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Genomic alterations recorded in two species of Chironomidae (Diptera) in the Upper Jurassic limestone area of the Ojców National Park in Poland attributable to natural and anthropogenic factors

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Key words. Diptera, Chironomidae, *Micropsectra pallidula*, *Polypedilum convictum*, anthropogenic, natural sources, genome alterations

Abstract. The Ojców National Park is situated in southern Poland in an area of Jurassic limestone, which determines the hydrochemistry of the water in the River Prądnik. The geochemical index of the sediment in the River Prądnik indicated it is moderately polluted with Pb and Zn, and heavily polluted with Cd compared to unpolluted sediment. The effect of natural and anthropogenic sources on the sediment in the River Prądnik and in appearing of structural and functional alterations in the salivary gland chromosomes of two species of Chironomidae, *Micropsectra pallidula* and *Polypedilum convictum*, was investigated. Two types of chromosomal rearrangements (inherited and somatic) were identified in the species studied. Inherited heterozygous inversions occurred at a higher frequency (between 5.55%–57.81%) and may have local adaptive value. In *M. pallidula* a karyotype divergence consisting of fixed chromosome inversions on arms B and E was recorded. As somatic chromosome rearrangements can be caused by stress agents, we suggest that the somatic aberrations in both of the species studied indicate the existence of pollution, i.e. induced stress. On the basis of these somatic rearrangements the somatic index of both species was defined: *M. pallidula* – 0.346, *P. convictum* – 0.555. In addition to these rearrangements functional alterations in key structures, Balbiani rings (BRs) and the nucleolar organizer region (NOR) located on chromosome EF, which significantly decreased their transcriptional activity, were recorded in *M. pallidula*. Changes in the appearance of the telomere region on chromosome G in *P. convictum* was considered to be a response to the environmental conditions in the River Prądnik. It was shown that polytene chromosomes are very sensitive to environmental changes and can be used to detect pollutants in aquatic ecosystems.

INTRODUCTION

The Ojców National Park is situated in the southern part of the Krakow-Częstochowa Uplands (Southern Poland) and includes two deeply carved limestone valleys, the 12 km long upper fragment of the valley of the River Prądnik and the lower stretch of the valley of the River Sąspówka (5 km) (Partyka & Klasa, 2008). The entire area consists of upper Jurassic limestone with a thickness of about 200 m consisting of three main types of limestone: massive, bedded and platy. Due to the geochemical characteristics of the region there are a lot of caves (more than 660) (Gradziński et al., 2008; Partyka & Klasa, 2008). This park is included in the "Natura 2000" program of the EU as an area of special habitat protection (SHP).

Physicochemical parameters of the water of rivers reflect the geochemical background and anthropogenic activity in the catchment basin (Miernik & Wałęga, 2008; Kostrakiewicz, 2001). High amounts of Ca^{2+} and HCO_3^- ions, originating from the Jurassic limestone, occur in the water (Siwek & Chełmicki, 2004). As it is a typical agricultural and rural settlement area, elevated amounts of other ions (NO_3^-, PO_4^{3-}, Cl^-, SO_4^{2-}, Na^+, and K^+) are also periodically recorded (Kostrakiewicz, 2001; Miernik & Wałęga, 2008). Atmospheric emissions are an important source of contamination in the Ojców National Park. High concentrations of metal ions in rainfall are associated with particular wind directions. During the years 1986–1990 there were high concentrations of Zn (400 µg dm^{-3}), Pb (7.2 µg dm^{-3}) and Cu (2.8 µg dm^{-3}) in the rainwater caused by emissions from the Upper Silesia Region. The Ojców National Park is the most heavy-metal-contaminated National Park in Poland and the majority of the trace metal pollution comes from the atmosphere (Grodzińska, 1980) .

The water quality does not affect the biodiversity of invertebrates that inhabit the River Prądnik, which is characterized by great abundance and diversity (about 400 taxa) (Dumnicka & Szczęsny, 2008) and the largest group of insects occurring in this river is the Chironomidae. However, as the biodiversity of invertebrates is not greatly affected it is a poor indicator of contamination with trace metals (Michailova et al., 2012b). Alterations

in the genome of some chironomids are better indicators of the presence of stress agents in the environment. Therefore, it is of interest to monitor the response of organisms at the genetic level to changes in the aquatic environment at this Special Habitat Protection (SHP) site. Good candidates for these studies are species of the family Chironomidae, a group of insects that inhabit a wide range of habitats, including the River Prądnik (Dratnal, 1976, 1977). Especially important is its larval stage, which is very sensitive to temperature, pH, amount of dissolved oxygen, ions, heavy metals and other contaminants in the water (Armitage et al., 1995).Their salivary gland chromosomes are very sensitive to various factors in the environment and previous studies (Michailova et al., 2009a, b) indicate that on these chromosomes there are cytogenetic biomarkers that can be used to measure the level of toxic agents in water ecosystems. By appearing of structural and functional alterations in the salivary gland chromosomes it is possible to determine the level of pollution in river basins (Michailova, 2011).

