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Genome Instability of *Chironomus riparius* Mg. (Diptera, Chironomidae) from Polluted Water Basins in Bulgaria

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Abstract. Larvae of *Chironomus riparius* Mg. (Chironomidae, Diptera) collected from two polluted water basins in Bulgaria, the Maritsa and Chaya Rivers (adjacent to Plovdiv and Asenovgrad respectively), a small pool (near Plovdiv) plus controls reared in the laboratory were studied. High concentrations of the heavy metals Pb, Cu and Cd were recorded in the sediments of the polluted stations. Marked somatic structural chromosome aberrations were found in *C. riparius* salivary polytene chromosomes from the field stations and their frequency was significantly higher ($p<0.01$) compared to the control. The observed somatic chromosome changes are discussed as a response of the chironomid genome to aquatic pollution. A new cytogenetic index based on the number of aberrations found in larvae from polluted regions in comparison with the control was applied to the data to more easily evaluate the degree of heavy metal pollution in aquatic ecosystems. Our study of a polluted site near the River Chaya showed that the somatic index was very high at 3.35 for 2010 and 11.66 for 2013 compared to 0.5 in the control. The cytogenetic index was effective in showing that all studied sites were highly polluted in comparison with the control. To determine the mechanism involved in the concentration of aberration breakpoints within specific regions of the chironomid polytene chromosome the FISH method was applied. The localization of a transposable element TFB1 along the polytene chromosomes of *C. riparius* was analyzed and the sites of localization were compared with breakpoints of chromosome aberrations. A significant correlation ($p<0.05$) was found which shows that most of the aberrations do not appear randomly but are concentrated in sites rich in transposable elements.

Key words: Chironomidae, polytene chromosomes, somatic and cytogenetic index, heavy metals.

Introduction

Pollution of aquatic ecosystems is a major ecological problem globally, coinciding with the rapid industrialization and urbanization that began in the early nineteenth century. In Bulgaria, numerous rivers have been classified as very polluted and shown to be contaminated with heavy metals by the National Biomonitoring Program (PEEV & GERASIMOV, 1999). The Maritsa and Chaya Rivers are polluted rivers in Bulgaria, with sediments highly contaminated with metals such as Cr, Cu, Mn, Pb and Cd (MICHAILOVA *et al*., 2012a). The biological effect of heavy metal exposure is of growing concern due to the increasing release of such metals into the environment via industrial and agricultural processes, exacerbated by their tendency to accumulate in biotic systems (WHITESIDE *et al*., 2010).

Biological indicators are useful in assessing the overall effect of environmental contaminations by virtue of their important role aquatic ecosystem structure and function (MENG *et al*., 2009). The larvae of

non-biting aquatic midge *Chironomus riparius* are dominant in many aquatic ecosystems, especially those with moderate to high levels of pollution (AL-SHAMI *et al*., 2010). *C. riparius* is considered as an ideal organism for ecotoxicological monitoring as the larvae spend most of their life cycle at the sediment-water interface where they therefore are exposed to toxicants both in the water and sediment. They have a short life cycle (MEBANE *et al*., 2008; AL-SHAMI *et al*., 2010; MICHAILOVA *et al*., 2012b) and are ideal organisms for comparative bioassays in the laboratory because they are relatively easy to culture (WARWICK, 1988). Moreover, our previous studies have showed that the polytene chromosomes of this species can be used as a bioassay of heavy-metal-induced genome instability (MICHAILOVA *et al*., 2012b).

In order to evaluate the impact of heavy metals on biota of the aquatic basins we used structural and functional alterations of the salivary gland chromosomes of Chironomids which provide early warning indicators of contaminants in the environment.

In this paper we outline a new, sensitive and a reliable method to assess the impact of toxicants, specifically heavy metals, on the genome of the larva stage of *C. riparius*. On the basis of different somatic chromosome alterations we propose a cytogenetic index as a sublethal method to assess the degree of pollution in freshwaters. For this purpose cytogenetic damage was investigated by analyzing the somatic alterations in individual *C. riparius* collected from the polluted Maritsa and Chaya Rivers and compare with mean somatic rearrangements found in laboratory reared control populations of the same species. The index, a ratio of somatic rearrangements in a control to potentially polluted environment is simple to apply and, by definition, provides as sublethal measure of pollution damage. We also performed in situ hybridization with selected transposable element in order to assess whether the somatic chromosome rearrangements are randomly distributed.

