

Biogeography and Karyotypes of Freshwater Planarians (Platyhelminthes, Tricladida, Paludicola) in Southern Brazil

Tanise Knakievicz^{1*}, Adriana Helena Lau², Daniel Prá¹ and Bernardo Erdtmann³

¹Programa de Pós-Graduação em Genética e Biologia Molecular. Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre-RS, Brasil

²Universidade Luterana do Brasil (ULBRA), Canoas-RS, Brasil

³Instituto de Biotecnologia da Universidade de Caxias do Sul (UCS), Caxias do Sul-RS, Brasil

In the Tricladida (Platyhelminthes), the incidence of different biotypes identified by several ploidy levels is very common. Planarians collected in the State of Rio Grande do Sul were identified using cytogenetics. Different species distributions were observed with respect to Rio Grande do Sul's geomorphology, which could have been caused by their different microhabitats. *Girardia tigrina* and *G. anderlani* consisted of diploid and triploid individuals, whereas *G. schubarti* showed diploids, triploids, and mixoploids; for all these species, individuals of different ploidies were sympatric. Only for diploid *G. anderlani* were B chromosomes observed. These B chromosomes seem to have an irregular segregational behavior during mitosis, and possibly also during meiosis. However the processes (e.g., selection, mutation) of maintaining 2n, 3n, and 2n/3n individuals within natural populations of *G. schubarti* remain to be clarified.

Key words: B chromosomes, karyotypes, mixoploidy, polyploidy, taxonomy

INTRODUCTION

In Tricladida, the presence of different biotypes identified by several ploidy levels is very common. Diploid and triploid individuals were reported in *Dugesia japonica* (Tamura *et al.*, 1995, 1998), *Schmidtea lugubris* (Gremigni *et al.*, 1980; Storhas *et al.*, 2000), *Girardia anceps* (Benazzi and Benazzi-Lentati, 1976), *Polycelis nigra* (Beukeboom *et al.*, 1998), and *Schmidtea polychroa* (Storhas *et al.*, 2000). Mixoploids were found in *Schmidtea polychroa* (Benazzi and Benazzi-Lentati, 1976), *Girardia schubarti* (Kawakatsu *et al.*, 1980), *G. anderlani* (Kawakatsu *et al.*, 1983), and *Dugesia ryukyensis* (Tamura *et al.*, 1998). Tetraploids and pentaploids occur in *Schmidtea polychroa* (Beukeboom *et al.*, 1996) and *G. schubarti* (Kawakatsu *et al.*, 1984). In the order Paludicola, B chromosomes are present in polyploid populations (Beukeboom *et al.*, 1994; Benazzi and Benazzi-Lentati, 1976). These data represent significant material for evolutionary studies on ploidy levels.

Six species of planarians have been identified in Rio Grande do Sul (Southern Brazil): *Girardia (Cura) schubarti* (Marcus, 1946), *Girardia tigrina* (Girard, 1850), *Girardia anderlani* (Kawakatsu *et al.*, 1983), *Girardia uroriogranda* (Kawakatsu *et al.*, 1992), *Girardia arndti* (Marcus, 1946), and *Girardia biapertura* (Sluys, *et al.*, 1997). The first three, *G. schubarti*, *G. tigrina*, and *G. anderlani*, have been

the subjects of studies on morphology, karyology, and taxonomy (Kawakatsu *et al.*, 1981, 1982, 1985, 1993; Vreys *et al.* 2002), reproduction and life-cycle (Knakievicz *et al.*, 2006), and ecotoxicology (Guecheva *et al.*, 2001, 2003; Preza *et al.*, 2001; Prá *et al.*, 2005). However, little is known about their biogeography and ecology.

This work aimed to obtain preliminary data on the karyotype variation and geographical distribution of *G. schubarti*, *G. tigrina*, and *G. anderlani* in the State of Rio Grande do Sul for future experimental approaches based on modern biomolecular techniques and/or applications to environmental monitoring.