The aim of this study was to determine the genome characteristics of two chironomid species that inhabit the River Prądnik in the Ojców National Park and the effect of natural and anthropogenic sources of pollution on genome rearrangements to be considered. In order to fulfil this task the chromosomal aberrations at structural and functional levels of the salivary gland chromosomes of two species were analyzed.

MATERIAL AND METHODS

Study area

The River Prądnik is a left-tributary of the Vistula River (Southern Poland). The total length of this river is 34 km, its catchment area is 141 km^2 and it flows through the Prądnik valley, which is built up of Upper Jurassic platy limestone. Samples of water, sediments and Chironomidae larvae were collected from the River Prądnik (50°12′28″N, 19°49′42″E) (Fig. 1) in spring or summer in 2008–2010.

Physicochemical characteristics of the water and sediment in the River Prądnik

The determination of water temperature and conductivity, and water and sediment pH were done in situ. Anions (Cl^-, SO_4^{2-}, HCO_3^-, and NO_3^-) and cations (Mg^{2+}, Ca^{2+}, K^+, and Na^{2+}) were analyzed using ion chromatography (DIONEX, IC25 Ion Chromatograph; ICS-1000, Sunnyvale, California, USA). Ammonia was determined using the nesslerization method, while dissolved oxygen and BOD_5 was determined using the Winkler method (APHA, 1992).

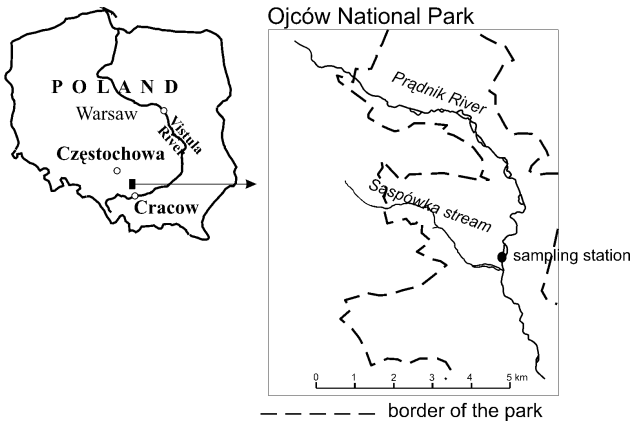

Fig. 1. Location of the sampling station on the Prądnik River and general view of the Ojców National Park.

Sediment samples were sieved using a sieve with a mesh of 0.063 mm. For the analysis of total metal (Cd, Pb, Cu, Zn, Mn, Fe, Ni, and Cr) concentrations, three subsamples were digested in 65% HNO_3 using a microwave Speed Wave (Berghof, Enningen, Germany). The operationally defined BCR (Community Bureau of Reference of the European Commission, now the Standards, Measuring and Testing Programme) procedure was used to study the fractionations of heavy metals in the sediments (Larner et al., 2006). This procedure allows the determination of four operationally defined phases of metals, i.e. (F1) acid extractable, (F2) reducible, (F3) oxidizable and (F4) residual. Fraction F1 includes exchangeable and carbonate bound metals; F2 includes iron and manganese oxide/hydroxide associated metals; F3 includes metals bound to sulphide and organic phases; F4 includes mineral phases. Tiurin's method was used to determine organic carbon content of the sediment samples (Ostrowska et al., 1991).

Heavy metals in the water (total concentrations) and sediment were analyzed using a Varian (Spectra AA – 20, Mulgrave, Victoria, Australia) atomic absorption spectrophotometer. There was a good agreement between the sum of the concentrations of heavy metals obtained using the BCR procedure and the total metal concentration. The SPS-SW1 Quality Control Material was analyzed to determine analytical accuracy for the water samples and Standard Reference Material (NCS DC 73308) for sediment samples. Comparisons of concentrations measured and certified concentrations of analytical standards are given in Table 1.

Cytogenetic method

For the cytogenetic studies larvae of Chironomidae were collected with a hand-net from the surface of the sediment in the river and picked up using fine forceps. In the laboratory, larvae

TABLE 1. Measured concentration and certified values for the standards used in the analysis of the samples of water SPS-SW1 Quality Control Material and sediment NCS DC 73308.

	Cd μg g^{-1}	Pb μg g^{-1}	Cu μg g^{-1}	Cr μg g^{-1}	Ni μg g^{-1}	Mn μg g^{-1}
Water (μg dm^{-3})						
Measured values	0.48 ± 0.01	4.9 ± 0.1	19.5 ± 0.05	2.08 ± 0.07	9.8 ± 0.15	10.4 ± 0.3
Certified values	0.5 ± 0.01	5.0 ± 0.1	20 ± 1	2.0 ± 0.02	10.0 ± 0.1	10.0 ± 0.1
Sediment (μg g^{-1})						
Measured values	1.21 ± 0.05	28.2 ± 0.20	21.2 ± 1.2	132.4 ± 4.8	28.9 ± 0.9	990 ± 8.2
Certified values	1.12 ± 0.08	27 ± 2	22.6 ± 1.3	136 ± 10	30 ± 2	1010 ± 29

TABLE 2. Number of individuals and cells studied in both species collected from the River Prądnik in 2008–2010.

