Research Article

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The Response of Genome of the Chironomidae (Diptera) to Heavy Metal Pollution in Two Rivers of Southern Poland

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Abstract: The effects of heavy metals pollution in two Polish rivers on the genome of phylogenetically distant species of the family Chironomidae were studied. The concentrations of Pb, Zn and Cd in the channel sediments of Chechło and Biała Przemsza Rivers were many times higher than reference data, as a result of over 50 years of the lead and zinc ores extraction. In the studied species *Prodiamesa olivacea* Meigen, *P. bureschi* Michailova, *Chironomus riparius* Meigen and *C. annularius* Meigen, genome instability was evaluated by somatic and inherited chromosome aberrations. On the basis of somatic alterations, the somatic index (S) has been calculated and demonstrated highest value in the genus *Chironomus* (S = 2 in *C. annularius*). This index is a good biomarker for assessing the genotoxic effect of contaminants in the sediments. Cells with aberrations in *P. olivacea* were significantly higher than those in *P. bureschi* (G=7.381, P<0.01). Different types of ectopic conjugations between chromosome arms appeared with very high frequency in *P. bureschi* (61.15%) and *P. olivacea* (81.95%) but in *Chironomus* spp. occurred in single cells only (about 3%). The mouthpart deformities were detected with highest frequency in *P. olivacea* (26.9%). The results showed that the species of different genera have a species-specific genome reaction to stress agents in the environment. The data obtained support the idea that these species can be useful in biomonitoring studies in aquatic ecosystems.

Key words: Chironomidae, polytene chromosomes, somatic index, heavy metals

Introduction

In southern Poland, the Chechło River and Biała Przemsza River, draining the Upper Silesian Industrial Region, have been affected for over 50 years by mine effluents from the Pb-Zn mines in Trzebinia and Bukowno. Discharge of mine waters changed physic-chemical conditions of river waters. The ore extraction in Trzebinia and discharge of mine waters to the Chechło River ceased 6 years ago, whereas the Pb-Zn mine in Bukowno still affects the Biała Przemsza River. The studies in both rivers carried out about 20 years ago (Ciszewski 1997, 1998) indicated very high contents of Zn, Pb, Cd in channel sediments. The data showed similar degree of sediment contamination in both rivers, which may be a good example to study effects of long-term metal toxicity for aquatic organisms. It is known that the long period of river contamination with different stress agents (heavy metals, organic pollution, etc.) both associated with sediments and released to interstitial waters can have toxic and genotoxic effect, damaging either the DNA or chromosome structures (Al-Shami *et al.* 2012, Michailova *et al.* 2015, 2016).

Among macroinvertebrate organisms, the family Chironomidae is one of the richest groups in species that are found in aquatic ecosystem (Kownacki 2011). Their larvae are very sensitive to environmental stress and we showed that they offer important cytogenetic biomarker test system, including many somatic chromosome changes at structural and functional level which are well defined due to the large salivary gland chromosomes with well visible and species specific band patterns. The new cytogenetic indices, somatic (S) and cytogenetic (C), calculated on the basis of the observed structural somatic chromosome rearrangements are very sensitive and reliable methodological approach for assessing the genotoxicity effect of contaminants in the environment (Ilkova *et al*. 2014, Michailova *et al*. 2015, 2016).

Having in mind the significance of the chironomid larvae, the aim of this study was to detect the effect of some trace metals in river sediment at mining and post-mining activities on the genome in some chironomid species. For this purpose the salivary gland chromosome rearrangements of four species of two genera *Chironomus* Meigen, 1803 (*Chironomus riparius* Meigen, 1804 and *Chironomus annularius* Meigen, 1818) and *Prodiamesa* Kieffer, 1906 (*Prodiamesa olivacea* (Meigen, 1818) and *Prodiamesa bureschi* Michailova, 1977) were analysed.

Material and Methods

Study area

The Chechło River drains the southern part of the Silesian-Cracow Upland. It is a small river with a catchment area of ca. 110 km^2 and length of 23 km. The dolomites located in the catchment basins of the river contain rich deposits of zinc and lead ores. The Zn-Pb mine in Trzebinia (Trzebionka) operated from 1962 but finally the mine was closed in 2010. The underground and postflotation waters were discharged into the Chechło River via the Luszówka stream. Currently, most of the municipal and industrial sewages from two towns Trzebinia and Chrzanów located in the catchment basin of the Chechło River are treated biologically (Ciszewski 1997).

