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Chromosome fission or duplication in *Macrostomum lignano* (Macrostomorpha, Plathelminthes) – remarks on chromosome numbers in ‘archoophoran turbellarians’

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Abstract

Mitotic metaphase plates of a new species of the genus *Macrostomum* were studied using conventional Giemsa staining, Orcein staining and semi-thin sections stained after Richardson. Because of the ease in culturing it, this species has the potential to become the new model organism for developmental and evolutionary studies among the lower metazoa. The chromosome number was found to be $2n = 8$. Reaching a relative length of 45.9% of the haploid genome size, the chromosomes of one pair were significantly larger than all other chromosomes. Of the smaller pairs, chromosomes of one pair were slightly larger (relative length of 21.3%) than the chromosomes of the remaining two pairs (each with a relative length of 16.4%). All chromosomes were metacentric ($2m + 2m + 2m + 2m$). For the first time, a diploid chromosome set of four pairs was described for the genus, as compared with previous studies showing predominantly $2n = 6$ for 20 different *Macrostomum* species.

Key words: Platyhelminthes – karyotype – idiogram – Giemsa – Orcein – phylogeny – evolution

Introduction

Macrostomum is a species-rich (about 130 described species) genus of the family Macrostomidae, which is the most advanced family in the order Macrostomorpha (Rieger 2001). Along with the Acoelomorpha, the Catenuclida and the Polycladida, the Macrostomorpha form the paraphyletic ‘archoophorans’, characterized through production of entolecithal eggs. All other plathelminths, the neophorans, develop from ectolecithal eggs. Among rhabditophoran plathelminths, encompassing all plathelminths except the Acoelomorpha and the Catenuclida, the Macrostomorpha are the most basal taxon (Ehlers 1995; Littlewood and Olson 2001; Rieger 2001; Tyler 2001). Recently, several molecular studies have been suggesting a position of the acoelomorphs outside the plathelminths, as a basal sistergroup of all other bilaterians (Ruiz-Trillo et al. 1999, 2002; Cook et al. 2004; Telford et al. 2004). These findings are supported by studies on the nervous system (Reuter et al. 1998; Raikova et al. 2000; Reuter et al. 2001) and the cleavage pattern (duet spiral cleavage, see Apelt 1969 and Henry et al. 2000) of acoelomorphs. The likely totipotent stem cell system, however, can be seen as a feature uniting all of the plathelminths, including acoelomorphs (Ehlers 1995).

Macrostomum lignano Ladurner et al. (2005) is easily kept in culture and is due to its size and transparency a suitable model organism for cytological, histological and developmental studies that occupies a distinctly more basal phylogenetic position than any of the presently used model organisms, such as planarians or *Caenorhabditis elegans* (Ladurner et al. 2005).

Until now, karyotype or chromosome number of 20 *Macrostomum* species have been determined (Table 1). A major part of these studies has been carried out by Ferguson (1937a,b,1939a,b,c,1940a,b), while other chromosome descriptions reach back to 1905 (Luther 1905). Recently and including the present paper, two new investigations about *M. gigas* (Yamamoto et al. 2003) and *M. lignano* add to the knowledge about *Macrostomum* chromosomes.

For another genus in the family Macrostomidae, *Promacrostomum*, chromosomes have been characterized for the species *P. gieysztori* (Papi 1951). A single member of the family

Microstomidae, namely *Microstomum bispinalis*, has been subjected to karyological observation as well (Stirewalt 1937).

In the light of taxonomic revisions and new chromosome findings, previous compilations/reviews by Ferguson (1940b) and Benazzi and Benazzi-Lentati (1976) on *Macrostomum* chromosomes need to be updated.

Materials and Methods

Macrostomum lignano was found in Lignano, Italy, at the shores of the Mediterranean, and has been cultured in the laboratory since 1995 in petri dishes with the feeding diatom *Nitzschia curvilineata* (Bacillariophyceae) at about 32‰ salinity, 20°C, 60% humidity and a light and dark cycle of 13–11 h. One litre of culture medium contains 32‰ NaCl, 75 mg NaNO₃, 5 mg NaH₂PO₄·H₂O, 25 mg NaSiO₃·5H₂O, 4.36 mg C₁₀H₁₄N₂O₈Na₂·2H₂O, 3.15 mg FeCl₃·6H₂O, 0.18 mg MnCl₂·4H₂O, 0.01 mg CuSO₄·5H₂O, 0.02 mg ZnSO₄·7H₂O, 0.01 mg CoCl₂·6H₂O, 0.01 mg Na₂MoO₄·2H₂O, 0.1 mg thiamine·HCl, 0.5 mg biotin and 0.5 mg vitamin B12.