Species	Date collected	Number of larvae studied	Number of cells studied
Micropsectra pallidula	05.vii.2008	19	163
Micropsectra pallidula	08.viii.2009	7	93
Polypedilum convictum	iv.2010	18	224

were dried using filter paper and preserved in Carnoy's solution (96% ethanol and glacial acetic acid, 3 : 1). The Carnoy's solution was changed three times at intervals of 15 min. All the samples were stored in a freezer until analyzed.

Stage IV larvae of *Polypedilum convictum* (Walker, 1856) and *Micropsectra pallidula* (Meigen, 1830) were studied cytogenetically. Fourth instar larvae, phase 6–7, can be recognized by the presence of the abdominal segment (Wülker & Götz, 1968). Preparations of salivary gland chromosomes were performed according to Michailova (1989) and larval morphology analyzed according to Schlee (1968). Both types of preparations are preserved in the Institute of Biodiversity and Ecosystem Research, Sofia, Bulgarian Academy of Sciences.

Somatic and inherited chromosome aberrations in both species were analyzed.

Following the idea advanced by Lagadic & Caquet (1998), Sella et al. (2004) and Michailova et al. (2012a) rearrangements of somatic chromosome were used as biomarkers indicating the presence of stress agents in the environment.

The chromosome rearrangements were defined as somatic when a salivary gland contained both nuclei with and without chromosome aberrations and as inherited when cells in both salivary glands showed the same chromosomal aberrations (Sella et al., 2004). Chromosomal aberrations in *M. pallidula* (syn. *Micropsectra viridiscutellata*, Goetghebuer, 1932) were detected by comparison with the standard pictures of chromosomes in Michailova (1989). The cytogenetic characteristics of *P. convictum* are presented for the first time in this paper. As a control population of one of the species of Chironomidae studied, *M. pallidula*, specimens from a Bulgarian population that occurs at the foot of Mt. Pirin, which is included in the National Biomonitoring Program of Bulgaria (Peev & Gerassimov, 1999) as an unpolluted site, were collected.

Functional alterations were determined by puffing of the key structures, the Balbiani rings (BRs) and nucleolar organizer region (NOR) in both species. The level of puffing (an indicator of the degree of transcription) of the NOR and BRs was scored according to Beermann (1971) as follows: (++/++) = high (each sign corresponds to a homologue), (+/+) = intermediate, (–/–) = little or none. The functional activity of BRs and NOR in the cells of the main lobe of the salivary glands was determined in both species. The polytene chromosomes from these cells have the highest degree of the polyteny with a well banded chromosome structure and easily visible BRs and NOR.

The number of larvae and cells of both species studied is given in Table 2.

Statistical analysis

The values of the geoaccumulation index were calculated according to Muller's (1981) formula. The frequency of chromosome aberrations (inherited and somatic) and functional alterations are presented as percentages. The comparative analysis of the frequencies of chromosome aberrations and level of puffing activity was done using G test (Sokal & Rohlf, 1995).

TABLE 3. Physicochemical parameters and heavy metals content of the water of the River Prądnik in 2009.

Parameter	Unit	13.viii.2009
Temperature	°C	11.6
pH		8.4
Conductivity	µS cm^{-1}	506
Dissolved oxygen	mg dm^{-3}	10.1
Chloride	mg dm^{-3}	14.4
Sulphate	mg dm^{-3}	17.2
Hydrocarbonates	mg dm^{-3}	226.2
Nitrate	mg dm^{-3}	17.6
NH_4^+	mg dm^{-3}	0.222
PO_4^-	mg dm^{-3}	0.082
Na^+	mg dm^{-3}	7.4
K^+	mg dm^{-3}	2.5
Ca^{2+}	mg dm^{-3}	90.1
Mg^{2+}	mg dm^{-3}	2.5
Cd	µg dm^{-3}	0.05
Pb	µg dm^{-3}	0.9
Cu	µg dm^{-3}	1.7
Zn	µg dm^{-3}	30
Mn	µg dm^{-3}	32
Fe	µg dm^{-3}	202
Cr	µg dm^{-3}	0.78
Ni	µg dm^{-3}	0.90