The Biała Przemsza River receives polluted waters from the Zn-Pb mine in Bukowno in the middle of its 66 km long course. The exploitation of the Zn-Pb ore in the Biała Przemsza River catchment basin started in the 16th century, but large-scale operation took place since the mid-20th century. Today, the mine is the largest source of heavy metals for the Biała Przemsza River whereas, ore processing and zinc smelter are the smallest ones (Pasieczna *et al*. 2010).

Chironomid larvae and samples of surface sediment were collected from the Chechło River about 10 km below the closed mine and from the Biała Przemsza River– about 5 km below the mine water discharge.

Chemical analysis

The sediment samples were dried at 105^o C. The two subsamples were digested in a mixture of 65 % HNO_3 (10 cm^3) and 30 % H_2O_2 (2 cm^3) in Teflon bombs using a microwave technique and then filtered. Concentrations of Zn, Cd, Pb and Cu in solutions were determined using a flame atomic absorption spectrometer (ICE 3500 Thermo Scientiffic). A reference river sediment materials IAEA-405 and SL-1 were analyzed to evaluate the accuracy of determinations. For the concentrations of studied metals, see Table 1.

Cytogenetic methods

Larvae (IVth instar) of *P. olivacea*, *P. bureschi* and *C. riparius* from the Biała Przemsza River and *C. annularius* from the Chechło River were used for cytogenetic and external morphological analysis (Michailova 1989). The standard chromosome maps of the four species (Hägele 1970, Kiknadze *et al*. 1991, 2012, Michailova 1989, Michailova & Hirvenoja 2015) were applied for both species identification and precise cytogenetic analysis detecting the chromosome rearrangements in the salivary glands. For the number of studied individuals and cells of each species, see Table 2. We considered somatic and inherited chromosome rearrangements (Sella *et al*. 2004). The levels of puffing of the Balbiani rings (BRc and BRb) and the nucleolar organizer (NOR) of chromosome G in *C. riparius* were estimated according to Beermann (1971).

Statistical analysis

The frequencies of chromosome aberrations and deformities were calculated as percentages. Comparative analyses between different species from different localities have been estimated by G-test (Sokal & Rohlf 1995). Probality $P<0.001$, $P<0.01$ and $P<0.05$ were considered as significant. Somatic index (S) was calculated as the ratio of number of different somatic aberrations relative to the number of individuals at that locality (Sella *et al*. 2004).

Table 1. Heavy metal concentrations (µg/g) in sediments in the Biała Przemsza River, Chechło River and the reference data.

Object	**Cd (µg g⁻¹)**	**Pb (µg g⁻¹)**	**Cu (µg g⁻¹)**	**Zn (µg g⁻¹)**
Chechło River	63.3	1932	94.0	9581
Biała Przemsza River	135.4	9674	39.4	25396
Sediment fossil river (Förstner & Salomons 1980)	0.3	30	51.0	115

Results

Chemical analysis

The sediments of the Biała Przemsza River and Chechło River were characterised by very high concentrations of Pb, Zn, Cd (Table 1) which exceeded many times the reference data (Förstner & Salomons 1980). The Chechło River shows lower pollution (2-5 times) than the Biała Przemsza River, which is still affected by mine activity. Inversely, Cu concentration was 2.4 times higher in the sediment of the Chechło River. Concentrations of Zn, Pb and Cd in both rivers exceeded the threshold effect concentration of SEL (*severe effect level*, Zn 820 µg g^{-1}, Pb 250 µg g^{-1}, Cd 10 µg g^{-1}) (Persaud *et al*. 1993).

Cytogenetic characteristics

Genus *Prodiamesa*

P. bureschi and *P. olivacea* are sibling species with chromosome set 2n = 6 and chromosome arm combinations AB CD EF plus a small compact chromosome "B". Chromosomes AB and CD are metacentric, while EF is submetacentric. Chromosome arm E has a Nucleolar Organizer (NOR). Both species can be distinguished by fixed homozygous inversions in chromosome AB and the band sequences in chromosomes CD and EF (Michailova 1989).

