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POLYPLOIDY IN PROSERIATA (PLATYHELMINTHES) AND ITS PHYLOGENETICAL IMPLICATIONS

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Considerable progress has been made in the last few years in elucidating the phylogenetic relationships between the Platyhelminthes and the other Bilateria (Ax, 1985; Rieger, 1985, 1986; Smith and Tyler, 1985; Smith et al., 1986) and among the major platyhelminth taxa (Karling, 1974; Ehlers, 1985, 1986*a*, 1986*b*). Most hypotheses on phylogeny have been based on morphological characteristics at the light-microscopic level, on developmental processes, and in particular, on ultrastructural data, which have increased tremendously in the last 15 years (see review by Rieger [1981] and the reference list by Tyler et al. [1986]).

Within the Platyhelminthes, the Tricladida and Polycladida have long been favorite subjects of karyologists (see Benazzi and Benazzi-Lentati, 1976; Galleni and Puccinelli, 1986). However, the use of karyological data has been generally limited to the elucidation of patterns of karyotypic evolution among closely related species or among various populations of the same species. Tricladida and Polycladida are mostly large free-living Platyhelminthes with high regeneration capacities; karyological studies on other Platyhelminthes (microturbellaria) are rather scanty or merely occasional observations (Ruebush, 1938; Reuter, 1961; Benazzi and Benazzi-Lentati, 1976; Heitkamp, 1978; L'Hardy, 1986).

Two of us (M.C.C.-G. and P.M.M.) are presently studying the karyotypes of Proseriata (sister taxon of the Tricladida + Bothrioplana [see Sopott-Ehlers, 1985]). Proseriata have a cosmopolitan distribution, with representatives in various marine habitats and brackish water and with a few freshwater species. In some cases, they can play a major role in benthic communities (Martens and Schockaert, 1986). More than 70 species have been studied so far (about one-fourth of all known species). For the Monocelididae, a clearly monophyletic taxon within the Proseriata (Martens, 1984), a basic karyotype with $n = 3$ could be recognized, consisting of one large metacentric, one medium-sized metacentric, and one small acro- or subtelocentric chromosome (Curini-Galletti et al., 1989).

Studying the karyotypes of the nonmonocelidid Proseriata (including the Unguiphora [Sopott-Ehlers 1985]) chromosome sets with $n = 6$ were frequently found. Not only the number of chromosomes, but also their morphology strongly suggested that the nonmonocelidid taxa might have originated by polyploidy from the same ancestor as the Monocelididae (i.e., with a chromosome set with $n = 3$).

To test the hypothesis of polyploidy, chromosome morphology and genome length in 35 nonmonocelidid proseriate species were compared with those of the Monocelididae. The genome sizes (DNA content) in five species of Monocelididae and in five species of the nonmonocelidid group were measured.

MATERIALS AND METHODS

Thirty-six species of the Monocelididae and 35 species of four nonmonocelidid taxa of the Proseriata were collected from various inter- or sublittoral stations in the Mediterranean region and along the coasts of northern Europe and eastern Canada (see Table 1). Species identification was based on living animals and, whenever necessary, on sectioned material. All unnamed species are new and will be described in a later publication.

For karyological purposes, whole living specimens were placed in a 0.2% isotonic solution of colchicine for 3–4 hr. Animals are then transferred into a 2% acetic-acid solution for about two minutes, stained for a few minutes in lactic-aceto-orcein, and squashed under a coverglass. The haploid chromosome number was ascertained from both primary and secondary spermatocytes, and the diploid number was ascertained from spermatogonial mitoses, which were also used for karyometric analysis. The idiograms in Figure 2 are based on absolute lengths obtained from measurements of 5–20 metaphase plates, with an estimated medium degree of coiling. The chromosome nomenclature employed is that of Levan et al. (1964), and the fundamental number (NF) is according to Matthey (1949).

The relative amount of DNA in the nuclei of different tissues was measured on Feulgen-stained serial paraffin sections with a digital scanning cytophotometer shaded by a small-band interference filter. Animals were fixed in Bouin's fluid; paraffin sections, 8–10 μm thick, were hydrolized in 4-N HCl at 28°C for one hour. DNA-Feulgen staining was performed for 30 minutes at room temperature with a 0.5% pararosaniline so-

TABLE 1. Karyological data including haploid chromosome number (n), mean haploid genome length, and fundamental number (NF) of the 71 investigated Proseriata species. Species marked with asterisks possess the Monocelididae basic karyotype.