Giemsa staining with air drying method (Imai et al. 1977 – modified for planarians)

For metaphase plates of neoblasts, adult animals were cut transversely and left to regenerate for 1 ($n = 5$), and 2 ($n = 3$) days in culture medium. For metaphase plates of blastomeres ($n = 13$), an embryo with about 40 cells was selected. Animals and the egg were then treated for 3–4 h in sea water with 10^{-6} – 10^{-4} M colchicine to arrest mitotic cells in metaphase, followed by 0.5–1 h in culture medium with 0.2% KCl. Specimens were washed with a freshly prepared mixture of acetic acid, ethanol and distilled water at the ratio of 3 : 3 : 4, respectively. In this solution, animals were dissected with needles to single out cells or small groups of cells on the slide. Cell associations were rinsed in a 1 : 1 mixture of acetic acid and ethanol, half dried and then washed with full-strength acetic acid and thoroughly dried overnight. Slides were stained with 4% Giemsa’s solution for 10–15 min on the next day, washed with tap water and air dried.

Orcein staining

Fifteen specimens were cut behind the pharynx and the tail pieces (anterior regenerants) were left to regenerate for 1 day, then treated with a 10^{-4} M colchicine solution for 4 h and washed in artificial sea

Table 1. All available information on *Macrostomum* chromosome numbers are shown here. Also, studies on the closely related genus *Promacrostomum* and one representative of the family Microstomidae are included

Name and authority	Chromosome number		Reference	Additional information
	Haploid	Diploid		
<i>M. hystricinum</i> Beklemishev 1951	2		Luther (1905)	Developing eggs; doubted by Luther (1947)
<i>M. thermale</i> Reisinger 1933	2		Reisinger (1933)	Developing eggs
<i>M. beaufortensis</i> Ferguson 1937	3	6	Ferguson (1937b)	Somatic cells
<i>M. fergussoni</i> Beklemishev 1951	3	6	Ferguson (1939a)	Meiotic metaphase in testis
<i>M. finlandense</i> (Ferguson 1940)	3	6	Luther (1947)	Spermatocytes and somatic cells
<i>M. gabriellae</i> Marcus 1949	3		Marcus (1949)	
<i>M. gigas</i> Okugawa 1930		6	Yamamoto et al. (2003)	Somatic cells; the taxonomic status of <i>M. gigas</i> is unclear; often regarded synonymous with <i>M. tuba</i> , it is kept here as a separate species; belongs to tuba species group
<i>M. glochistylum</i> Ferguson 1939	3	6	Ferguson (1939b)	Germinative and somatic cells
<i>M. granulophorum</i> (Ferguson 1940)	3	6	Ferguson (1940a)	Spermatogones
<i>M. lewisi</i> Ferguson 1939	3	6	Ferguson (1939c)	Spermatogones in metaphase; belongs to tuba species group
<i>M. reynoldsi</i> Ferguson 1939	3	6	Ferguson (1939c)	Germinative and somatic metaphase; belongs to tuba species group; in Benazzi and Benazzi-Lentati (1976), this species is missing; instead, the chromosome number for <i>M. phillipsi</i> is given, for which no chromosome description is available
<i>M. riedeli</i> Ferguson 1940	3	6	Ferguson (1940a)	Germinative (telophase in meiosis) and somatic cells
<i>M. ruebushi crenatostylum</i> (Ferguson 1940)	3	6	Ferguson (1940a)	Somatic metaphase
<i>M. shenandoahense</i> (Ferguson 1940)	3	6	Ferguson (1940a)	
<i>M. tenuicauda</i> Luther 1947		6	Luther (1947)	Spermatogones in metaphase; belongs to tuba species group
<i>M. truncatum</i> (Ferguson 1940)	3	6	Ferguson (1940a)	Somatic metaphase
<i>M. tuba</i> (Graff 1882)	3	6	Phillips (1936)	somatic mesenchyme cells, oogones, spermatogones, developing egg; belongs to tuba species group
<i>M. virginianum</i> Ferguson 1937	3	6	Ferguson (1937a)	Spermatogonial tissue
<i>M. lignano</i>		8	Present paper	Belongs to tuba species group
<i>M. hustedi</i> Jones 1944	6	12	Jones (1944)	Somatic cells, spermatocytes, oocytes; probably polyploid (tetraploid)
other Macrostomidae				
<i>Promacrostomum gieysztorii</i> (Ferguson 1939)	3	6	Papi (1951)	1. Meiotic metaphase in oocyte, spermatogones
other Macrostomorpha: Microstomidae				
<i>Microstomum bispiralis</i> Stirewalt 1937	8	16	Stirewalt (1937)	Somatic cells; this species is similar to <i>M. jenseni</i> described in Riedel, 1932 and <i>M. tortipenis</i> (Steinböck, 1938)

water for 25 min. After relaxation with 7.14% MgCl₂, specimens were fixed with Carnoy (ethanol : chloroform : acetic acid = 6 : 3 : 1) and put for 2 min in 1 M HCl. Staining was conducted in 1% Orcein solution (2.2 g Orcein dissolved in 100 ml hot glacial acetic acid and diluted with distilled water 4.5 : 5.5) for 10 min, specimens were then washed in 5% acetic acid and mounted with coverslips.

Richardson staining (Richardson et al. 1960)

Egg shells of eggs with early cleavage stages were punctured with Wolfram needles and the eggs then fixed in Bouin and embedded in Spurr's resin. Semi-thin sections (0.5 and 1 µm) were prepared and stained after Richardson (1 g Azur II, 1 g methylene blue and 1 g borax in 100 ml a.d., filter before use).