Prodiamesa bureschi Michailova

Cytogenetic analysis showed 12 somatic chromosome alterations (inversions and deficiencies), located in all chromosomes (Fig.1a) and 2 inherited chromosome aberrations, found in chromosomes AB, EF (28.57%). The value of S index is 1.71. Ectopic conjugations were detected in most of the cells (61.15%) in various combinations (Fig.1a). A malformation in mentum was seen in one individual only (14%).

Prodiamesa olivacea Meigen

In *P. olivacea*, 18 somatic heterozygous inversions, located in all chromosome arms are found (Fig. 1b). The S index is 0.69. Cells with aberrations in *P. olivacea* were significantly higher than those in *P. bureschi* (G = 7.381 P<0.01). Inherited heterozygous inversions have been observed in chromosomes AB, CD and EF (23.08%). Ectopic contacts have been found in 81.95% of the studied cells (Fig. 1b). Malformations were found in 7 (26.9%) individuals (mentum - 11.54% and mandibles - 15.38%).

Genus *Chironomus*

C. riparius and *C. annularius* belong to thummi cytocomplex (Keyl 1962) with chromosome set 2n = 8 and arm combinations AB CD EF G. Chromosomes AB and CD are metacentric, chromosome EF – submetacentric and chromosome G – acrocentric in *C. riparius* and telocentric in *C. annularius*. In *C. riparius*, chromosome G has a nucleolar organizer (NOR) and three Balbiani rings (BRa, BRb, BRc). *C. annularius* has three Balbiani rings (BR) in chromosome G and five Nucleolar organizers (NOR) in chromosome arms A, C, E and G (Kiknadze *et al*. 1991, Michailova & Hirvenoja 2015).

Chironomus riparius Mg.

Only somatic chromosome aberrations were detected: 29 heterozygous inversions (pericentric and paracentric) distributed in all chromosomes (Fig. 1c) and amplification in arm F, in homo and heterozygous state.

A change the activity of the key structures was seen. In the most of the cells BRc/BRb showed high level of activity ++/+- and ++/++ (80.7% and 14.04%). Intermediate activity (+/+) was detected in single cells only (5.26%). On the contrary NOR were found in suppressed +/+ state in 63.2% of the cells, followed by ++/++ (22.81%) and ++/+- (14%). Ectopic contacts were observed between arm C and arm A as well as arm B and arm D in single cells only (3.5%). No morphological deformities were found in *C. riparius*.