Species	n	Genome length (μm)	NF
Monocelididae:			
*Boreocelis filicauda**	3	8.99	5
Boreocelis urodasyoides	4	11.77	7
Monocelis sp. 1	2	7.89	4
Monocelis sp. 2	2	7.19	3
Monocelis lineata	3	6.34	6
Monocelis fusca	3	6.30	6
Monocelis longistyla	3	6.44	4
Pseudomonocelis ophiocephala	3	13.89	4
Pseudomonocelis agilis	3	11.83	4
*Duplominona longicirrus**	3	13.00	5
*Duplominona paucispina**	3	8.63	5
*Duplominona corsicana**	3	13.93	5
*Duplominona septentrionalis**	3	10.96	5
Duplominona sp. 1*	3	10.01	5
*Duploperaclistus circocirrus**	3	8.64	5
Minona sp.*	3	8.88	5
Pomonotus sp. 1*	3	8.29	5
*Promonotus schultzei**	3	9.08	5
*Promonotus ponticus**	3	11.31	5
Promonotus arcassonensis	4	8.99	6
Promonotus marci	5	10.04	6
Promonotus sp. 2	5	9.03	6
*Archilina endostyla**	3	6.80	5
Archilina sp. 1*	3	8.64	5
Archilina sp. 2*	3	8.15	5
Archilina sp. 3*	3	10.85	5
Archiloa sp. 1	4	12.35	6
Archiloa rivularis	4	12.54	6
Archiloa petiti	4	12.06	5
Archiloa westbladi	4	12.91	5
Monocelopsis otoplanoides	5	9.34	5
Mesoda septentrionalis	5	8.56	9
Archilopsis unipunctata	5	13.55	6
Archilopsis arenaria	5	12.05	6
Archilopsis spinosa	5	13.68	5
Archilopsis marifuga	5	13.12	5
Archimonocelididae:			
Archimonocelis oostendensis	6	17.82	10
Archimonocelis sp. 1	6	21.02	10
Archimonocelis sp. 2	12	16.92	16
Archimonocelis mediterranea	12	19.53	19
Nov. gen. sp. 1	6	17.80	11
Nov. gen. sp. 2	6	16.99	7
Coelogynoporidae:			
Coelogynopora forcipis	8	18.79	12
Coelogynopora biarmata	11	15.68	15
Carenscoilia bidentata	6	17.62	12
Carenscoilia biforamen	8	15.85	13
Invenusta sp. 1	10	22.73	13
Vannuccia campana	6	11.28	11
Cirrifera aculeata	9	19.27	12
Cirrifera cirrifera	10	14.25	18
Cirrifera sp. 1	11	16.49	12
Otoplanidae:			
Archotoplana holotricha	7	15.30	11
Otoplana truncaspina	9	16.89	15

TABLE 1. Continued.

Species	n	Genome length (μm)	NF
Monostichoplana sp. 1	5	14.27	8
Otoplanella baltica	8	12.78	14
Otoplanella schultzi	8	18.41	13
Pseudorthoplana foliacea	4	15.21	7
Xenotoplana acus	4	17.85	7
Parotoplana sp. 1	5	10.70	10
Parotoplana renatae	6	13.73	11
Parotoplana macrostyla	6	16.85	11
Parotoplana procerostyla	7	11.95	13
Parotoplana sp. 2	7	13.23	13
Parotoplana multispinosa	8	21.63	14
Parotoplana papii	8	16.07	12
Postbursoplana propontica	8	17.16	14
Triporoplana synsiphonioides	8	19.58	14
Parotoplanide sp. 1	9	18.10	17
Unguiphora:			
Nematoplana sp. 1	6	15.50	12
Nematoplana coelogynoporoides	11	13.62	22
Polystyliphora sp. 1	9	20.08	17

lution (Graumann, 1952). Three destaining baths (5 minutes each) were used (consisting of 180 ml water + 10 ml 10% sodium metabisulfite + 10 ml 1-N HCl), and the slides were subsequently washed in running tap water for 30 minutes.