Material and Methods

Material

Chironomus riparius larvae were collected from the three stations in Maritsa and Chaya Rivers (Kemera, Asenovgrad and Katuniza) (in 2009, 2010, and 2013) and a farm near Plovdiv (in 2010). On the basis of data from the Bulgarian National Biomonitoring Program (1999), the Maritsa and Chaya Rivers are subject to industrial pollution, including the heavy metals Pb and Zn, and also $S0_2$, $N0_2$ (MICHAILOVA *et al*., 2012a). The pollution originates from producing metal products. A further site near Plovdiv is subject to domestic sewage and animal wastes.

The control (lab. stock) *C. riparius* larvae were reared from an existing laboratory population in pure mash filter paper under standard conditions (temperature 18 - 20°C, photoperiod 16h light & 8h dark; feeding 2 times a week and constant aeration).

Methods

Cytogenetic analysis. For cytogenetic analysis, the larvae of *C. riparius,* collected in the field and those from the lab. stock were fixed in alcohol: acetic acid (3:1). Polytene chromosome preparations were prepared using conventional cytogenetic method (MICHAILOVA, 1989). Chromosome maps of HäGELE (1970) and KIKNADZE *et al*. (1991) were used as a standard and applied to localize the somatic chromosome rearrangements.

In situ hybridization. A probe of fold-back transposable element was used. The TFB1 326 bp probe was obtained by using the following primers:

TFB1311-F (5'-GCAACGACTATTCCTACCTTGCC-3') and TFB1636-R (5'-TCACACCGTTTTCACGTGTGAATCT-3') and labeled with Digoxigenin. The hybridization signals were detected by the anti-digoxigenin antibody (Roche).

The locations of the TE copies along the salivary gland chromosomes were determined by FISH, following the methods of SCHMIDT (1992) and HANKELN *et al*.

(1993). The signals found in all studied cells and individuals were considered as fixed signals; variable signals appeared in all or almost all cells but not in all studied individuals (MICHAILOVA *et al*., 2009).

Water and sediment metal analysis. Two samples of water and sediment were collected from each site. The water samples were acidified on site using trace metal grade nitric acid. Acidification ensured that there was no adsorption on to the walls of the container and that the metals remained in their dissolved state for later analysis. Sediment samples were stored on plastic containers and frozen until analyzed.

Sediment samples were placed in an oven until dry, passed through a 250 micron sieve and a known amount in the range of 0.08-0.15g from each site transferred to glass vials for immediate analysis. Five ml of trace metal grade nitric acid was added to each sediment sample and digested on a hotplate. A marble was placed on top of each glass vial to facilitate reflux and the samples boiled gently until fumes of NO_2 were no longer produced. Once the digest had been completed the solutions were transferred to 10ml volumetric flasks and made up to volume using deionized water. Any remaining particulate in the samples was then removed by filtering through a 45 micron Whatman glass fiber filter paper.

After appropriate dilution in deionised water, metal (Cu, Cd, Pb, Cr and Mn) analysis of both water samples and sediment digests was carried out by inductively coupled plasma atomic emission spectroscopy (ICP-AES) using a Perkin-Elmer Optima 5300. The spectroscope was calibrated using an internal standard solution, which was a matrix matched serial dilution of Specpure multi element plasma standard solution 4 (Alfa Aesar).

Data analysis. We compared the somatic alterations in the polytene chromosomes of *C. riparius* from polluted stations with those from the laboratory stock used as a control. The types and localization of chromosome alterations of the polytene chromosomes of *C. riparius* from Maritsa and Chaya Rivers collected in 2009, 2010 and the Plovdiv farm collected in 2010 were analyzed as reported previously (MICHAILOVA *et al*., 2012a). In this paper we analyzed somatic chromosome rearrangements, their types and localization in *C. riparius* from Chaya River (2013) and the control.

Somatic aberrations affect only a few cells of the salivary glands of *C. riparius* and these were used for analyzing the somatic index (SELLA *et al*., 2004) by dividing the number of aberrations to the number of analyzed individuals at each site.

Here we present a new cytogenetic index of the polytene chromosomes of *C. riparius* collected from the Chaya River in 2013 and the control and further statistical analysis of somatic chromosome rearrangements found in this species from the Maritsa and Chaya Rivers collected in 2009, 2010 and from the Plovdiv farm in 2010. We applied the cytogenetic index as the ratio of the percentage aberrations in control to percentage aberrations in polytene chromosome of *C. riparius* from each polluted site.