MATERIALS AND METHODS

Sampling

Specimens were collected in lotic and lentic freshwater environments at various sites in Rio Grande do Sul from 1996 to 2000. Worms were detached from pebbles with small brushes and transported to the laboratory in plastic containers filled with water. In some cases, different sites of the same lake or creek were sampled. Previously published data on the distribution of species of *Girardia* Genus in Brazil are shown in Table 1.

Culturing

In the laboratory, planarians were cultivated in atoxic plastic containers filled with reconstituted water (0.228 mM NaHCO₃, 0.013 mM KCl, 0.060 mM MgSO₄, 0.090 mM CaSO₄) (Cabridenc, 1979) and fed weekly with small chunks of bovine liver. The water was immediately changed after feeding. Populations were kept in a dark environment at the optimal temperature for each species, 18–22°C for *G. tigrina* (Gee *et al.*, 1998) and 13–15°C for *G. schubarti* (Knakievicz, *et al.*, 2006). Only *G. anderlani* did not adapt to laboratory conditions before karyotyping.

* Corresponding author. Phone: +51-3308-6070;
Fax : +51-3308-7309;
E-mail: tanisek@cbiot.ufrgs.br

Karyology

Regenerating tissue obtained as described by Baguña *et al.* (1989) was used for karyotyping. The regenerating tissue (postblastema) was dissected and treated with colchicine (0.2% for 3 h). The colchicine-treated blastemas were minced and further disaggregated by pipetting several times. They were then incubated for 20 min in a hypotonic KCl solution (0.075 M). Free cells and tissue fragments were sedimented by centrifugation and resuspended in 5 ml of cold Carnoy's solution. Slides were prepared by the air-drying method and stained with 10% Giemsa (Lamatsch *et al.*, 1998) for standard anal-

ysis under a ZEISS Axiophot optical microscope. At least 10 mitotic cells were analyzed to determine the ploidy level of each individual. However, the average was 26 mitotic cells analysed per individual.

RESULTS

Biogeography of *Girardia* in the State of Rio Grande do Sul

For biogeographical and ecological studies, a total of 140 freshwater planarians were karyotyped from 16 areas in Rio Grande do Sul (Table 2). The samples comprised *G.*

Table 1. Data published about the distribution of the species of *Girardia* Genus in Brazil.

City/State ^a	Species	Reference
Arroio do Meio/RS	<i>G. ururiograndeana</i>	Kawakatsu <i>et al.</i> , 1992
Arroio Grande/RS	<i>G. ururiograndeana</i>	Kawakatsu <i>et al.</i> , 1992
Caxias do Sul/RS	<i>G. schubarti</i>	Kawakatsu <i>et al.</i> , 1980
Constantina/RS	<i>G. sp</i>	Kawakatsu <i>et al.</i> , 1986
Dois Irmãos/RS	<i>G. anderlani</i> , <i>G. schubarti</i> and <i>G. sp</i>	Hensel, 1980; Kawakatsu <i>et al.</i> , 1976, 1980, 1986
Encruzilhada do Sul/RS	<i>G. anderlani</i>	Kawakatsu <i>et al.</i> , 1986
Montenegro/RS	<i>G. schubarti</i>	Hensel, 1980; Kawakatsu <i>et al.</i> , 1980
Nova Petrópolis/RS	<i>G. sp</i>	Kawakatsu <i>et al.</i> , 1980
Novo Hamburgo/RS	<i>G. schubarti</i>	Kawakatsu <i>et al.</i> , 1980
Pelotas/RS	<i>G. anderlani</i> , <i>G. schubarti</i> and <i>G. tigrina</i>	Kawakatsu <i>et al.</i> , 1986
São Francisco de Paula/RS	<i>G. schubarti</i> and <i>G. tigrina</i>	Kawakatsu <i>et al.</i> , 1986
São Leopoldo/RS	<i>G. schubarti</i> and <i>G. tigrina</i>	Kawakatsu <i>et al.</i> , 1980
Sapiranga/RS	<i>G. schubarti</i>	Hensel, 1980
Botucatu/SP	<i>G. schubarti</i>	Kawakatsu <i>et al.</i> , 1983
Campos do Jordão/SP	<i>G. schubarti</i>	Kawakatsu <i>et al.</i> , 1976
Cantareira/SP	<i>G. schubarti</i>	Kawakatsu <i>et al.</i> , 1983
Cidade Universitaria/SP	<i>G. tigrina</i>	Kawakatsu <i>et al.</i> , 1981
Monte Alegre/SP	<i>G. schubarti</i>	Kawakatsu <i>et al.</i> , 1976
Salesópolis/SP	<i>G. schubarti</i>	Kawakatsu <i>et al.</i> , 1976
São Carlos/SP	<i>G. schubarti</i> and <i>G. tigrina</i>	Kawakatsu <i>et al.</i> , 1982
Salvador/BA	<i>G. tigrina</i>	Preza and Smith, 2001
Serra da Mantiqueira/RJ	<i>G. schubarti</i>	Kawakatsu <i>et al.</i> , 1976