Results

Observation of embryonic blastomeres stained with Giemsa (Fig. 1) and neoblasts stained with Orcein (Fig. 2), supported by semi-thin sections of embryos stained with Richardson (Fig. 3) have revealed the karyotype of *M. lignano* to be

$2n = 8 = 2m + 2m + 2m + 2m$ (nomenclature following Levan et al. 1964). All chromosomes are metacentric. The first chromosome pair is considerably larger than the second, third and fourth pair. The mean relative length of the largest chromosomes compared to the haploid genome size is $45.9 \pm 1.7\%$ ($n = 16$). The chromosomes of the second largest pair are to an average relative length of $21.3 \pm 2.1\%$ ($n = 16$) slightly larger than the chromosomes of the two smallest pairs which are about the same size, each chromosome with an average length of $16.4 \pm 1.4\%$ ($n = 32$) of the haploid genome size. The statistical analysis was performed after a preselection of the chromosomes into different categories. While there is always the possibility that such a preselection groups non-homologous chromosomes together, non-overlapping standard deviations support our preselection, indicating an actual length difference between the selected chromosome categories. Measuring the absolute chromosome size did not yield consistent data, the absolute chromosome size varied with different staining methods and

Fig. 1. (a–b) Mitotic metaphase plates of *Macrostomum lignano* chromosomes from embryonic blastomeres stained with Giemsa. The diploid chromosome number is $2n = 8$

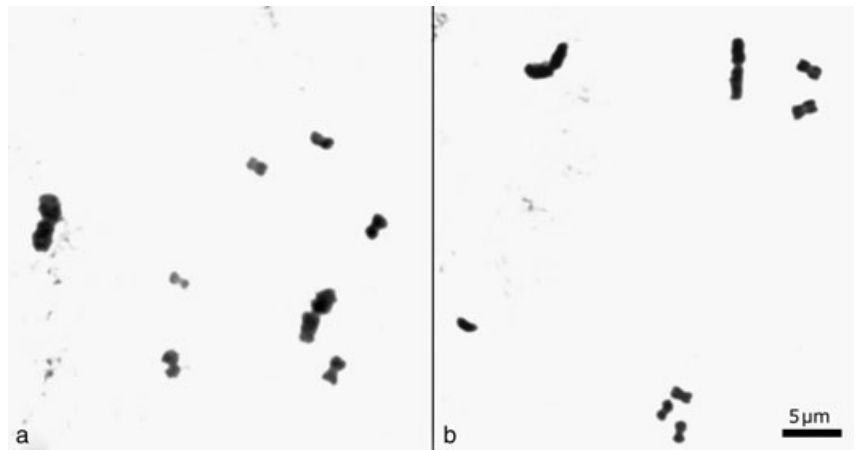


Fig. 2. (a–d) Mitotic metaphase plates of *Macrostomum lignano* chromosomes from neoblasts stained with Orcein. The diploid chromosome number is $2n = 8$

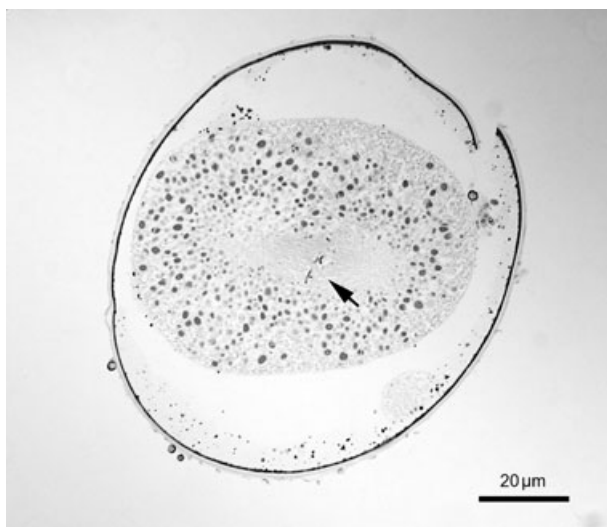
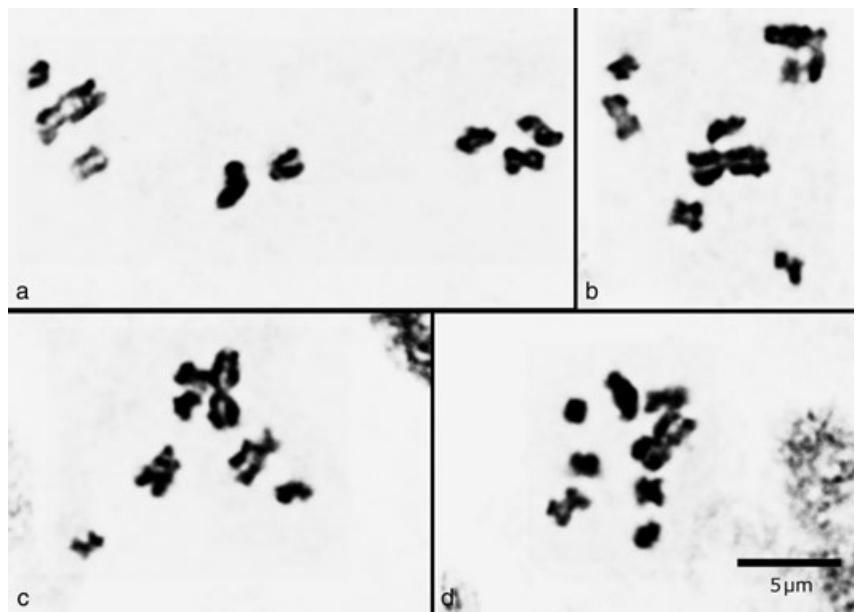


