Karyotype characteristics of the different biotypes in the '*D. lugubris-polychroa* group' and in *D. gonocephala* s.l. The European *D. lugubris-polychroa* group comprises four sibling species corresponding to the A–D, E, F, G biotypes established by Benazzi[10]. The A–D biotypes make up an autopolyploid series starting with the A biotype (2n = 8; n = 4); they can interbreed, and correspond to the species *D. polychroa* (O. Schmidt). The E biotype is diploid (2n = 8; n = 4), and the F biotype is again diploid but its chromosome number is 2n = 6, n = 3; the latter should derive from the former mainly through a Robertsonian mechanism[7,8]. Both biotypes were tentatively assigned to *D. lugubris* (O. Schmidt), although Benazzi[2], on the basis of some morphological differences of the copulatory apparatus, and the fact that the two biotypes are genetically isolated, has proposed for the F biotype the specific name *D. nova*. The G biotype is diploid, too, with chromosome number 2n = 8, n = 4; it corresponds to *D. mediterranea*[11].
In *D. gonocephala* s.l., which comprises various species or microspecies widely distributed through the Old World[12], the original karyotype should be the one of *D. gonocephala* s.s. of Central Europe (2n = 16, n = 8). From this karyotype, polysomic and

polyploid complements have originated; moreover, variations in the basic set have taken place, with n = 7 or 9. In Italy the following species of the *gonocephala* group have been recognized: 1) *D. etrusca* Benazzi, with three races, *monoadenodactila* (constantly diploid), and *biadenodactila* and *labronica* (frequently polysomic); 2) *D. ilvana* Benazzi; 3) *D. sicula* Lepori, with n = 9; 4) *D. benazzii* Lepori, with a diploid biotype and two polyploid biotypes; 5) *D. hepta* Pala et al. with n = 7; 6) *D. brigantii* De Vries and Benazzi with chromosome number about 24 (karyological studies are in progress).

Materials and methods. The evaluations in situ of genome size were carried out on metaphase plates from regenerative blastemas of several individuals of the species *D. polychroa* (A biotype), *D. lugubris* (E biotype) and *D. benazzii* (diploid biotype). All the animals used in the present study had been kept for some months in the laboratory.

The planarians were cross-sectioned in their middle zone; after 4 days' regeneration, the portions containing the blastemas were removed and transferred to 0.3% colchicine (Merck) for 3 h; blastemas were then hypotonized in distilled water for 10 min at 37 °C, then fixed at 4 °C with 3:1 methanol:acetic acid for 15 min, and finally disaggregated in 45% aqueous acetic acid by means of a Pasteur pipette. The suspension of cells and metaphases was repeatedly allowed to evaporate on slides kept at 50 °C on a slide warmer. Further details of the preparation method have been reported in a previous paper[13].

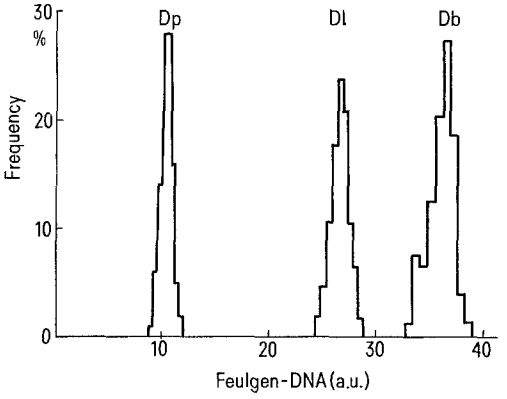
All the specimens of the three planarian species were simultaneously processed, and particular care was taken to standardize strictly the times and temperatures of all the preparation steps, starting with the colchicine treatment, so as to avoid as much as possible differences in chromosome condensation due to the length of colchicine treatment and to the fixation procedure.

The amount Feulgen-DNA in pg in *D. lugubris* has already been determined to be 1.75 pg by calibration of its Feulgen-DNA amount against the Feulgen-DNA amounts of several animal species of which the absolute DNA content is known[7]; in the present paper *D. lugubris* has been used as the reference species for evaluating the DNA amount in pg of *D. polychroa* and *D. benazzii*.