^aRio Grande do Sul state (RS), São Paulo state (SP), Rio de Janeiro state (RJ), and Bahia state (BA).

Table 2. Sampling sites and karyotypic classification of the collected planarians in Rio Grande do Sul state.

Site	Species/ karyotype ^a						
	<i>G. schubarti</i>			<i>G. tigrina</i>		<i>G. anderlani</i>	
	2n=8	3n=12	2n/3n	2n=16	3n=24	2n=18	3n=27
Arambaré	–	–	–	3	–	–	–
Caçapava do Sul	–	–	–	–	–	5	–
Dois Irmãos	2	–	–	–	–	–	–
Erechim	1	1	1	–	–	1	2
Flores da Cunha	6	–	–	–	–	7	–
Glorinha	–	–	–	1	–	–	–
Jacutinga	–	12	–	–	–	–	–
Porto Alegre	–	–	–	13	–	–	–
Salvador do Sul	–	–	–	1	2	5	–
Santa Maria	6	–	2	–	–	2	–
Santo Ângelo	–	9	6	–	–	–	–
São Francisco de Paula	16	–	3	–	–	1	–
São José dos Ausentes	6	–	–	–	–	–	–
Severiano de Almeida	10	–	1	–	–	3	–
Uruguaiana	5	–	1	–	–	2	–
Viamão	–	–	–	–	–	1	–
Total	54	22	15	18	2	27	2

^anumber of planarians (identified from of the analyses of at least ten metaphasic cells per individual).

schubarti, *G. tigrina*, and *G. anderlani*, taken from distinct geomorphological regions of Rio Grande do Sul.

Girardia schubarti occurred under stones in well-oxygenated lotic water bodies. It was found predominantly in

creeks of the Basaltic Rock Plateau, a geomorphological region extending from São Paulo State to the northern half of Rio Grande do Sul (Tables 1 and 2; Fig. 1b).