Fig. 3. Metaphase in a 1-cell stage embryo of *Macrostomum lignano* stained with Richardson. Not all chromosomes can be seen on a single section

also with the same staining in different experiments (see also Bennett, 1970).

Discussion

Albeit a fair number of *Macrostomum* species has undergone chromosomal analysis, recently only two more studies have been undertaken to examine the chromosome number.

With few exceptions, all studies on chromosome number in the genus *Macrostomum* report six chromosomes for diploid cells and three chromosomes for haploid cells (see Table 1). Only in *M. lignano* eight chromosomes in mitotic metaphases could be found, whereas *M. hustedi* with 12 somatic chromosomes is probably polyploid (Jones 1944). In *M. thermale* (Reisinger 1933), merely two chromosomes have been reported in mature eggs. Ferguson (1940b) gives a haploid chromosome number for *M. orthostylum* of $n = 2$ and refers to Braun as the authority, but in neither of the listed references (Braun 1885, 1894) is information about *M. orthostylum* chromosomes available. The respective chromosome number is therefore considered to be unknown. In *M. finlandense* and *M. hystricinum*, Luther (1905) has seen two chromosomes, but later

corrected these findings with new data for *M. finlandense* in Luther (1947) to three haploid chromosomes and doubted the presence of only two chromosomes in *M. hystricinum*, although without undertaking new experiments to confirm this presumption.

In most of the studied *Macrostomum* species where a chromosome description or picture is given, one chromosome pair is larger than the other ones. Exceptions are *M. truncatum* (Ferguson 1940a) and *M. riedeli* (Ferguson 1940b) with two large chromosome pairs and *M. ruebushi crenatostylum* (Ferguson 1940a) with three large chromosomes and three small ones, according to the author's descriptions. Looking at his Fig. 11, depicting a metaphase of somatic chromosomes of *M. ruebushi crenatostylum*, one could see two large chromosomes, two medium-sized and two small ones as well. Ferguson's karyotype description of *M. beaufortensis* is inconsistent, the first source (Ferguson 1937b) claims that there is a large, a medium-sized and a small pair, the second one (Ferguson 1940b) describes two large pairs and one small pair.

Neither picture nor description is given for the karyotype ($x = 2$) of *M. hystricinum* and *M. thermale*.

Macrostomum lignano is a member of the *Macrostomum tuba* species group (Table 1, see Ladurner et al. 2005), a classification which subsumes species with similar male copulatory stylets. All members of the *M. tuba* species group have stylets with a blunt distal opening. Judging from the stylet appearance and other morphological characteristics, *M. tenuicauda* is the closest relative of *M. lignano*. Luther's (1947) description of the *M. tenuicauda* karyotype distinguishes a large pair, a medium-sized pair and a small pair of chromosomes. The magnification given in the original work does not seem to reflect the actual chromosome size, though, as a comparison with other *Macrostomum* karyotypes suggests. Yamamoto et al. (2003) published karyotypes of *M. gigas* also showing a large pair of chromosomes, a medium-sized pair and

a slightly smaller pair. In *M. tuba* (Phillips 1936), there is one large pair of chromosomes and two slightly smaller pairs, a similar situation as in *M. lewisi* and *M. reynoldsi* (Ferguson 1939c, see Fig. 4). Closely related species often show different karyotypes (Puccinelli et al. 1990) and chromosome numbers (Curini-Galletti and Puccinelli 1994), which can be interpreted as a post-mating isolation mechanism for sympatric speciation.

The size difference between the large chromosome pair and the smaller ones is much more pronounced in *M. lignano* than in other members of the *M. tuba* species group. Regarding that size difference, it appears to be possible that one medium-sized chromosome pair has split into two smaller pairs, thus giving rise to the extra pair in *M. lignano* (Robertsonian or centric fission and subsequent pericentric inversion). The total amount of DNA in *M. lignano* could therefore be comparable with other closely related species, the additional chromosome pair notwithstanding. A similar situation can be found in *Bothromesostoma personatum* (Typhloplanoida) with $2n = 8$ (Valkanov 1938) and *B. essenii* with $2n = 10$ (Papi 1950, both reviewed in Benazzi and Benazzi-Lentati 1976). While $2n = 8$ is believed to be the basic chromosome number in this genus, the chromosome morphology indicates the fragmentation of a pair of homologous chromosomes in *B. essenii*.