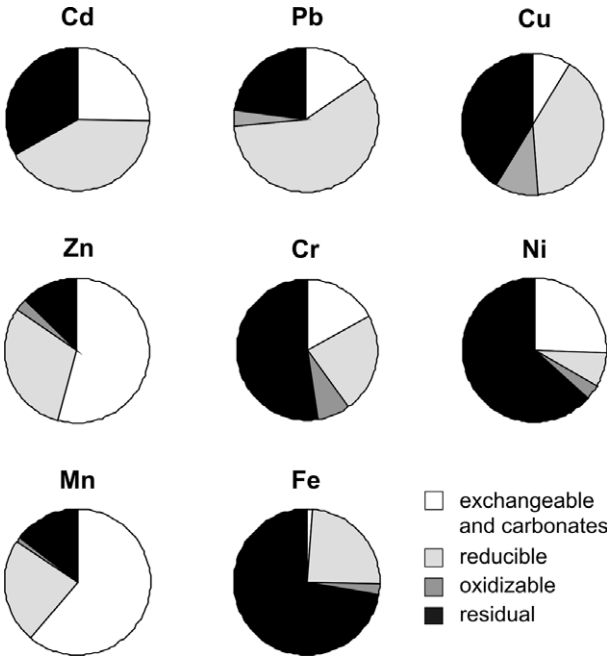

Fig. 2. Binding form of the heavy metals recorded in the sediment of the River Prądnik.

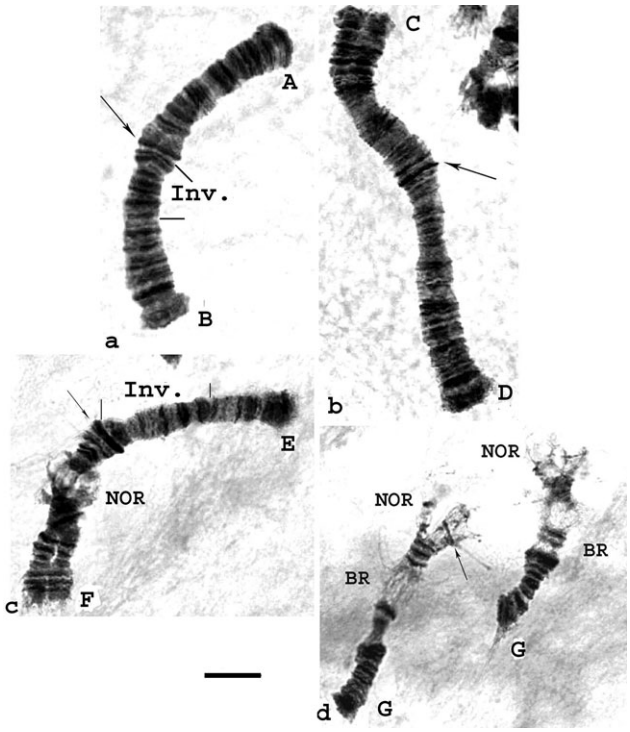

Fig. 3. Salivary gland chromosomes of *Micropsectra pallidula*. a – chromosome AB; b – chromosome CD; c – chromosome EF; d – chromosome G; Inv. – region with fixed homozygous inversion; BR– Balbiani ring; NOR – nucleolar organizer region; arrows – centromere region. Bar = 100 µm.

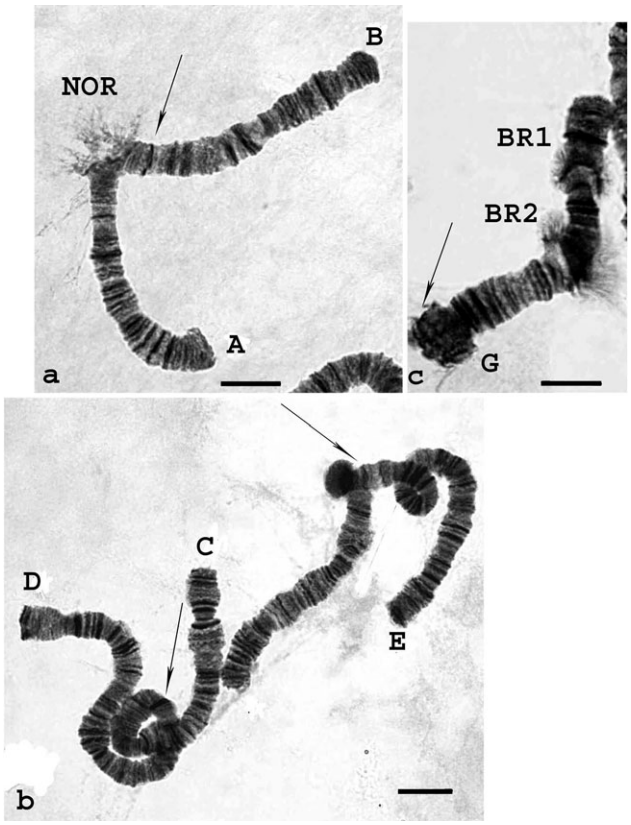

Fig. 4. Salivary gland chromosomes of *Polypedilum convictum*. a – chromosome AB; b – chromosomes CD and EF; c – chromosome G; BR1 and BR2 – Balbiani rings; NOR – nucleolar organizer region; arrows – centromere region. Bar = 100 µm.

The somatic index was calculated by dividing the number of somatic alterations by the number of individuals studied (Sella et al., 2004).