We statistically analyzed somatic aberrations to make a comparative analysis between polluted and unpolluted material (control), using contingency G test (SOKAL & ROHLF, 1995). The values $p<0.05$ was considered as significant.

Results and Discussion

1. Water and sediment analysis

Concentrations of Cu, Cd, Pb, Cr and Mn in the water collected in 2013 were not elevated when compared to water quality standards (data not shown). However, in the four sites the concentrations of trace metal in the sediments were higher than those of reference data (FÖRSTNER & SALOMONS, 1980) and, as previously shown (MICHAILOVA *et al*., 2012a) in 2009 and 2010 (Table 1). The concentrations of Cu in the Maritsa River sediment collected in 2009 and 2010 were 5.8 and 4.8 times higher than the reference data; in Chaya River the concentration was between 3 to 12 times higher in 2009 and 2010; the Farm - 2.6 times. Lead in the Maritsa River sediments in 2009 and 2010 were 24 and 35 times

higher respectively; in the Chaya River (2009, 2010 and 2013) between 24-36 times higher and 7.8 times greater at the farm site. Cadmium concentrations in the Maritsa River - 84-94 times higher in 2009 and 2010; concentrations in the Chaya River (2009, 2010, 2013) were between 14-105 times higher; the farm site contained 14 times more metal than the reference data reported in FÖRSTNER & SALOMONS (1980). The concentrations of Pb, Cu and Cd in the sediments of the field stations were all higher than the control sediment (Table 1).

2. Cytogenetic characteristics and somatic chromosome alterations

Chironomus riparius has a chromosome set 2n = 8, belonging to the thummi cytocomplex (KEYL, 1962) with chromosome arm combination AB, CD, EF, G. Chromosomes AB, CD are metacentric, chromosome EF – submetacentric and chromosome G acrocentric (Fig. 1). Three Balbiani rings (BRa, BRb, BRc) and a Nucleoli Organizer (NOR) are localized in chromosome G. BRa is expressed in only a few cells of the salivary glands.

Table 1. Concentration (μg/g) of trace metals in the sediments of the each station

Stations	**Cr**	**Cu**	**Mn**	**Pb**	**Cd**
Fossil sediment	59	25	406	16	0.2
Maritsa River, 2009	45.19	120	1885.4	388.2	16.83
Maritsa River, 2010	0.173	144	63.8	559.78	18.81
Chaya River, 2009	70.4	314.3	434.7	585.9	7.3
Chaya River, 2010	0.114	213.8	60.21	536.75	2.86
Chaya River, 2013	18.4	75.23	765.87	383.41	21.03
Plovdiv-farm, 2010	0.127	66.44	74.05	293.07	2.84
Control, 2013	45.76	134.97	720.35	125.94	1.99

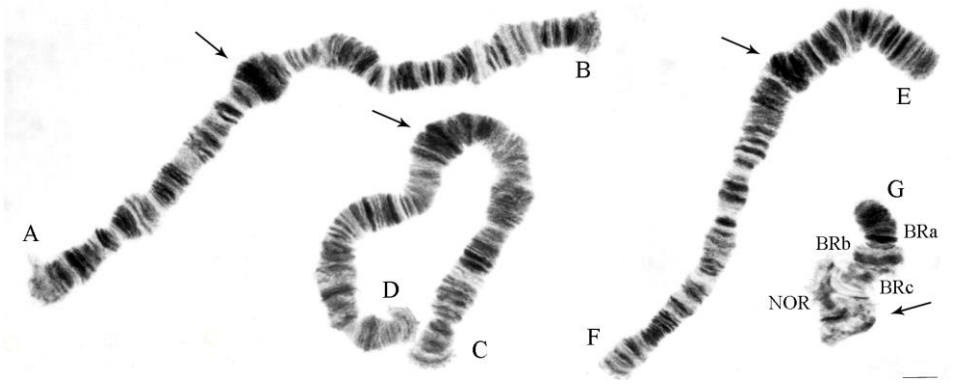

Fig 1. Standard karyotype of *C. riparius;* Balbiani Ring (BR); Nucleolar Organizer (NOR). The arrow indicates the centromere region in each chromosome. Bar - 10μm

A number of different somatic chromosome rearrangements (para- and pericentric heterozygous inversions, amplifications, deletions and deficiencies) were observed in the polytene chromosomes of *C. riparius* from all sites on the Chaya and Maritsa Rivers (Fig.2). The highest somatic index was calculated in *C. riparius* from the Chaya River (2013, 2010 – 11.66 and 3.25) (Table 2) where the concentrations of trace metals were highest.