Girardia tigrina was detected among vegetation in lentic

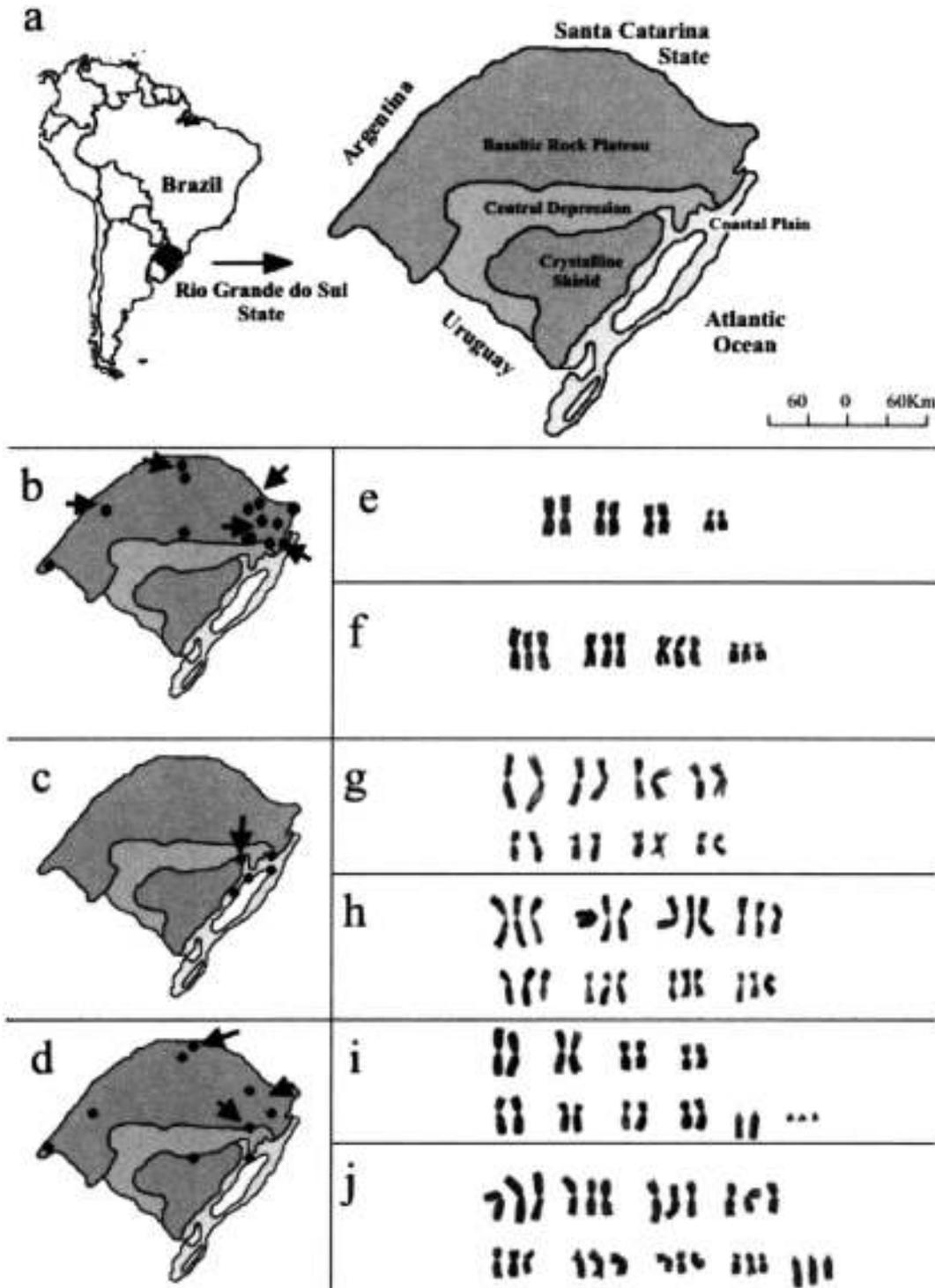


Fig. 1. Geographic distribution and karyotypes of freshwater planarians in the State of Rio Grande do Sul, Brazil. The black dots indicate where diploid planarians were found. The black arrows indicate where triploids or mixoploids were also found. (a) Morphogeological regions of Rio Grande do Sul State (map adapted from Ramgrab *et al.*, 2000). (b) Distribution of collected *Girardia schubarti* specimens. (c) Distribution of collected *Girardia tigrina* specimens. (d) Distribution of collected *Girardia anderlani* specimens. (e, f) 2n and 3n karyotypes of *G. schubarti*. (g, h) 2n and 3n karyotypes of *G. tigrina*. (i, j) 2n and 3n karyotypes of *G. anderlani*.

water bodies. It was found in lakes of the Serra Geral's scarp, the Central Depression, the Coastal Plain (Fig. 1c), and northern Uruguay (Table 1).

Girardia anderlani was detected in sympatry with *G. schubarti* in some places located at the borders of lakes and streams. *Girardia anderlani* was also found in the Central Depression, Coastal Plain, and Crystalline Shield regions (Figs. 1d).

These geographical distributions indicated by our data (Table 2) and previous studies (Table 1) suggest a rough association between species and geomorphological regions in Rio Grande do Sul.

Cytogenetics of *Girardia* in southern Brazil

Planarians were checked cytogenetically for ploidy levels. Diploid ($2n=8$; Fig. 1e), triploid ($3n=12$, Fig. 1f), and mixoploid ($2n/3n$) individuals of *G. schubarti* were detected at different sampling sites (Fig. 1b). The sample population consisted of 54 diploids (68%), 22 triploids (14%), and 15

mixoploids (18%) (Table 2).