RESULTS

Physicochemical characteristics of the water and sediment in the River Prądnik

The water in the River Prądnik was cold, slightly alkaline and had a conductivity of ca. 500 µS cm^{-1} (Table 3). Ions of HCO_3^- and Ca^{2+} were predominant in the water, while Cl^-, SO_4^{2-}, Na^+, K^+ and Mg^{2+} occurred in lower amounts. There were small concentrations of heavy metals in the water (Table 3).

Sediment from the River Prądnik was approximately neutral, pH 7.3, and contained little organic carbon (TOC 2.6%). The concentrations of Cu, Cr, Ni, Mn and Fe in the sediment were low, while that of Cd, Pb and Zn were elevated (Table 4). The geoaccumulation index (Müller, 1981) indicated that the sediment was not polluted by Cu, Cr, Ni, Mn and Fe, moderately polluted by Pb and Zn and strongly polluted by Cd (Table 4).

TABLE 4. Heavy metal concentrations recorded in the sediment of the River Prądnik.

		Ojców		Igeo		PEL	SFF
		September 2008	August 2009	September 2008	August 2009		
Cd	µg g^{-1}	3.5	2.9	3.0****	2.7***	3.53	0.3
Pb	µg g^{-1}	72	72	1.3**	1.3**	91.3	30
Cu	µg g^{-1}	41.5	14.7	–0.7*	–2.2*	197	51
Zn	µg g^{-1}	510	356	1.8**	1.3**	315	115
Mn	µg g^{-1}	325	230	–2.0*	–2.5*		960
Fe	mg g^{-1}	9.7	8.2	–2.9*	–3.1*		32.35
Ni	µg g^{-1}	19.6	11.5	–2.4*	–3.1*	36	46
Cr	µg g^{-1}	32.6	13.2	–2.1*	–3.4*	90	47

* unpolluted, ** moderately polluted, *** moderately to strongly polluted, **** strongly polluted; Igeo – geoaccumulation index (Müller, 1981); PEL – probable effect level (Smith et al., 1996); SFF – sediment fossil river (Förstner & Salomons, 1980).

Sequential extraction of the sediment indicated that the majority of the Zn (54%) and Mn (61%) was bound to mobile F1 (exchangeable and carbonated phase) (Fig. 2). Considerable percentages of Cd (42%), Pb (58%), Cu and Cr (40%) were bound to F2 (reducible phase). In total, a great percentage of the Zn (85%), Mn (84%), Pb (73%), Cd (67%) and Cu (50%) were bound to these two fractions (F1 and F2). Thus, these elements were characterised by a potentially high mobility. The total amount of the heavy metals studied in F3 (oxidizable phase) was small (below 10%). A large part of Fe (72%), Ni (63%), Cu and Cr (41%) was associated with the immobile F4 (residual phase) (Fig. 2).

Cytogenetic characteristics of the species studied

Microspectra pallidula (Meigen, 1830) (syn. *M. viridiscutellata* Goetghebuer, 1932)

The chromosome set is 2n = 8, with chromosome arm combinations: AB, CD, EF and G. Chromosomes AB, CD and EF are metacentric, while chromosome G is acrocentric (Fig. 3a–d). Two NORs are located on chromosomes EF and G respectively and a BR on chromosome G; however, there are no records of BR in the Bulgarian population (Michailova, 1989). The homologues of chromosome G were always unpaired.

The band sequences of arms A, C, D, F and G coincided with that of individuals from Bulgaria. Arm B of individuals from the River Prądnik (8–7–6–5–**2–3–4–1)** differed from those from the Bulgarian population (8–7–6–5–1–2–3–4) (Michailova, 1989) by a fixed homozygous inversion (Fig. 3a). Arm E of individuals from the River Prądnik had a homozygous inversion (1–2–3–4–**9–8–7–6–5),** which distinguished them from

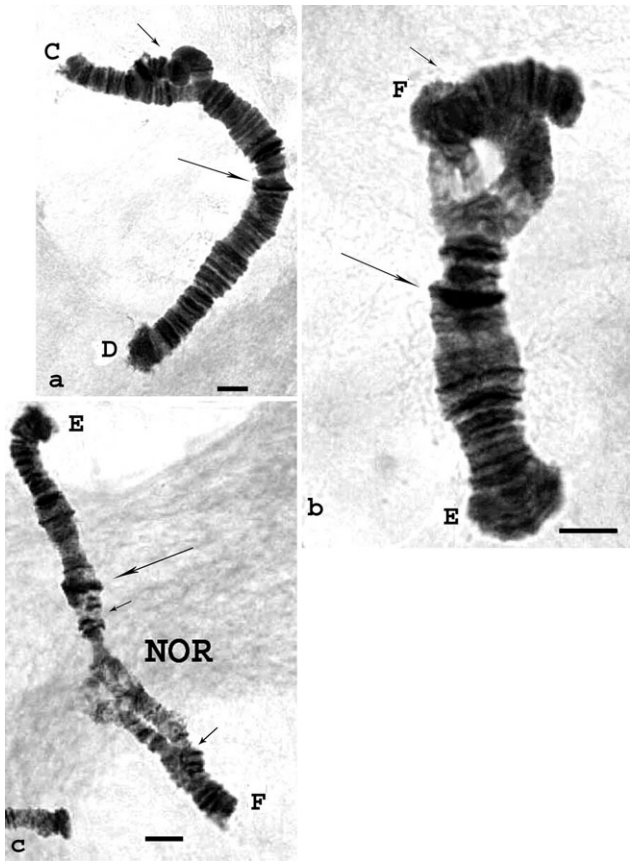

Fig. 5. Chromosome rearrangements in *Micropsectra pallidula*. a – inherited heterozygous inversion in arm C; b – inherited heterozygous inversion in arm F; c – somatic heterozygous inversions in arm F; long arrows – centromere region; short arrows – inversion. Bar = 100 μm.