Chironomus annularius sensu Strenzke, 1959

Comparative analysis with chromosome map done

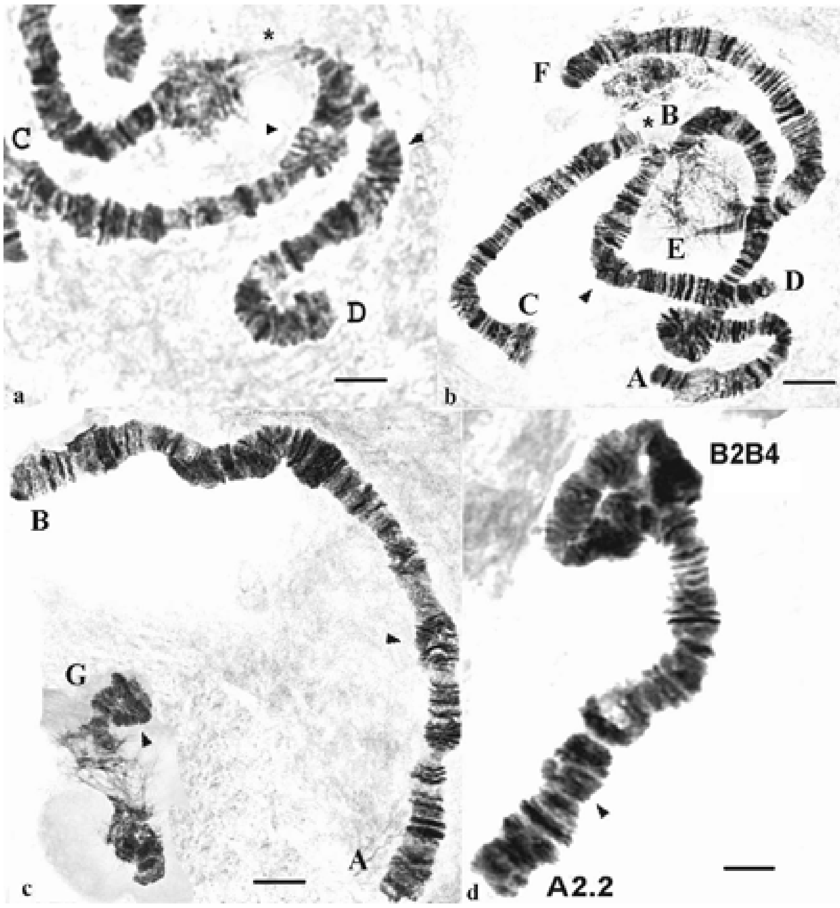

Fig. 1. Somatic chromosome aberrations in polytene chromosomes of the studied species: a. Somatic chromosome aberrations (►) and ectopic pairing (*) in polytene chromosomes of *Prodiamesa bureschi* Michailova; Bar – 10 µm. b. Somatic chromosome aberration (►) and ectopic pairing (*) in polytene chromosomes of *Prodiamesa olivacea* Meigen; Bar – 10 µm. c. Somatic chromosome aberrations (►) in *Chironomus riparius* Mg.; Bar – 10 µm. d. Somatic chromosome aberration (►) in *Chironomus annularius* sensu Strenzke; Bar–10 µm.

Table 2. Number of studied individuals and cells and somatic (S) index

Locality	Species	N of individuals	N of cells	N of somatic aberrations	S index
Biała Przemsza River	*Prodiamesa olivacea*	26	543	18	0.69
Biała Przemsza River	*Prodiamesa bureschi*	7	224	12	1.71
Biała Przemsza River	*Chironomus riparius*	1	57	30	
Chechło River	*Chironomus annularius*	5	127	10	2.00

by Kiknadze *et al*. (2012) showed that in the studied material the chromosomes have the following band sequences: A2.2 B1.1, B2.4 C1.1 D1.1 E1.1 F1.1 F1.2 G1.1. No standard band sequences were detected in arm A.

Two types of chromosome rearrangements were detected: 10 somatic, located in arms A, B, C, D, F, G (Fig. 1d) and 2 inherited (B2.4, 60%), F1.2(40%). S index is 2.

For the first time the ectopic conjugations (arm C and arm E) were established (3.94%). A heterozygous state of NOR (19.68%) in arm A (19.68%) was found. In one individual (16.67%) malformation on teeth of mentum has observed.

Discussion

The concentrations of Cd, Pb and Zn in the sediments of the Chechło River and Biała Przemsza River were extremely high in comparison to in a small degree polluted water bodies (Szarek-Gwiazda 2008, Polechońska & Samecka-Cymerman 2016). The metal concentrations in both rivers exceed many times the background values for shales given by Turiekian & Wedepohl (1961) and the background concentrations for river sediments given by Förstner & Salomons (1980). The metal concentrations in the sediments of both rivers also exceeded the threshold effect concentration of SEL (*severe effect level*) above which adverse effects on the majority of sediment dwelling organisms are expected (Persaud *et al*. 1993). As it was expected, the Biała Przemsza River still affected by discharge of mine effluents from the Pb-Zn mine showed considerable higher pollution of sediment by Cd, Pb and Zn than the Chechło River.

From all studied species, *C. riparius* genome shows the highest sensitivity, in spite of that one individual from the Biała Przemsza River was studied only. No cell with standard karyotype was detected. Besides of heterozygous inversions, amplification in arm F of *C. riparius* was seen. Similar results were established in *C. riparius* from Italian population (Michailova *et al*. 2012). It is quite possible, that this aberration increases the adaptive potential of the species having in mind the significance of this aberration in the process of adaptation. The *C. riparius* genome appeared to have the highest sensitivity to genotoxic agents, as shown by its high genome instability at the cytogenetic level in different polluted European population (Sella *et al*. 2004, Ilkova *et al*. 2014). Genome instability in all cases has been provoked by stress factors influence (mostly trace metals ions) and it can be used as valuable biomarker for heavy metal pollution in aquatic ecosystems (Michailova *et al*. 2015). *C. annularius* is other species which shows a high sensitivity of its genome. In spite of that, the discharge of mine waters in Chechło River was closed some years ago, the somatic index in *C. annularius* is quite high ($S = 2$) and indicated presence of pollution in the sediment of the studied river as the chemical analysis supported. It should be known that this index is a valuable tool to detect some genotoxicity in the sediments (Michailova *et al*. 2015).