For *G. tigrina*, diploid ($2n=16$; Fig. 1g) and triploid ($3n=24$; Fig. 1h) specimens were collected (Fig. 1c). The sample population consisted of 18 diploids (65%) and two triploids (35%) (Table 2).

Diploid ($2n=18$; Fig. 1i) and triploid ($3n=27$; Fig. 1j) specimens of *G. anderlani* were collected (Fig. 1d). The sample population consisted of 27 diploids (93%) and two triploids (7%) (Table 2). B chromosomes were found in a diploid *G. anderlani* population from Salvador do Sul. The Bs are small, acrocentric chromosomes, approximately one-quarter of the size of the smallest pair (Fig. 1i). When present, they range in number from zero to three in the somatic cells of diploid individuals. This pattern is not constant for all cells of the same planarian (Table 3). In one of the planarians, it was possible to observe six meiotic cells; all revealed only one B chromosome, whereas in somatic cells from the same animal the B chromosomes varied from zero to three.

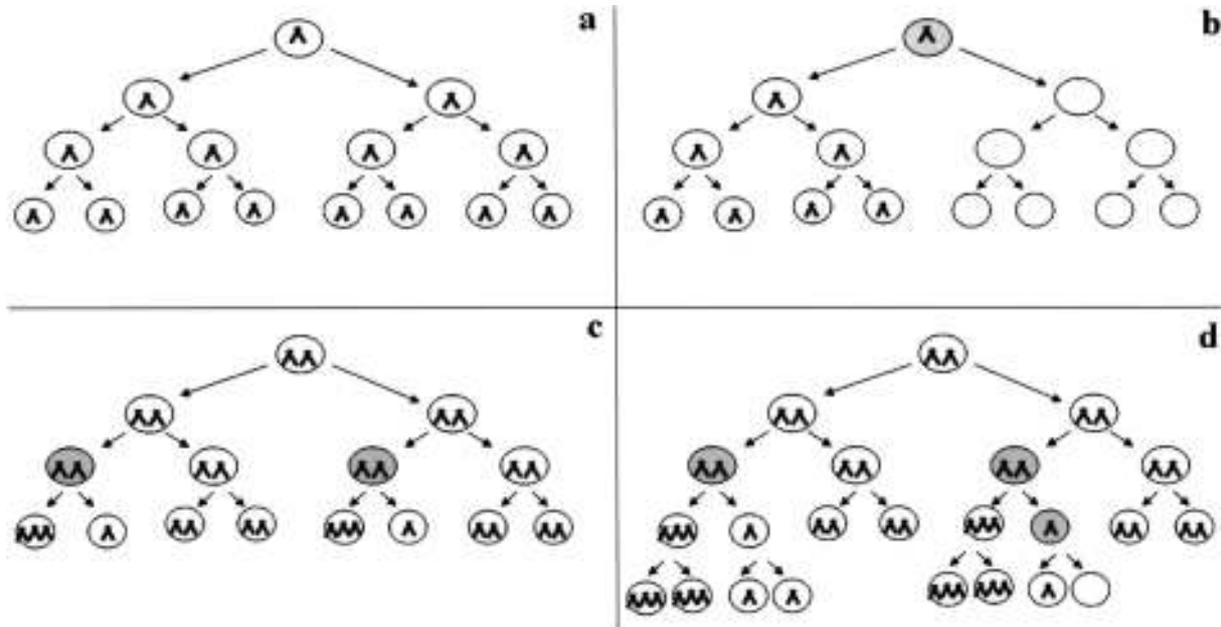


Fig. 2. Hypothetical schemes, for four of the specimens studied, of irregular segregational behavior of B chromosomes during mitosis in *G. anderlani* that explain the observed number of B chromosomes. The zygotes originated from fusion of one gamete with and the other without B chromosomes. Planarians with irregular segregation during mitosis resulted in different distributions of B chromosomes. **(a)** Specimen PLA 45. **(b)** Specimen PLA 38. **(c)** Specimen PLA 44. **(d)** Specimen PLA 1000. In the schematic diagrams, the symbol of an acrocentric chromosome indicates a B chromosome.