TABLE 5. Structural chromosome aberrations recorded on polytene chromosomes of *M. pallidula.*

Arm	Aberration	2008				2009			
		Individuals with aberrations		Cells with aberrations		Individuals with aberrations		Cells with aberrations	
		Number	%	Number	%	Number	%	Number	%
A	Het.inv.inherit	1	5.26	18	100	1	14.28	13	100
B	Het.inv.inherit	2	10.52	21	100	–	–	–	–
C	Het.inv.inherit	1	5.26	8	100	3	42.85	33	100
D	Het.inn.inhetit	5	26.37	58	100	–	–	–	–
F	Het.inv.inherit	4	21.05	47	100	4	57.14	51	100
A	Het.inv.somatic	–	–	–	–	1	14.28	1	1.07
A	Het.def.somatic	1	5.26	1	0.61	–	–	–	
B	Het.inv.somatic	–		–		2	28.57	2	2.15
AB	Pericentric inv.somatic	3	15.78	3	1.84	2	28.57	2	2.15
C	Het.inv.somatic	1	5.26	1	0.61	–		–	
D	Het.inv.somatic	1	5.26	1	0.61	–		–	
E	Het.inv.somatic	–		–		1	14.28	1	1.07
F	Het.inv.somatic	–		–		2	28.57	3	3.22
F	Het.def.somatic	1	5.26	1	0.61	–		–	

Het. inv. – heterozygous inversion; Het. def. – heterozygous deficiency.

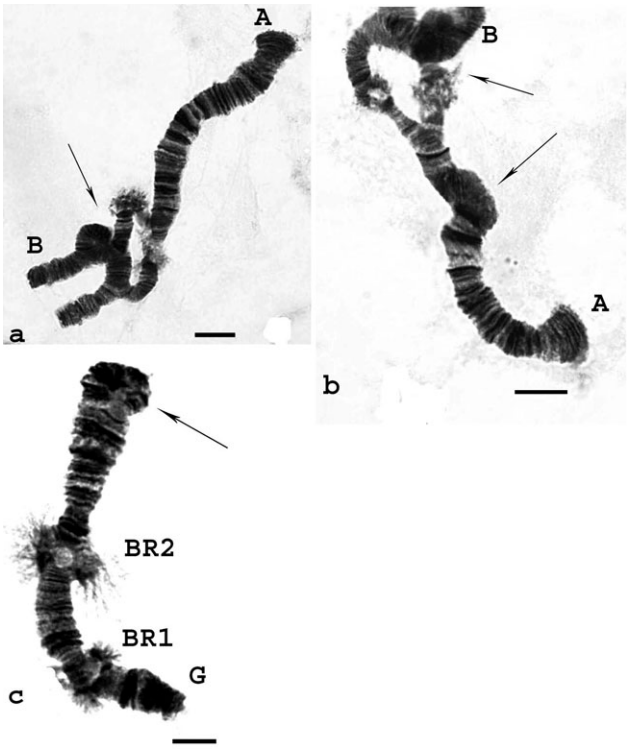

Fig. 6. Chromosome rearrangements in *Polypedilum convictum*. a – inherited heterozygous inversion in arm B; b – inherited heterozygous inversion in arm B and somatic inversion in arm A; c – somatic heterozygous inversion in arm G; BR1 and BR2 – Balbiani rings; arrows indicate inversions. Bar = 100 µm.

those from the Bulgarian population (1–2–3–4–5–6–7–8–9) (Michailova, 1989) (Fig. 3c) (bold indicates the position of the homozygous inversion).

Polypedilum (*Uresipedilum*) *convictum* (Walker, 1856)

The chromosome set is 2n = 8. Chromosomes AB, CD and EF are metacentric, while chromosome G is telocentric (Fig. 4a–c). Chromosome AB can be identified by an NOR located in the middle of the chromosome (Fig. 4a). Chromosome CD has a constriction near to the telomere of arm C (Fig. 4b). Chromosome EF can be recognised by the banding patterns close to both telomeres (Fig. 4b). Chromosome G is the smallest chromosome and has two BRs and a dark, compact centromere region, resembling a dark knob. The telomeres appeared different in every individual and in many cases formed Balbiani-like structures (Figs 4c, 7a–d).