The technical details for carrying out the Feulgen reaction were as follows[14]: 60 min hydrolysis with 5 N HCl at 23 °C; staining with Schiff's reagent for 45 min; 30 min differentiation in sulphurous water (10 ml of 10% sodium bisulfite; 10 ml of 1 N HCl; 200 ml of distilled water); 30 min washing in running tap water; dehydration and mounting in Eukitt (Vitromed).

Assessment of the quantity of Feulgen-positive material was carried out with a Vickers M85 scanning microdensitometer at a wavelength of 545±5 nm. This instrument was used under the following conditions: 100 × oil immersion objective (N.A. 1.25); 10 × eyepiece; dry condenser; area of the measuring spot projection 0.4 µm; 1 × 1 scanning frame. Photometric errors due to glare and to nonspecific light loss were evaluated as suggested by Bedi and Goldstein[15]; since they proved to be steady and negligible (< 3%), no instrument adjustment was introduced. The Feulgen-DNA amout of the least 100 metaphasic plates was recorded for each planarian species.

Morphometric measurements of the total genome length were performed on photomicrographs taken from the same slides used for microdensitometry. Single chromosome (and chromosome arm) lengths were recorded by means of a graphic tablet connected with an Apple II microcomputer; data were stored and successively retrieved by using a computer programme (written by D. Formenti) which finally gives the total length of the karyotype, the lengths of single chromosomes and of chromosome arms (expressed both in µm and as the percent of the total chromosome set), and the centromeric index. 15–30 metaphases were measured for each species studied.



Distribution histograms of Feulgen-DNA contents in the three species of planarians studied; Dp = *D. polychroa*; Dl = *D. lugubris*; Db = *D. benazzii*.

Results. The distribution histograms of the Feulgen-DNA contents for the three species examined are reported in the figure. The corresponding mean values (table) are significantly different, the trend of genome sizes being, from the smallest to the largest, *D. polychroa*, *D. lugubris* and *D. benazzii*. It is rather surprising that there is a difference between *D. lugubris* a *D. polychroa* (both with 2n = 8) which are thought to be closely related[2,10]: in fact the former has about twice as much Feulgen-DNA as the latter.

The same trend of genome size variability was observed after morphometric analysis (table); however, the relative ratios are very different, the karyotype of *D. lugubris* being only 20% longer than that of *D. polychroa*. The mean ratios between Feulgen-DNA amount and karyotype length showed noticeable differences of DNA quantity per unit length of the chromosomes; this observation points out that a different degree of DNA supercoiling is present in the chromosomes of the three species, even under our standardized conditions of metaphase preparation.

Discussion. The three species of the planarian genus *Dugesia* we have studied here exhibit great differences after both microdensitometric and morphometric analysis of the genome size on Feulgen-stained metaphase plates. *D. benazzii* showed the greatest genome size, and this finding was to be expected, taking into account the chromosome number (2n = 16 instead of 2n = 8 in the other species). On the other hand, the significant difference of DNA-content between *D. lugubris* and *D. polychroa* suggests that the karyotypes of these species cannot be closely related, and surely rules out the hypothesis that one may derive from the other through simple chromosome rearrangements. If a common origin of the two species is to be maintained, a significant variation of DNA-amount must be hypothesized, during the

Mean values (± SD) of the Feulgen-DNA amount (both in arbitrary units and pg) and the karyotype length (in µm), and quantity of Feulgen-DNA per karyotype unit length for the three species studied

Species	Feulgen-DNA content		Karyotype length (µm)	Feulgen-DNA (AU)
	Arbitrary units	pg		Karyotype length (µm)
D. polychroa	10.40 ± 0.53	0.70	27.06 ± 2.28	0.38
D. lugubris	26.72 ± 0.90	1.75	32.96 ± 3.20	0.81
D. benazzii	36.04 ± 1.16	2.36	69.70 ± 6.43	0.51

All the differences among species are statistically significant ($p < 0.01$) after both microdensitometry and karyometry.

speciation process, to account for the present quantitative differences.