Other than fission of a larger pair into two smaller pairs, a duplication of a small chromosome pair might have occurred as well. This notion is supported by slight differences in the three smaller chromosome pairs, two of which are of an approximately equal size. The two smallest pairs are possibly the result of a chromosome duplication. Interestingly, *M. lignano* has proven to be the most robust *Macrostomum* species so far to culture in the laboratory. It can only be speculated if this feature is related to an additional chromosome pair derived from chromosome duplication (and thus, gene duplication).

Last, the diploid number of eight can be seen as the plesiomorphic condition, from which through deletion of one

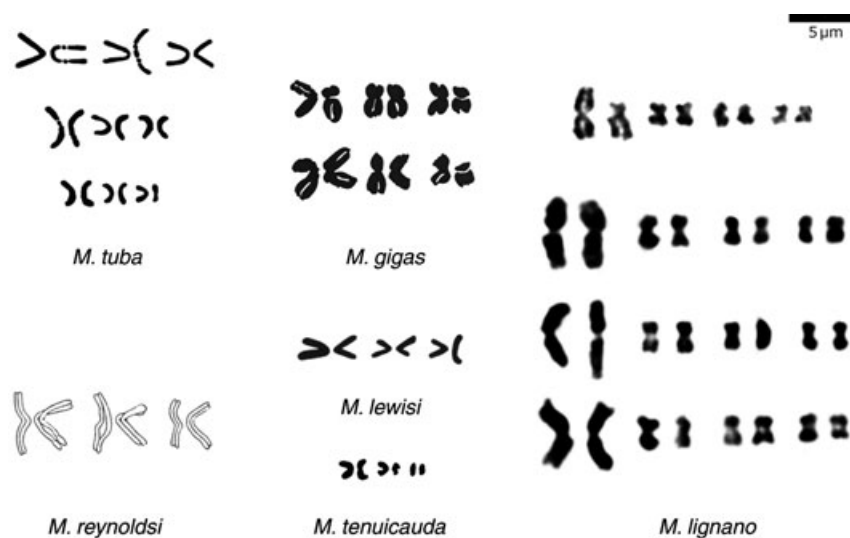


Fig. 4. Karyotypes of members of the *Macrostomum tuba* species group. *Macrostomum tuba* is based on three metaphase plates from Phillips (1936), *M. gigas* is taken from two idiograms from Yamamoto et al. (2003, Fig. 4), *M. tenuicauda* is based on a metaphase plate from Luther (1947), *M. lewisi* and *M. reynoldsi* are based on a metaphase plate from Ferguson (1939c). Chromosomes of *M. tuba*, *M. reynoldsi*, *M. lewisi* and *M. tenuicauda* have been rearranged from the original figures according to size. Note the size difference of *M. tenuicauda* chromosomes to all other karyotypes, this might be due to an incorrect magnification given in Luther (1947). The first karyotype of *M. lignano*, stained with Orcein, is somewhat smaller (largest chromosome is about 4 μm in length) than the three karyotypes stained with Giemsa (largest chromosome is about 5.3 μm in length)

chromosome pair most other *Macrostomum* species were derived. Such an assumption would not be supported by morphological characteristics, though. Also, considering that the closely related genus *Promacrostomum* showed the existence of six diploid chromosomes (Papi 1951) as is the case with most studied *Macrostomum* species, it is likely that $2n = 6$ is a basal, not a derived chromosome number in the Macrostomidae.

Using molecular techniques like FISH could be applied to help distinguish which mechanism actually led to the super-numeral chromosome pair (Lizarralde et al. 2003).

Establishing phylogenetic relationships between taxa on the level of orders or above based on chromosome number is problematic. Not only is there a high variation in chromosome number within higher taxonomic levels, but also the ancestral chromosome number for many taxa is uncertain. Thus, there is a large gap between different authors' estimations for the ancestral chromosome number in 'turbellarians'. According to Imai et al. (2002), $2n = 2$ comprises the ancestral chromosome number of various eukaryotic taxa including 'turbellarians', as opposed to Birstein (1991), who proposes $2n = 16$ – 20 for all 'turbellarian' taxa. The latter number is in accordance with chromosome findings in many taxons, including *Microstomum bispiralis* with $2n = 16$ (Stirewalt 1937). The family Microstomidae is a primitive taxon within the Macrostomorpha (Rieger 2001), further supporting an ancestral chromosome number of $2n = 16$.

Chromosome numbers in the sponge *Suberites domuncula* ($2n = 32$, Imsiecke et al. 1995) and the cnidarian *Hydra* ($2n = 30$, Alexandrova et al. 2003) could suggest either a high diversity in chromosome numbers also in metazoans below the bilaterian level, or a higher ancestral chromosome number than $2n = 2$ in these taxa.