As shown in Fig. 3, a significantly higher frequency of somatic alterations was found in larvae collected from all polluted sites when compared with larvae reared under laboratory conditions and hence not exposed to toxicants (Maritsa River, 2009 – $G = 66.631$, $df = 1$, $p<0.001$; Maritsa River,

2010 – G = 36. 889, df = 1, $p< 0.001$; Chaya River, 2009 - G = 73.766, df = 1, $p<0.001$; Chaya River, 2010 - G = 40.802, df = 1, $p< 0.001$; Chaya River, 2013 – G = 97.496, df = 1, $p<0.001$; Plovdiv, farm 2010 – G = 39.447, df = 1, $p<0.001$). Our comparative study of this biomarker revealed marked differences in the natural populations of *C. riparius* larvae collected from different polluted habitats. However, it is important to bear in mind that, under field conditions, it is difficult to attribute observed changes to a single metal because synergistic and antagonistic interactions may occur. Therefore, on the basis of applying, somatic and cytogenetic index we are not able to ascertain which trace metal specifically affects the genome structure, but we can show the degree of pollution at the different sites.

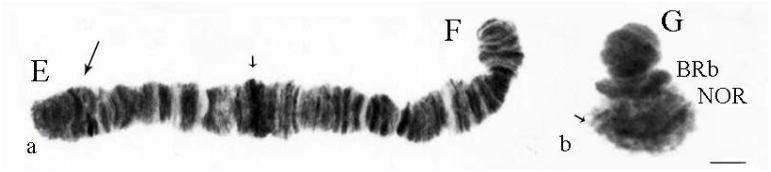

Fig. 2. Somatic aberrations in *C. riparius* polytene chromosomes; Balbiani Ring (BR); Nucleolar Organizer (NOR). The large arrow indicates the somatic aberration; the small arrow shows the localization of the centromere region. Bar - 10μm; a) Somatic heterozygous inversion in arm E; b) deletion of BRc

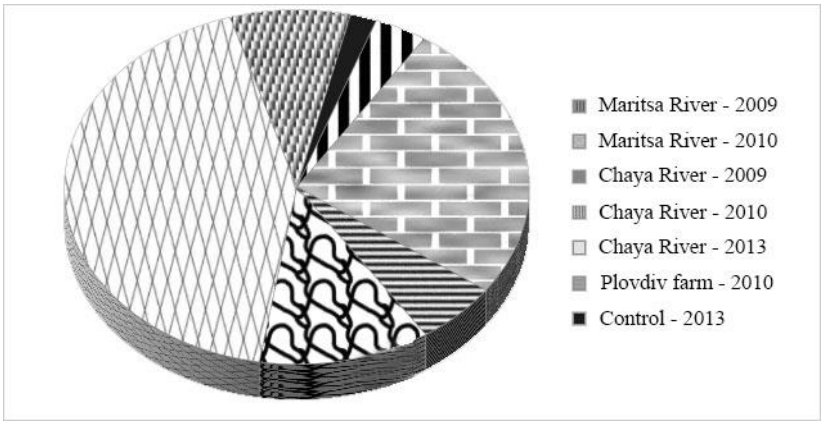

Fig. 3. Somatic index at each site studied

For the first time we suggest to use a cytogenetic index as a biomarker for evaluating the degree of environmental pollution (Table 2). The laboratory reared stock, which we used as a control therefore has a cytogenetic index of 1. On the basis of this index we proposed three levels of pollutants: slight pollution with a cytogenetic index near to the control (0.9-0.8), moderate polluted (0.7-0.5) and highly polluted (<0.5). Comparing our data using this cytogenetic index we can conclude that the all stations of both rivers are highly polluted. The Maritsa River (2010) and the Chaya River (2010, 2013) are the most heavy metal polluted, followed by the farm near Plovdiv and the Maritsa and Chaya Rivers in 2009 (Table 3).

The present results indicate that heavy metal pollutants at sublethal concentration influence the genome of the ubiquitous aquatic insect *C. riparius*. Our study suggests a relationship between concentrations of trace metals and a cytogenetic index of somatic chromosome rearrangements in *C. riparius* and this approach therefore merits further investigation. This could include examining the response to other stressors such as pesticides. We further suggest that our cytogenetic index using this model chironomid species would be a useful approach to be included in the suite of bioassessment methods.