The microphotometric apparatus used was a Leitz MPV3 microscope coupled to a PDP 11/03 computer (Digital equipment). All measurements were performed at 556 nm and with scanning steps of 0.1 μm using a 100× oil-immersion objective. The measuring diaphragm was 0.5 μm. The scanning software has been described in detail by Van Oostveldt et al. (1985) and by Broeckaert et al. (1986).

About 40 *C*, 2*C*, and 4*C* nuclei were measured for each species (*C* being the amount of DNA in a haploid nucleus). The *C* and 4*C* nuclei were taken from the male germinal line (spermatids and spermatogonia, respectively). The 2*C* nuclei were from pharynx or body-wall musculature. The measurements of the different types of nuclei may be regarded as a control for the method. Absolute amounts of DNA were calculated from parallel measurements on human lymphocytes (Olmo, 1983). In some instances the *C* values are lower than those predicted based on the 2*C* and 4*C* values. As *C* nuclei (spermatids) are so small and condensed, their integrated absorbance is often underestimated (Goldstein, 1970). For this reason only the measured or calculated values of 2*C* cells will be mentioned in the Discussion.

RESULTS

A summary of the karyological data for all 71 studied Proseriata species is given in Table 1. Within the Monocelididae, the chromosome number varies between $n = 2$ and $n = 5$, with 55.5% of the species having $n = 3$ and only two species having $n = 2$ (Fig. 1A). Those species marked with an asterisk in Table 1 have the basic karyotype of the Monocelididae referred to in the introduction. Karyotype lengths range from 6.30 μm to 13.93 μm, with a mean of 10.2 (Fig. 1C). Karyotype lengths of the species with the basic set range from 6.80 μm to 13.93 μm ($\bar{x} = 9.71$ μm). Within the non-Monocelididae the chromosome number varies from $n = 4$ to $n = 12$ (Fig. 1B). A karyotype with $n = 6$ occurs in nine species (25.7%) of all non-monocelidids studied and is the only one occurring in all four non-Monocelididae taxa. Karyotype lengths range from 10.70 μm to 23.73 μm ($\bar{x} = 16.6$; Fig. 1D); the mean for the nine species with $n = 6$ is 15.4 μm.

Idiograms of four species with $n = 6$ are given in Figure 2A (one example from each taxon of the non-Monocelididae). The two largest chromosomes are very similar in length and centromere position. *Nematoplana* sp. n. (Fig. 2A) is the only exception, with chromosome 2 being distinctly shorter than chromosome 1. The two medium-sized chromosomes in the four species are also of almost the same length and they are mostly metacentric. The two small chromosomes are also equal to each other in length and are metacentric to telocentric.

The amounts of DNA (genome size) in five Monocelididae and in five non-Monocelididae have been measured. Karyotypic data, relative amounts of DNA (in arbitrary units [AU]), absolute amounts of DNA in pg, and the relation AU/μm are reported in Table 2. For two species, the relative amounts of DNA in 2*C* nuclei were interpolated from the values of *C* and 4*C* nuclei. Within the Monocelididae, the relative amount of DNA in 2*C* cells varies from 116.8 AU to 257 AU, with a mean of 176 AU, while within the non-Monocelididae, it ranges from 269.9 AU to 531.4 AU, with a mean of 402 AU.

DISCUSSION

Previous research on the Monocelididae has shown the basic karyotype to be one with $n = 3$, consisting of one large and one medium-sized metacentric and one small acro- to subtelocentric chromosome. By in-