Table 3. Frequency of B-chromosomes in somatic cells of *Girardia anderlani* collected at Linha Júlio de Castilho site of Salvador do Sul.

Individual	Cell Number	Percentage of cells with different number of B Chromosomes (%)			
		0	1	2	3
PLA 37	6	100	–	–	–
PLA 38	7	57	43	–	–
PLA 44	23	4	30	43	22
PLA 45	9	–	100	–	–
PLA 100*	14	–	35	50	15

* In six meiotic cells analyzed all contained only one B-chromosome.

DISCUSSION

Gamo and Noreña-Janssem (1998) found turbellarians to be widely distributed in unpolluted streams and lakes, and to be sensitive to environmental variation and pollution. Similarly, we did not find any species of *Girardia* in water bodies polluted by domestic sewage, either in urban or rural areas of Rio Grande do Sul State. This knowledge of planarian ecology, in addition to their easy experimental manipulation, makes them potential candidates as bioindicator organisms.

Species of planarians have distinct biogeographic distributions in the southernmost Brazilian state of Rio Grande do Sul (Fig. 1), except in regions of geomorphological transition (Table 1; Kawakatsu *et al.*, 1992; Sluys *et al.*, 1997). *Girardia (Cura) schubarti* is native to Brazil (Benazzi and Benazzi-Lentati 1976), and its occurrence has been reported in several Brazilian states, including São Paulo, Santa Catarina, and Rio Grande do Sul (Table 1). Its populations vary in ploidy and reproductive behavior (Kawakatsu *et al.*, 1984; Knakievicz *et al.*, 2006). *Girardia schubarti* is found in freshwater lentic streams on elevated basaltic plains (700 m above sea level, on average), usually beneath stones.

Girardia tigrina was first described in Girard (1850), and was later redescribed by Kawakatsu *et al.* (1981). It is a cosmopolitan species distributed in North America (Gee *et al.*, 1998), Brazil (Bahia, São Paulo, Santa Catarina, and Rio Grande do Sul; Table 1), Uruguay (Kawakatsu *et al.*, 1992), and Europe (Benazzi *et al.*, 1971; Benazzi and Benazzi-Lentati 1976; Ribas *et al.*, 1989), and has recently been reported from Japan (Tamura *et al.*, 1998). It is possible that its worldwide dispersal was mediated by aquatic plants and ships' ballast water. In Rio Grande do Sul, *G. tigrina* was typically found attached to aquatic plants in freshwater lentic habitats such as the ponds and lakes of the sedimentary Central Depression and Coastal Plain (200 m above sea level, on average). *Girardia tigrina* mixoploids were not collected in our study.

The distributions of planarians were in accordance with the geomorphology of Rio Grande do Sul; however, this could have been due to their different microhabitats. Our results corroborated previous data on the species' distribution in Rio Grande do Sul (Kawakatsu *et al.*, 1992; Sluys *et al.*, 1997). Planarians use spatial and temporal separation to reduce competition, and these involve responses to physical aspects of their microhabitats (Reynoldson, 1981). However, *G. anderlani* has so far been found only in Rio Grande do Sul, in all types of geomorphological formations, and whether or not its distribution is flexible has yet to be studied.

Despite optimal reproduction temperatures of 18–22°C for *G. tigrina* (Gee *et al.*, 1998) and 13–15°C for *G. schubarti* (Knakievicz *et al.*, 2006), with the optimal temperature unknown for *G. anderlani*, no seasonal variation was detected in any of the three species collected. However, mixoploid (2n/3n) individuals of *G. schubarti* are more frequent in warmer seasons and usually die at temperatures below 6°C. Surprisingly, in Rio Grande do Sul, mixoploid individuals are able to constantly reemerge as offspring from sexual reproduction (Knakievicz *et al.*, 2006).