All studied 'archoophoran' orders (Acoela, Catenulida, Macrostomorpha and Polycladida) show a large range in chromosome numbers. Whereas chromosome counts in the Catenulida revealed between 16 and 40 diploid chromosomes (Ruebush 1938; reviewed in Benazzi and Benazzi-Lentati 1976), the Acoela come up with six to 34 diploid chromosomes (Gardiner 1898; Ruebush 1938; Marcus 1952, 1954; Costello 1970; reviewed in Benazzi and Benazzi-Lentati 1976; see also Apelt 1969; Crezee 1975; Birstein 1990). The variation is similar in chromosome numbers for haploid chromosomes in the Polycladida ranging from 2 (*Notoplana necta*, Marcus 1947) to 10 (*Stylochus ellipticus*, v. Name 1899; Bahr and Hillman 1966; *Notoplana igiliensis*, L. Galleni and I. Puccinelli, unpublished data; *Hoploplana inquilina*, Patterson and Wieman 1912; all reviewed in Benazzi and Benazzi-Lentati 1976). Remarkable is the variation in the genus *Notoplana* with two to 10 haploid chromosomes. These data on 'archoophoran turbellarians' suggest that chromosome number is not a phylogenetic signal stable enough to warrant useful comparisons beyond the taxonomic level of families and genera. On the other hand, more studies on chromosome numbers within the genus *Macrostomum* could help to evaluate the affiliation of species to different species groups proposed by Ladurner et al. 1997 and Ladurner et al. (2005). Considering the significance of chromosomes in the evolution of the eukaryotic genome and the basal position of some 'archoophoran' flatworms in the evolution of the Bilateria, it seems to be desirable to increase the database on chromosomes in the acelomorpha, macrostomids and catenulids.

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Zusammenfassung

Chromosomenteilung oder -verdopplung in Macrostomum lignano (Macrostomorpha, Plathelminthes) – Bemerkungen zur Chromosomenzahl in 'archoophoren Turbellarien'

Mitotische Metaphasen einer neuen Art der Gattung *Macrostomum* wurden mit Hilfe konventioneller Giemsa-Färbung, Orcein-Färbung und semi-dünnen Schnitten, gefärbt nach Richardson, untersucht. Dank der problemlosen Haltung in Kultur hat diese Art das Potential, ein neuer Modellorganismus unter den niederen Metazoen für entwicklungsbiologische und evolutionäre Fragestellungen zu werden. Die Chromosomenzahl wurde als $2n = 8$ bestimmt. Mit einer relativen Länge von 45,9% des haploiden Genoms waren die Chromosomen eines Paares deutlich länger als alle übrigen Chromosomen. Von den kleineren Paaren waren Chromosomen eines Paares etwas länger (relative Länge von 21,3%) als die Chromosomen der restlichen zwei Paare (relative Länge von jeweils 16,4%). Alle Chromosomen waren metazentrisch ($2m + 2m + 2m + 2m$). Zum ersten Mal wurde ein diploides Chromosomen-Set von 4 Paaren für diese Gattung beschrieben, während frühere Studien vorwiegend $2n = 6$ für 20 verschiedene *Macrostomum*-Arten ergeben haben.

References

- Alexandrova, O.; Solovei, I.; Cremer, T.; David, C. N., 2003: Replication labeling patterns and chromosome territories typical of mammalian nuclei are conserved in the early metazoan *Hydra*. *Chromosoma* **112**, 190–200.
- Apelt, G., 1969: Fortpflanzungsbiologie, Entwicklungszyklen und vergleichende Frühentwicklung acoeler Turbellarien. *Mar. Biol.* **4**, 267–325.
- Bahr, L. M. Jr; Hillman, R. E., 1966: Chromosome number of *Stylochus ellipticus* (Girard). *T. Am. Microsc. Soc.* **85**, 323–324.
- Benazzi, M.; Benazzi-Lentati, G., 1976: Platyhelminthes. In: John, B. (ed.) *Animal Cytogenetics*, Vol 1: Berlin, Stuttgart: Gebrüder Bornträger, pp. 1–182.
- Bennett, M. D., 1970: Natural variation in nuclear characters of meristems in *Vicia faba*. *Chromosoma* (Berlin) **29**, 317–335.
- Birstein, V. J., 1990: First contribution to karyology of two acoels (Turbellaria) and a dinophilid (Annelida). *Biol. Zbl.* **109**, 169–174.
- Birstein, V. J., 1991: On the karyotypes of the neorhabdocoela species and karyological evolution of Turbellaria. *Genetica* **83**, 107–120.
- Braun, M., 1885: Über die Turbellarien Livlands. *Zool. Anz.* **8**, 696.
- Braun, M., 1894: Die rhabdocoelen Turbellarien Livlands. *C. Mattiesen Co. Dorpat, Arch. Naturkunde-Naturf. Ges., Ser. 2. Biol. Naturkunde* **10**, 131–259.
- Cook, C. E.; Jimenez, E.; Akam, M.; Salò, E., 2004: The Hox gene complement of acoel flatworms, a basal bilaterian clade. *Evol. Dev.* **6**, 154–163.
- Costello, D. P., 1970: Identical linear order of chromosomes in both gametes of the acoel turbellarian *Polychoerus carmelensis*: a preliminary note. *Proc. Natl. Acad. Sci. U. S. A.* **67**, 1951–1958.
- Crezee, M., 1975: Monograph of the Solenofilomorphae (Turbellaria: Acoela). *Int. Rev. ges. Hydrobio.* **60**, 769–845.
- Curini-Galletti, M.; Puccinelli, I., 1994: The *Gytrix hermaphroditus* species complex (Platyhelminthes, Kalyptorhynchia) in marine tropical areas: first data from the Caribbean. *Belg. J. Zool.* **124**, 157–166.
- Ehlers, U., 1995: The basic organization of the Plathelminthes. *Hydrobiologia* **305**, 21–26.