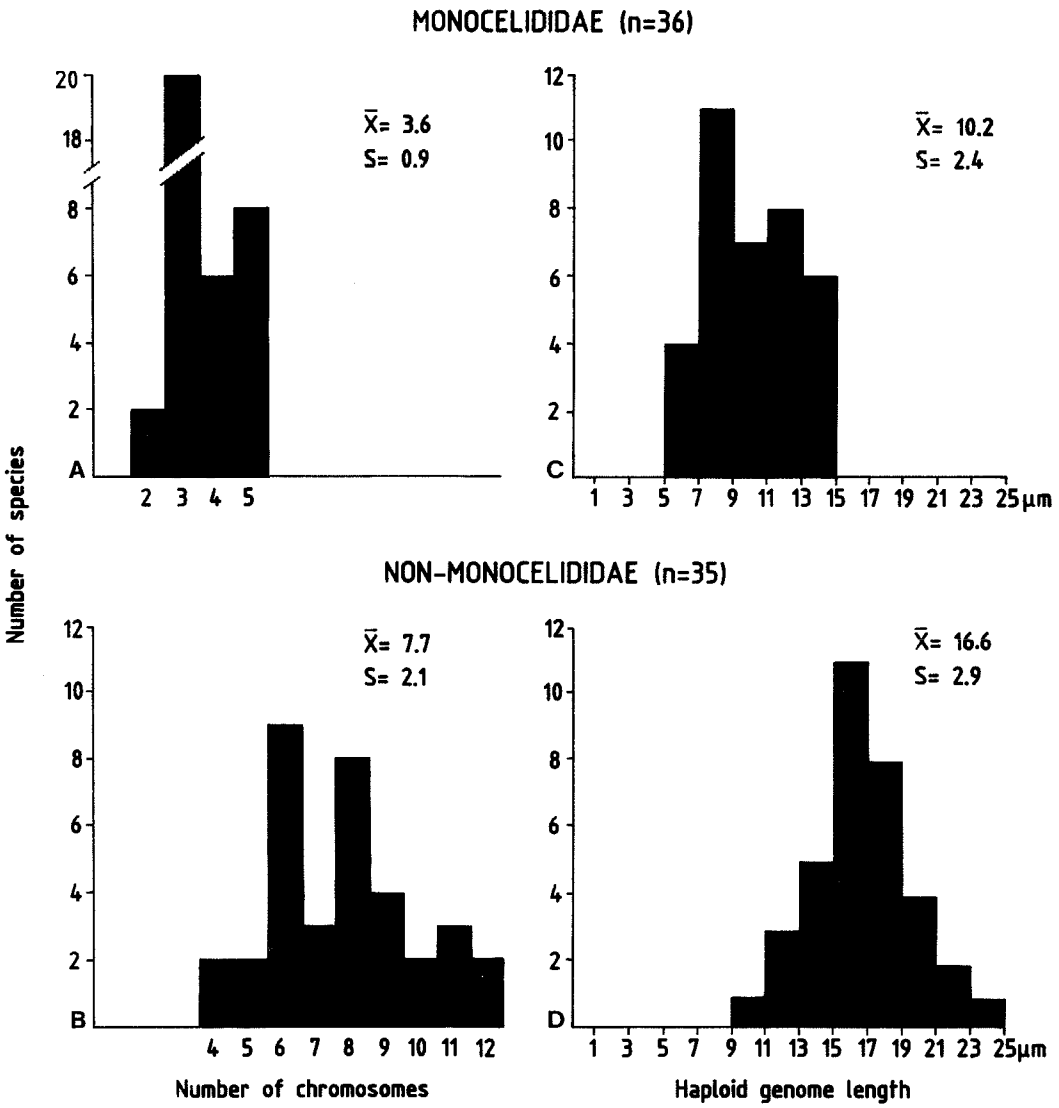

FIG. 1. Frequency distributions of the haploid number of chromosomes in species of A) the Monocelididae and B) the non-Monocelididae; frequency distributions of the genome length in species of C) the Monocelididae and D) the non-Monocelididae.

group comparison, this karyotype can be considered plesiomorphic within the Monocelididae (Curini-Galletti et al., 1989). All other monocelidid karyotypes can be explained as being derived from the basic type by chromosome mutations, such as pericentric inversion, translocation, fission, and fusion (Curini-Galletti et al., 1985, 1989; Martens and Curini-Galletti, 1987; Martens et al., 1989).

Species with $n = 6$ are found in all four nonmonocelidid taxa. In a number of these species, the chromosomes are arranged in three sets, each comprising two chromosomes of similar size. The three sets are distinctly different from one another in length. In addition, in most species the largest four chromosomes are isobrachial, but the smallest two are more variable. By ingroup comparison, this karyotype may be postulated as the basic set (i.e., plesiomorphic) for the nonmonocelidids.