To explain the significantly different Feulgen-DNA contents per unit length of chromosome which we observed in the three species of planarians, it may be hypothesized either 1) that metaphase condensation and coiling of DNA is actually different in the species examined; or 2) that there are differences of the M-phase length during the cell cycle, so that colchicine treatment always for the same length of time (as we carefully used) induced a different chromosome condensation at the moment of slide preparation. In the present state of the research, neither hypothesis can be conclusively ruled out; however, both explanations point to the existence of species-specific cytologic peculiarities within the genus *Dugesia*, besides the genome size differences observed.

In a previous paper[7], a fairly good correspondence was found between karyometric and microdensitometric values for the relative sizes of single chromosomes of the E and F biotypes of *D. lugubris*; however, some discrepancies between the results obtained with the two methods were noticed for the smallest elements of both karyotypes, so that the possibility was underlined that, in these chromosomes, DNA could be differently packed. That evidence and the findings of this paper show that no direct correlation may exist between Feulgen-DNA amount and linear karyotype length, and suggest that particular attention must be paid when comparing genome size estimates obtained by these two methods.

From a methodological standpoint, once more it is apparent that for the purpose of genome size determination the cytochemical approach is definitely preferable, both because it is more directly related to the actual amount of genetic material (which can be measured also on interphase nuclei) and because it is much less affected than the karyometric methods by the degree of chromatin condensation (or DNA coiling).

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Regression of genetically determined polycystic kidney disease in murine organ culture

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Summary. Cystic kidneys from the mutant CPK strain of C57BL/6J mice were cultured in serum-free organ culture. During 120 h of incubation in chemically-defined medium, CPK cystic tubular changes underwent complete regression. Environmental factors regulate the expression of genetically determined polycystic kidney disease in this model.
Key words. Polycystic kidney disease; organ culture.

Genetically determined polycystic kidney diseases (PKD) cause significant morbidity and mortality in both children and adults[1,2]. Despite the clinical importance of such disease states, the basic pathogenetic mechanisms operative in human renal cyst formation have not been clearly identified. Further, the factors responsible for the wide variation in the clinical expression of PKD remain unknown. Recent studies of toxin-induced renal cystic disease in rats have demonstrated that renal cyst formation may be totally reversible under certain experimental conditions, and that environmental factors may modulate the progression of cystic tubular changes[3,4]. Such data raise the possibility that the expression of genetically determined renal cyst formation may be regulated by the biochemical and microbial environment in which such kidneys develop. We therefore studied the development of autosomal recessive polycystic disease in kidneys from the mutant. CPK strain of C57BL/6J mice[5,6] under the highly controlled conditions of our previously described serum-free metanephric organ culture system[7,8]. The organ culture system permits organotypic growth and differentiation of renal tissue in chemically defined medium without perfusion, filtration, or urine formation. It was thus possible to experimentally isolate the process of genetically determined renal cystic maldevelopment from flow-related phenomena or the presence of cyst-promoting substances in CPK serum or urine.

Materials and methods. Our basic method of intact metanephric organ culture has been described in detail[7,8]. In the current study, newborns from matings of control C57BL/6J mice as well as newborns from matings of heterozygotes for the CPK trait were sacrificed by decapitation. Paired kidneys of each newborn were aseptically removed and one was placed in organ culture medium at 4 °C while the other was processed for light microscopy. The organ culture medium consisted of equal volumes of Dulbecco's modified essential medium and Ham's F-12 medium supplemented with selenium, 6.8×10^{-9} M; insulin, 8.3×10^{-7} M; triiodothyronine, 2×10^{-9} M; transferrin, 6.2×10^{-8} M; and prostaglandin E_1, 7.1×10^{-8} M. For organ culture, the kidney was cut into explants of 100–120 µm thickness utilizing a custom designed microslicer. Explants were trimmed and transferred onto a 0.8-µm Millipore filter sitting atop a Trowell-type double-welled organ culture assembly. The assemblies were incubated at 36 ± 0.5 °C and 95% humidity in the mixed air-5% CO_2 environment of a water-jacketed incubator. Culture medium was replenished every 24 h, and tissue was sampled daily for histological analysis and viability measurements as previously described[7,8].

For each kidney explanted into organ culture, its paired mate was processed for light microscopy and served as histological control. This assured clear identification of cystic kidneys from