Interesting results we obtained for both species of the genus *Prodiamesa*. Their genome showed instability confirmed by somatic index: higher S in *P. bureschi* ($S = 1.71$) in comparison with *P. olivacea* ($S = 0.69$), in spite of that the both species were from the polluted Biała Przemsza River. These results indicate the presence of genotoxic agents inducing the expression of spontaneous somatic aberrations and different response depending on DNA organization of the species. When compared our results from the Biała Przemsza River for both species with the data from unpolluted regions (Michailova *et al*. 2003), the significantly higher number of aberrant cells were established ($G = 13.78$, $P<0.001$ for *P. bureschi* and $G = 12.936$, $P<0.001$ for *P. olivacea*) in the species from the studied polluted regions. Szarek-Gwiazda *et al*. (2013) also examined the same *Prodiamesa* species from strongly polluted with Cd, Cu, Pb and Zn sediment in Matylda stream in southern Poland. *P. bureschi* genomes reacted to metal influence in Matylda stream with significantly higher number of aberrant cells ($G=4.149$ $P<0.05$) compare with the Biała Przemsza River even though lower concentration of Pb (2 times) and Cd (1.2 times) in the Matylda stream than the Biała Przemsza River. In Matylda sediment only Cu has higher concentration (5 times) than in the Biała Przemsza River. One possible explanation is that copper ions have stronger toxic effect on organisms as it was described previously (Lencioni *et al*. 2016). But it is not easy to find which one of the trace metals exactly influence on the biota. On the other hand, the trace metals ions can react to each other and formed complex which affect the biota (Satheeswaran & Thatheyus 2015). Some studies found that accumulation of metals by chironomids relatively decreased when they were exposed to mixture of the metals as compared when exposed to individual metal of similar concentration. This may be due to the competition of the metals for binding sites on the cell surface (Lagrana *et al*. 2011).

Except for the somatic chromosome alterations inherited inversions were established in *C. annularius*, *P. bureschi* and *P. olivacea*, occurred in high frequency. Also, in arms A, C, F of *C. annularius* we found a process of homozygotization of band sequences. These homozygous inversions indicated a process of differentiation of the population. They are the main markers of the early stages of species divergence (Keyl 1962) and together with high frequency of heterozygous inversions might be related to adaptive process in this species.

Together with chromosome rearrangements some changes in functional level was detected. Good example are the key structures (BRc/BRb and NOR) in *C. riparius* genome. Very slight changes have observed in functional activity of BRc/BRb: they decreased their activity in 5% of the studies cells.

Unlike BRs, the high activity of NOR in *C. riparius* was suppressed and low activity in majority of cells was detected plus heterozygous activity, well seen in arm A in *C. annularius*. Decreasing of NOR activity has been reported in *Chironomus* species inhabiting strongly metal polluted water basins (Ilkova *et al*. 2014, Michailova *et al*. 2015, 2016) as specific reaction to the stress influence. Changes in transcription activity of these key structures can be considered as one of the biomarker for genotoxicity in aquatic ecosystems. These structures supports homeostasis in the cell and their response to stress factors are specific and can serve as a cell activity indicator at the cellular and subcellular level and as a sensor of many stress factors (Gussev *et al*. 2012).

The ectopic pairing between chromosomes of the studies species is the other cytogenetic sign often observed in these species. It's occurred with high frequency in *Prodiamesa* and very rarely in *Chironomus* species. It is quite possible the ectopic contacts to be caused by stress agents in the environment and influence on the genome mobilization in these conditions.

Results obtained in the present study together with previously established data about genome instability of chironomids indicated that under stress conditions species-specific reaction of the genome exist and the appearance of chromosome alterations could be used as early warning signals for pollution in the aquatic ecosystems. The species of different genera react to stress agents in the environment in different way depending on their genome structure. Chironomidae spp. turned out very sensitive to pollution and could be usefully explored in biomonitoring studies in aquatic ecosystems.

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