- Ferguson, F. F., 1937a: The morphology and taxonomy of *Macrostomum virginianum* n. sp. *Zool. Anz.* **119**, 25–32.
- Ferguson, F. F., 1937b: The morphology and taxonomy of *Macrostomum beaufortensis* n. sp. *Zool. Anz.* **120**, 230–235.
- Ferguson, F. F., 1939a: A monograph of the genus *Macrostomum* O. Schmidt, 1848. Part III. *Zool. Anz.* **128**, 49–68.
- Ferguson, F. F., 1939b: A monograph of the genus *Macrostomum* O. Schmidt, 1848. Part IV. *Zool. Anz.* **128**, 188–205.
- Ferguson, F. F., 1939c: A monograph of the genus *Macrostomum* O. Schmidt, 1848. Part V. *Zool. Anz.* **128**, 274–291.
- Ferguson, F. F., 1940a: A monograph of the genus *Macrostomum* O. Schmidt, 1848. Part VI. *Zool. Anz.* **129**, 21–48.
- Ferguson, F. F., 1940b: A monograph of the genus *Macrostomum* O. Schmidt, 1848. Part VIII. *Zool. Anz.* **129**, 244–266.
- Gardiner, E. G., 1898: The growth of the ovum, formation of the polar bodies, and the fertilization in *Polychoerus caudatus*. *J. Morph. Physiol.* **15**, 73–110.
- Henry, J. Q.; Martindale, M. Q.; Boyer, B. C., 2000: The unique developmental program of the acoel flatworm, *Neochildia fusca*. *Dev. Biol.* **220**, 285–295.
- Imai, H. T.; Crozier, R. H.; Taylor, R. N., 1977: Karyotype evolution in Australian ants. *Chromosoma* **59**, 341–393.
- Imai, H. T.; Satta, Y.; Wada, M.; Takahata, N., 2002: Estimation of the highest chromosome number of eukaryotes based on the minimum interaction theory. *J. Theor. Biol.* **217**, 61–74.
- Imsiecke, G.; Custodio, M.; Borojevic, R.; Steffen, R.; Moustafa, M. A.; Müller, W. E. G., 1995: Genome size and chromosomes in marine sponges (*Suberites domuncula*, *Geodia cydonium*). *Cell Biol. Int.* **19**, 995–1000.
- Jones, W. A., 1944: *Macrostomum hustedi*, n. sp.; a morphological and cytological study of a rhabdocoel turbellarian. *J. Morphol.* **75**, 347–359.
- Ladurner, P.; Mair, G. R.; Reiter, D.; Salvenmoser, W.; Rieger, R. M., 1997: Serotonergic nervous system of two macrostomid species: recent or ancient divergence?. *Invertebr. Biol.* **116**, 178–191.
- Ladurner, P.; Schärer, L.; Salvenmoser, W.; Rieger, R. M., 2005: *Macrostomum lignano*, n. sp. (Rhabditophora, Macrostromorpha): A new model organism among the lower Bilateria and the use of digital video microscopy in taxonomy of meiobenthic Platyhelminthes. *J. Zool. Syst. Evol. Res.* **43**, 114–126.
- Levan, A.; Fredga, K.; Sandberg, A. A., 1964: Nomenclature for centromeric position of centromeres. *Hereditas* **52**, 201–220.
- Littlewood, D. T.; Olson, P. D., 2001: Small subunit rDNA and the Platyhelminthes: signal, noise, conflict and compromise. In: Littlewood, D., Bray, R. A. (eds), *Interrelationships of the Platyhelminthes*. London: Taylor & Francis, pp. 262–278.
- Lizarralde, M.; Bolzán, A.; Bianchi, M., 2003: Karyotype evolution in South American subterranean rodents *Ctenomys magellanicus* (Rodentia: Octodontidae): chromosome rearrangements and (TTAGGG)_n telomeric sequence localization in 2n = 34 and 2n = 36 chromosomal forms. *Hereditas* **139**, 13–17.
- Luther, A., 1905: Zur Kenntnis der Gattung *Macrostoma*. *Festschrift für Palmén* **5**, 1–66.
- Luther, A., 1947: Untersuchungen an rhabdocoelen Turbellarien. VI. Macrostromiden aus Finnland. *Act. Zool. Fenn.* **49**, 1–40.
- Marcus, E., 1947: Turbellarios marinhos do Brasil. *Bol. Fac. Ci. Letr. Zoologia* **12**, 99–215.
- Marcus, E., 1949: Turbellaria Brasileiros (7). *Bol. Fac. Ci. Letr. Zoologia* **14**, 7–156.
- Marcus, E., 1952: Turbellaria Brasileiros (10). *Bol. Fac. Ci. Letr. Zoologia* **17**, 5–188.
- Marcus, E., 1954: Turbellaria Brasileiros, Vol. 11. Sao Paulo, Brasil, Departamento de Zoologia, Secretaria da Agricultura, 419–489.