It is interesting to speculate on the advantages of simul-

taneously maintaining 2n, 3n, and 2n/3n individuals in natural populations, as has been observed for *G. schubarti* (Knakievicz, *et al.*, 2006), *G. tigrina* (Ribas *et al.*, 1989), *Dugesia japonica*, and *D. ryukyuensis* (Tamura *et al.*, 1995). In mixoploid individuals of *G. schubarti*, the proportion of 2n to 3n cells can vary in response to environmental conditions, including food abundance (data not shown), which causes an increase in mitotic response (Baguña, 1974); in our study, the ratio of 2n:3n cells showed no correlation with the frequency of fissiparity (data not shown).

Our data corroborate the hypothesis that ploidy variation may be an important determinant of morphological and physiological plasticity (Gregory, 2002) and, at least in some cases, may favor speciation events (Gregory, 2003). However the processes (e.g. selection, mutation) that maintain 2n, 3n, and 2n/3n individuals in natural populations remain to be clarified. It is known that different environmental factors such as dissolved oxygen, the types and amounts of other chemicals, and water temperature are relevant to platyhelminth species distributions (Reynoldson, 1965, 1981). Since a strong correlation exists between chromosomal polymorphism and asexuality, it could be argued that in these populations fission has been induced by gene imbalance due to intra-arm duplication somehow affecting fission-controlling genes (Vries *et al.*, 1984). Although a few of these factors were assessed in another study (Knakievicz, *et al.*, 2006), we intend to address such issues in future research.

From zero to three B chromosomes were found in diploid *G. anderlani* individuals. B chromosomes are defined as dispensable supranumerary chromosomes that do not recombine with A chromosomes and follow their own evolutionary pathway. B chromosomes have been described in several planarian species: *D. benazzi*, *Polycelis tenuis* (Benazzi and Benazzi-Lentati, 1976), *P. nigra* (Beukeboom *et al.*, 1996), *Dugesia estrusca*, *D. gonocephala* (Deri *et al.*, 1999), and *D. japonica* (Tamura *et al.*, 1998). B chromosomes are commonly more frequent in polyploid than in diploid strains and in species with multiples ploidies (Palestis *et al.*, 2004). They do not seem to show any geographical restriction (Camacho *et al.*, 1996) and probably occur in all planarian taxa. The major obstacle to comparative studies of the distribution of B chromosomes has been variation in the intensity of cytogenetic study among species (Palestis *et al.*, 2004).

In *G. anderlani*, variation in the number of B chromosomes was observed among cells of the same individual, suggesting irregular segregational behavior during mitosis (Table 2). For one individual, just six meiotic cells were analysed, and all of them contained only one B-chromosome (PLA 100, Table 3). If the behavior of B chromosomes during meiosis favored regular segregation, the individuals (Table 3) could each have received one maternal and/or one paternal B chromosome. In this case, zygotes could have contained one or two B chromosomes. Variation in the number of B chromosomes among cells of the same individual can be explained by irregular segregational behavior during mitosis (Fig. 2). However, it is unknown whether the behavior of B chromosomes in meiosis of *G. anderlani* is regular or irregular.

In the *P. nigra* polyploid lineage (Benazzi and Benazzi-

Lentati, 1976), which can reproduce either sexually or asexually through pseudogamous parthenogenesis, B chromosomes were unstable during meiosis, and their dynamics appear to comply with the selfish DNA theory (Beukeboom *et al.*, 1997). B Chromosomes as selfish DNA have two possible distinct properties: a) they arise when a DNA sequence spreads by forming additional copies of itself within the genome, and b) they make no specific contribution to the phenotype (Orgel and Crick, 1980). These properties suggest the need for further studies to clarify the role of B-chromosome DNA (Beukeboom, 1994; Palestis *et al.*, 2004).

Since B chromosomes can be present or absent in diploid populations of *G. anderlani*, this appears to be a good opportunity to evaluate B chromosome fitness and stability under the same environmental conditions in the laboratory and their possible involvement in speciation events. The presence of an acrocentric chromosome pair and acrocentric B chromosomes suggests that B chromosome segregation during meiosis could have facilitated the speciation of *G. anderlani*. The available evidence reinforces that such things as chromosomal evolution (Batistoni *et al.*, 1999), reproductive strategy (Kobayashi *et al.*, 1999), and micro-evolutionary change play a major role in the process of speciation of these planarians (Baguñá *et al.*, 1999).

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