Both the Monocelilidae and the nonmocelidids are monophyletic, and they are sister groups (Martens and Schockaert, 1988; see below for further discussion). A comparison of the basic karyotypes indicates that various mechanisms of karyological evolution can be hypothesized. Among these, reduction of chromosome number by fusion or chromosome loss, or increase by fission or polyploidy are the most likely. It has been shown that $n = 3$ is the basic karyotype for the Proseriata (Curini-Galletti et al., 1985), and hence, the polarity of evolution must have been from $n = 3$ to $n = 6$. Our data on the morphology of the chromosomes,

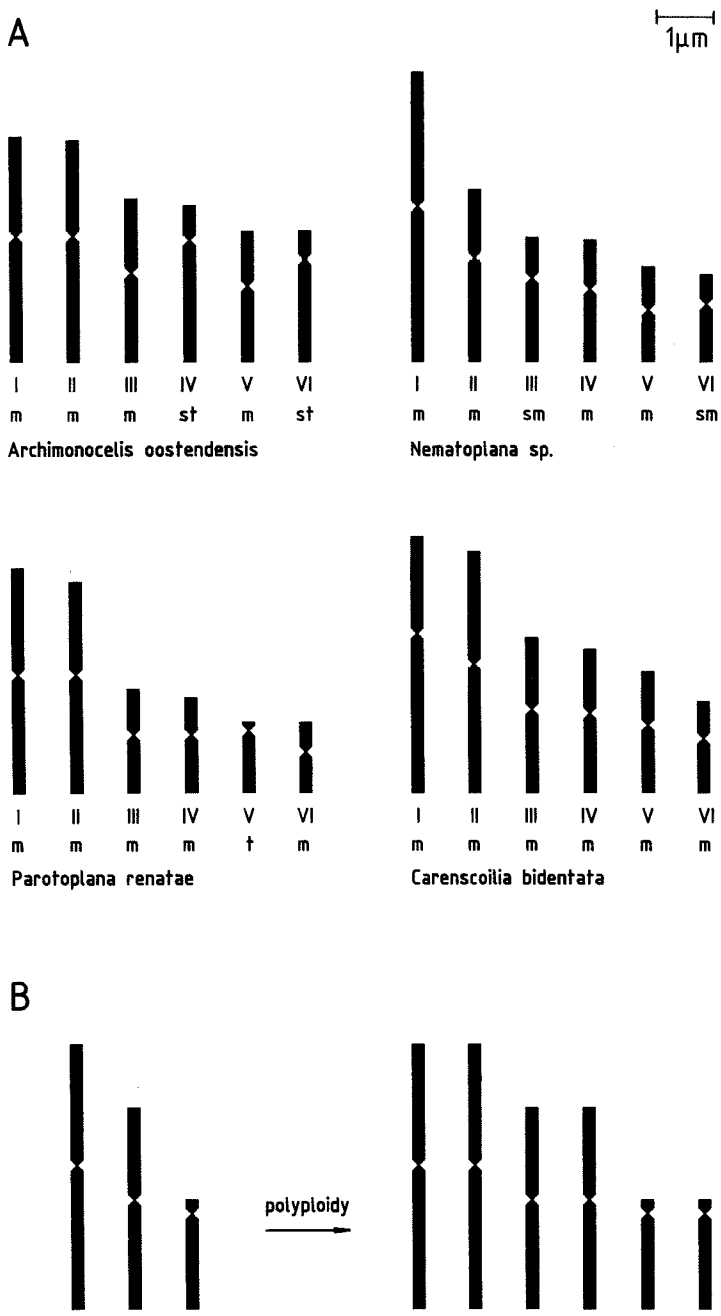

FIG. 2. A) Idiograms of the haploid set of chromosomes based on the absolute genome length of four representatives of the non-Monocelididae; B) hypothesized scheme for the evolution of polyploidy from the basic karyotype of the Monocelididae.

the genome length, and the genome size are highly in favor of the hypothesis that polyploidy (the doubling of the $n = 3$ genome) has taken place. Consequently, a karyotype with $n = 6$ must be an apomorphy, most probably a synapomorphy for the nonmonocelidid taxa.