Structural chromosome alterations

Two types of chromosomal rearrangements occurred in both the species studied: inherited and somatic. The aberrations in *P. convictum* were recorded in 2010 and in *M. pallidula* in 2008 and 2009 (Tables 5 and 6).

Inherited aberrations

Inherited heterozygous inversions in *M. pallidula* affected arms A, B, C, D and F. Their frequency is given in Table 5 and examples in Fig. 5a, b. Inherited aberrations in *P. convictum* occurred as heterozygous inversions on chromosome arms B and C (Table 6, Fig. 6a, b).

Somatic aberrations

Paracentric and pericentric heterozygous inversions and heterozygous deficiencies were found in both species (Tables 5 and 6). They occurred in few cells of the salivary glands and on a small region of the chromosome arms of both species. In *M. pallidula*, paracentric heterozygous inversions and deficiencies occurred on arms A, C, D and F in 2008 and arms A, B, E and F in 2009 (Table 5, Fig. 5c). A somatic pericentric inversion was observed on chromosome AB in both years (Table 5). In *P. convictum,* paracentric heterozygous inversions and deficiencies occurred on chromosome arms A, C, D, F and G (Fig. 6b, c). A pericentric heterozygous inversion was observed on chromosome CD (Table 6). The somatic index of *M. pallidula* was 0.346 and that of *P. convictum* 0.555.

TABLE 6. Structural chromosome aberrations recorded on polytene chromosomes of *P. convictum* from the River Prądnik in 2010.

Arm	Aberration	Individuals with aberrations		Cells with aberrations	
		Number	%	Number	%
B	Het.inv.inherit	5	27.8	25	100
C	Het.inv.inhetit	1	5.55	48	100
A	Het.inv.somatic	1	5.55	1	0.45
A	Het.def.somatic	1	5.55	1	0.45
B	Het.def.somatic	1	5.55	1	0.45
CD	Pericentric het.inv.somatic	2	11.11	2	0.89
C	Het.inv.somatic	3	16.67	3	1.34
D	Het.inv.somatic	3	16.67	3	1.34
D	Het.def.somatic	1	5.55	1	0.45
F	Het.inv.somatic	3	16.67	3	16.67
F	Het.def.somatic	1	5.55	1	0.45
G	Het.inv.somatic	2	11.10	2	0.90

Het. inv. – heterozygous inversion; Het. def. – heterozygous deficiency.

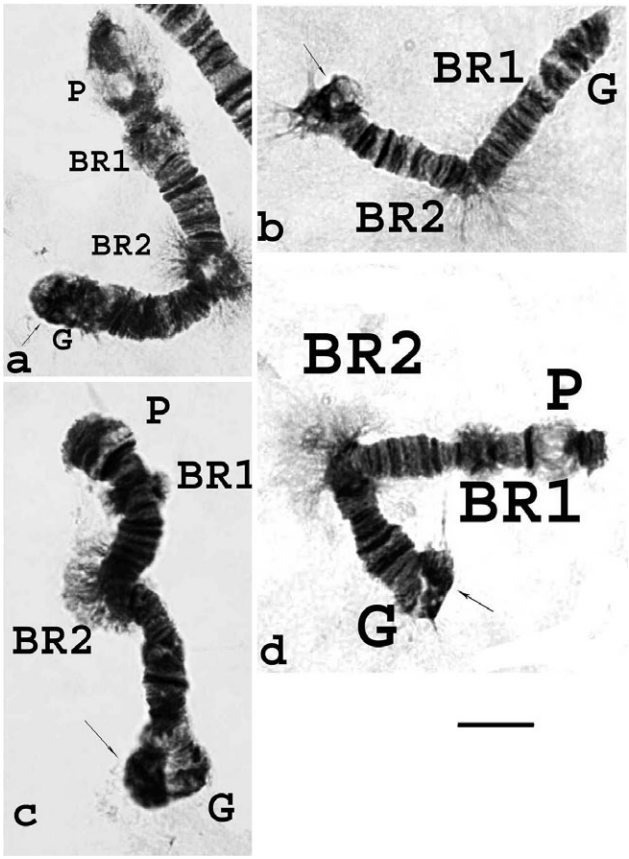

Fig. 7. Changes recorded in the functional activity of BRs, centromere region and telomeres on chromosome G of *Polypedilum convictum*. a – activity of BRs (+/++), centromere (–/–), puff on telomere (++/++); b – activity of BRs (–/++), centromere (–/+), puff on telomere (–/–); c – activity of BRs (+/++), centromere (–/+), puff on telomere (+/+); d – activity of BRs (+/++), centromere (–/+), puff on telomere (+/+). Bar = 100 μm.