3. In situ hybridization

The localization of TFB1 copies along polytene chromosomes of *C. riparius* from

the Chaya River (2010) and the farm adjacent to Plovdiv was studied and compared with observed breakpoints of somatic aberrations. The number of observed TFB1 signals is shown in Table 3. The fixed signals of the TE were concentrated in the proximal parts of the chromosomes (centromere regions) and variable signals were localized along the chromosome arms.

Table 2. Somatic chromosome rearrangements in the polytene chromosome of *C.riparius*, collected from polluted stations in Bulgaria and control plus the cytogenetic index for each site studied

Site /year	No indiv-iduals	No cells	No /% indv. with aberr.	No / % cells with aberr.	No somatic aberr.	S. index	Average No aberr. per indv. ($\bar{x}$ ±SD)	Cyto-genetic index	Reference
Maritsa River (2009)	13	**394**	12/92.31	90/22.84	13	1	22.30±10.61	**0.0968**	MICHAILOVA *et al*., 2012a; this study
Maritsa River (2010)	1	38	1/100	14/36.84	7	7	37±15.10	**0.0584**	MICHAILOVA *et al*., 2012a; this study
Chaya River (2009)	14	347	14/100	87/25.07	22	1.57	23.88±9.63	**0.0904**	MICHAILOVA *et al*., 2012a; this study
Chaya River (2010)	4	44	4/100	16/36.36	13	3.25	33.33±29.16	**0.0648**	MICHAILOVA *et al*., 2012a; this study
Chaya River (2013)	3	106	3/100	46 / 43.4	35	11.66	34±7.72	**0.0635**	This study
Plovdiv farm (2010)	7	111	7/100	26/23.42	16	2.28	28.71±16.67	**0.0742**	MICHAILOVA *et al*., 2012a; this study
Control (2013)	14	282	5	7/ 2.48	7	0.5	2.16±3.22	**1**	This study

Table 3. Number of TFB1 signals and common breakpoints of chromosome aberrations in *C. riparius* polytene chromosomes collected from two polluted stations

Station	Number of breakpoints	Common breakpoints of chromosome aberrations	Number of signals	
			Fixed signals	Variable signals
Plovdiv farm, 2010	65	46	17	135
Chaya River, 2010	85	60	13	94

In the larvae from the farm site, 22 of common breakpoints coincided with sites of TFB1. The correlation between frequency of common breakpoints and frequency of insertion sites of the TE was significant (rs = 0.53; df = 10; p<0.05). In the individuals from the other polluted sites on the Chaya River (Asenovgrad) we found 18 common breakpoints which significantly coincided with the TFB sites (rs = 0.85, df = 10; p<0.01) (ILKOVA *et al*., 2013).

These data support the hypothesis that aberration breakpoints do not appear randomly in the *C. riparius* genome and some sites on the chromosomes, called by us "hot spots", are more sensitive to various

environmental stress factors. BOVERO *et al*. (2002), MICHAILOVA *et al*. (2007), ILKOVA *et al*. (2007) found at these sites different mobile elements which are involved in producing the somatic chromosome rearrangements. The same effect have been established for other transposable elements (NLRCth1, CTRT) in the *C. riparius* genome (ILKOVA *et al*., 2013), in *C. piger* genome (ILKOVA *et al*., 2007; MICHAILOVA *et al*., 2009), plus in the fruit fly *Drosophila* (ZELENTSOVA *et al*., 1999, CASALS *et al*., 2005). These results support the hypothesis that stress agents in the environment activate transposable element mobility in the genome and influence genome instability, resulting in numerous somatic chromosome rearrangements (GUERREIRO, 2012; KALE *et al*., 2005).

In summary, our study has shown that the *C. riparius* genome is very sensitive to heavy metals stress and a number of different chromosome changes can be observed. On the basis of these changes and using somatic and cytogenetic index we are able to estimate the degree of pollution in river basins. Further work is required to establish a clearer relationship between individual heavy metal contaminants and the cytogenetic index over a range of metal concentrations and conditions, plus an examination of other anthropogenic stressors such as anoxia arising from pesticide release, organic pollution and eutrophication. The cytogenetic index could then prove to be a reliable and effective biomarker tool to assess the degree of genotoxicity of different toxicants.

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