The theoretical doubling of the chromosome number in the genome of the Monocelididae results in a karyotype which is very similar in chromosome number and morphology to that assumed to be basic for the non-monocelidids (Fig. 2B). In the Monocelididae the mean relative amount of DNA in $2C$ nuclei is 176 AU; the mean value in the non-Monocelididae is 402 AU, which is close to double of that in the Monocelididae (ratio = 2.28). Similar ratios are found when data for species

TABLE 2. Karyotypic data and DNA measurements for the analyzed species. The genome sizes marked with an asterisk are recalculated from the *C* and 4*C* values. The value given for the absolute genome size of humans (for lymphocytes) is taken from Olmo (1983).

Species	Karyotypic data			Genome size ± SD (AU)			Absolute size (pg)	2*C* genome size/haploid genome length (AU/μm)
	n	Haploid genome length ± SD (μm)	NF	*C*	2*C*	4*C*		
Monocelidid species:								
Monocelis lineata	3	6.34 ± 0.79	6	43.4 ± 7.6	116.8 ± 14.4	224.6 ± 51.4	1.0	18.42
Duplopera clistus circocirrus	3	8.64 ± 0.40	5	64.1 ± 11.3	130.3 ± 12.8	265.1 ± 24.1	1.2	15.08
Archilina sp. 3	3	11.94 ± 2.96	5	68.5 ± 8.2	164.9 ± 21.8	340.9 ± 24.2	1.5	13.81
Archilopsis marifuga	5	13.21 ± 2.39	5	—	213.2 ± 19.9	—	1.9	16.13
Archiloa petiti	4	12.06 ± 1.42	5	121.0 ± 13.5	257*	543.4 ± 63.7	2.3	21.31
Nonmonocelidid species:								
Archotoplana holotriche	7	15.30 ± 0.70	11	105.7 ± 14.8	269.9 ± 19.2	495.1 ± 66.3	2.4	17.64
Nov. gen. sp. 1	6	17.80 ± 2.04	11	—	362.4 ± 44.6	766.6 ± 73.9	3.3	20.36
Carenscoilia biforamen	8	15.85 ± 1.47	13	—	390.1 ± 40.6	—	3.5	24.61
Otoplana truncaspina	9	16.98 ± 0.41	15	213.0 ± 13.3	454*	964.8 ± 178.0	4.1	26.74
Archimonocelis sp. 2	12	16.92 ± 1.39	16	221.9 ± 30.1	531.4 ± 71.4	1,090.1 ± 149.2	4.8	31.41
Homo sapiens	—	—	—	—	778.8 ± 54.4	—	7.0	—

with the basic karyotypes in each group (*Duplopera clistus circocirrus* or *Archilina* sp. 3 vs. nov. gen. sp. 1) are compared (2.78 and 2.20, respectively). The relationship between genome size and genome length (Table 2) indicates that there is increased genome condensation (DNA-packing) with an increase in genome length. When the genome length is plotted against the log(genome size), a highly significant linear correlation is found ($y = -23.782 + 15.471[\log x]$, $r = 0.9334$, $t = 7.3572$). It appears that a doubling of the genome size is associated with a karyotype lengthening of 4.66 μm. This may explain why the total genome length in the species with $n = 6$ (15.40 μm) is not exactly double the mean value found in Monocelididae with the basic karyotype (9.71 μm).

This hypothesis is also congruent with morphological data. The nonmonocelidid proseriates all have a connective-tissue capsule surrounding the brain; such a capsule is absent in Monocelididae, Tricladida and all other platyhelminth taxa. This brain capsule is another synapomorphy, suggesting that the nonmonocelidid Proseriata comprise a monophyletic taxon, the Paramonocelida, sister taxon of the Monocelididae (Martens and Schockaert, 1988).

Our conclusions about the phylogenetic relationships within the Proseriata is in contrast with the point of view of Sopott-Ehlers (1985). She considered the Unguiphora to be the sister taxon of all remaining Proseriata, the synapomorphy being the statocyst, which is lacking in the Unguiphora. The consideration of the Paramonocelida as monophyletic implies that the statocyst must have been secondarily lost in the Unguiphora. In our opinion, this is a more parsimonious assumption that to suppose that the doubling of the chromosomes and the development of the brain capsule has occurred twice during evolution (Martens and Schockaert, 1988).