- Name, G. V., 1899: The maturation, fertilization and early development of the planarians. *Trans. Conn. Acad. Sci.* **10**, 263–300.
- Papi, F., 1950: Ricerche cariologiche sui Rabdoceli. I. Sopra alcuni rappresentanti della Fam. Typhloplanidae. *Caryologia* **2**, 113–126.
- Papi, F., 1951: Ricerche sui Turbellari Macrostromidae. *Arch. Zool. Ital.* **36**, 288–341.
- Patterson, J. T.; Wieman, H. L., 1912: The uterine spindle of the polyclad *Planocera inquilina*. *Biol. Bull.* **23**, 271–293.
- Phillips, H. M., 1936: A cytological study of *Macrostomum tuba* von Graff. *Zool. Anz.* **114**, 322–330.
- Puccinelli, I.; Curini-Galletti, M.; Mariotti, G.; Moretti, I., 1990: Chromosomal evolution and speciation in the *Gyratrix hermaphroditus* species complex (Platyhelminthes, Kalyptorhynchia): Karyometric and morphological analysis of 15 fresh-water populations from West-Europe. *Hydrobiologia* **190**, 83–92.
- Raikova, O. I.; Reuter, M.; Jondelius, U.; Gustafsson, M. K., 2000: The brain of the Nemertodermatida (Platyhelminthes) as revealed by anti-5HT and anti-FMRamide immunostainings. *Tissue Cell* **32**, 358–365.
- Reisinger, E., 1933: Turbellaria der Deutschen Limnologischen Sunda-Expedition. *Arch. Hydrobiol., Suppl.-Bd. XII. "Tropische Binnengewässer, Band IV"*, pp. 239–262.
- Reuter, M.; Raikova, O. I.; Gustafsson, M. K., 1998: An endocrine brain? The pattern of FMRamide immunoreactivity in Acoela (Platyhelminthes). *Tissue Cell* **30**, 57–63.
- Reuter, M.; Raikova, O. I.; Jondelius, U.; Gustafsson, M. K. S.; Maule, A. G.; Halton, D. W., 2001: Organisation of the nervous system in the Acoela: an immunocytochemical study. *Tissue Cell* **33**, 119–128.
- Richardson, K. C.; Jarret, L.; Finke, E. H., 1960: Embedding in epoxy resins for ultrathin sectioning in electron microscopy. *Stain. Technol.* **35**, 313–323.
- Rieger, R. M., 2001: Phylogenetic systematics of the Macrostromorpha. In: Littlewood, D., Bray, R.A. (eds), *Interrelationships of the Platyhelminthes*. London: Taylor & Francis, pp. 28–38.
- Ruebush, T. K., 1938: A comparative study of the Turbellaria chromosomes. *Zool. Anz.* **122**, 321–329.
- Ruiz-Trillo, I.; Riutort, M.; Littlewood, D. T.; Herniou, E. A.; Baganà, J., 1999: Acoel flatworms: earliest extant bilaterian metazoans, not members of Platyhelminthes. *Science* **283**, 1919–1923.
- Ruiz-Trillo, I.; Paps, J.; Loukota, M.; Ribera, C.; Jondelius, U.; Baganà, J.; Riutort, M., 2002: A phylogenetic analysis of myosin heavy chain type II sequences corroborates that Acoela and Nemertodermatida are basal bilaterians. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 11246–11251.
- Stirewalt, M. A., 1937: *Microstomum bispiralis* n. sp. *Zool. Anz.* **119**, 314–321.
- Telford, M. J.; Lockyer, A. E.; Cartwright-Finch, C.; Littlewood, D. T. J., 2004: Combined large and small subunit ribosomal RNA phylogenies support a basal position of the acoelomorph flatworms. *Proc. R. Soc. Lond., B, Biol. Sci.* **270**, 1077–1083.
- Tyler, S., 2001: The early worm – origins and relationships of the lower flatworms. In: Littlewood, D., Bray, R.A. (eds), *Interrelationships of the Platyhelminthes*. London: Taylor & Francis, pp. 3–12.
- Valkanov, A., 1938: Cytologische Untersuchungen über die Rhabdocoelen. *Jb. Univ. Sofia, Physiko-Math. Fak.* **34**, 321–402.
- Yamamoto, K.; Sasaki, G.; Kawakatsu, M., 2003: Chromosomes of a macrostomid species from Central Japan: *Macrostomum gigas* Okugawa, 1930? Available at: <http://planarian.net/kswp/39/mgi-gas.pdf>.

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