Other karyological mechanisms are not supported by our data. Reduction of chromosome number by centric fusion should not result in differences in genome size of the magnitude we measured, even taking into account the loss of centromeres and adjacent heterochromatin. Reduction by loss of three chromosomes (half of the genome size) seems to be an exceedingly drastic phenomenon. Reduction by itself is not supported by outgroup comparisons from which $n = 3$ appears to be the plesiomorphic condition (Curini-Galletti et al., 1985). Increase from $n = 3$ to $n = 6$ by fission is not supported by the measurements of genome size or by genome morphology, since the basic karyotype of the Paramonocelida should then consist of only acrocentric chromosomes. Though our data support the hypothesis of polyploidy, other evidence is still missing: multivalents have not been observed during meiosis (probably because a reorganization of the loci has occurred during further evolution), and other techniques, such as chromosome banding and isozyme data, may confirm or reject our hypotheses in the future.

Within the Paramonocelida, further karyological evolution has taken place through various small chromosome rearrangements and more drastic changes in the karyotypes, mainly Robertsonian fission. The complexity of the karyological evolution within this group is reflected by the wide ranges of chromosome numbers and fundamental numbers (see Table 2). However, a

detailed discussion of karyological evolution within the Paramonocelida is not within the scope of this paper.

Polyploidy has been reported in several species of Platyhelminthes, mainly in Tricladida (Dham, 1958; Gourbault, 1981; Oki et al., 1981), but also in Macrostomida and Typhloplanoida (Jones, 1944; Luther, 1950; Papi, 1950; for a review see Benazzi and Benazzi-Lentati [1976]). In freshwater triclads, polyploidy may even have been an important factor in the process of speciation (Benazzi, 1982). The hermaphroditic state (with the possibility of self-fertilization) makes polyploidy more likely to occur in platyhelminths than in dioecious taxa. Polyploidy has indeed been reported in other hermaphroditic taxa, such as oligochaetes and pulmonate mollusks. In taxa in which parthenogenesis occurs, polyploidy has also been observed (e.g., in triclads, lizards, blackflies, and weevils [White, 1978; Lokki and Saura, 1979; Gibby, 1981; Benazzi, 1982]). Polyploidy is also well documented in fishes and amphibians (Schultz, 1979; Bogart, 1979).

In spite of the many reports of polyploidy, White (1978), Jackson (1976) and Stebbins (1977) believe that it has played a secondary role in animal evolution. White (1978 p. 285) stated that "polyploidy has been so rare that its overall evolutionary role has been fairly insignificant." However, many other authors are convinced that polyploidy must have played an important role in animal evolution and even may have allowed the evolution of higher taxa, such as some Archaeogastropoda taxa, the amphibians, and the mammals (see Ohno, 1970, 1974; Schultz, 1979; Nakamura, 1986).

Positive evidence for polyploidy in higher taxa is, however, generally lacking, being overshadowed perhaps by subsequent karyological events. It is therefore not surprising that most reports of polyploidy are limited to karyotypic comparisons at the species level and, sometimes, at the genus level. Formulation of the hypothesis of polyploidy for the origin of the Paramonocelida was possible here mainly because the karyology of the Proseriata is exceptionally well known, and because Proseriata presumably have a slow rate of karyological evolution.

Genetical advantages of polyploidy are often explained in terms of gene duplication and an increase in heterozygosity (Ohno, 1970; Ralin and Selander, 1979). This can lead to increased morphological and ecological variation with a higher tolerance for stress than in the progenitor species (Grant, 1971; Bogart, 1979; Lokki and Saura, 1979; Schultz, 1979; Gibby, 1981). Within the Proseriata, the Monocelididae inhabit muddy to fine-medium sand of sublittoral waters or littoral sheltered areas (often brackish). Nearly all species with the basic karyotype inhabit sublittoral localities (primitive habitats). Members of the Paramonocelida mostly live in coarser sediments (even gravel) of highly dynamic littoral or sublittoral localities. At some very exposed beaches (i.e., stressful environments), they have been observed to be the dominant turbellarian taxa (Martens and Schockaert, 1986). Some Paramonocelida are even considered to be indicators for the most dynamic zone in a sandy beach (the "Otoplana-zone" of Remane [1933] and the "Nematoplana-zone" of Rieger and Ott [1971]). Therefore, it seems that polyploidy may have had ecological significance, allowing a new adaptive area to be inhabited.

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