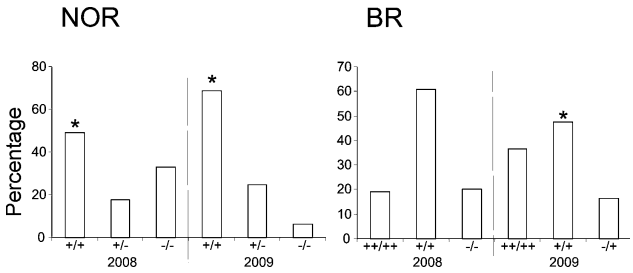

Fig. 8. *Microprescta pallidula* – activity of NOR and BR on chromosomes EF and G, respectively. (*) statistically significant differences ($P < 0.05$ or $P < 0.01$). ++/++ – high activity; +/+ – intermediate activity; +/– – heterozygous activity; –/– – no activity.

Functional alterations

In both species, the functional alterations affected the key structures, BRs and NOR. In *M. pallidula*, high activity of the NOR on chromosome G was recorded in both the years studied. In 2008, three types of activity were detected: high, 93 cells (++/++, 57.05%); intermediate, 63 cells (+/+, 38.65%); and heterozygous (one homologue with intermediate activity, others no activity), 2 cells (+/–, 1.23%). In 2009, the NOR on chromosome G was highly active (100%) in all cells. The activity of the NOR on chromosome EF differed in both years (Fig. 8). No high activity was observed in 2008. The intermediate activity (+/+) occurred at a high frequency and was significantly higher than the other two activities, i.e. heterozygous and no activity (G = 5.036, df = 1, $P < 0.05$). A similar trend was observed in 2009 (G = 8.630, df = 1, $P < 0.01$).

BR on chromosome G also changed in activity from high to intermediate or complete collapse (Fig. 8). The incidence of intermediate activity differed significantly from other activities in 2009 but not in 2008 (2009: G = 4.97, df = 1, $P < 0.05$; 2008: G = 0.046, df = 1, $P > 0.1$).

The NOR on chromosome AB in *P. convictum* was very highly active in almost all cells (++/++, 214 cell, 95.53%); the NOR appeared in a heterozygous state in only 10 cells (+/–, 4, 45%). However, the BR_1 on chromosome G was very sensitive. Also, it is interesting to note that the changes in the appearance of the centromere region and telomere of this chromosome occurred together with changes in the activity of BR_1. Several levels of activities of these structures were detected (Table 7, Fig. 7a–d). Also, near to the telomeres, in more than 70% of the cells studied, there was a puffed structure that looked like a Balbiani ring.

DISCUSSION

The alterations in the genome of the chironomid species studied reflect the concentrations of heavy metals in the water of the River Prądnik flowing through the Ojców

TABLE 7. The changes recorded in the functional activity of chromosome G in *P. convictum.*

Numbers of cases	BR_1/BR_2	Centromere region (homologous I/homologous II)	Telomere (homologous I/homologous II)	Percentage
1	+/++	–/–	++/++	23.21
2	–/++	+/+	++/++	14.73
3	+/++	–/+	++/++	20.08
4	–/++	–/+	–/–	8.93
5	+/++	++/++	–/+	11.60
6	+/++	+/+	+/+	8.04
7	+/++	–/+	+/+	13.39

+ intermediate activity, ++ high activity, – no activity, –/+ heterozygous activity.

National Park, which in turn reflect the geochemical background and anthropogenic activity in its catchment basin. The most relevant cytogenetic response to these pollutants is functional and structural changes in the salivary gland chromosomes.

Species specific changes in functional activity of chromosome G of *M. pallidula* and *P. convictum* were recorded. Balbiani rings (BRs) were not found in *M. palidulla* collected from the control Bulgarian population but were present in all the individuals of this species studied from the River Prądnik, which might be linked to the specific environmental conditions at this locality. BRs control the synthesis of high molecular weight proteins used in the production of the tubes in which larvae live (Wieslander, 1994). Generally they are sites of intensive transcription and can be detected by their characteristic large puffs at 6–7th phase of the IVth instar larva and are highly active in both homologues. Decreases in the level of transcription by BRs are reported in other chironomids after treatment with different concentrations of chemicals, including trace metals (Beermann, 1973; Diez et al., 1990; Aziz et al., 1991; Michailova at al., 2012a). BR in *M. pallidula* (IVth instar larva, 6–7 phase) changed its activity from very high to intermediate and low. In both years studied intermediate activity (+/+) occurred at a high frequency, even more in 2009 it has significant value which might be linked to the anthropogenic pollutants found in the National Ojców Park in Poland (Grodzińska, 1980). Also, the toxicity of aluminium for some species of Simuliidae may be related to specific gene expression (Sanderson et al., 1982). A new BR is recorded for *Chironomus acidophilus* Keyl, a species whose larvae are found in an acidic metal rich environment in Afon Goch, UK (Michailova et al., 2009a).The same response was recorded in *M. pallidula* collected from the River Prądnik in the Ojców National Park. However, the mechanisms resulting in this specific response require further investigation.

Essential for cellular maintenance, NOR is the site of high transcription (Hudson & Ciborowski, 1996). The level of transcription is related to variation in the protein requirements of cells. In the majority of cells in a salivary gland NORs are highly active at the 6–7th phase of IVth larva stage (Kiknadze, 1978). Hudson & Ciborowski (1996) suggest that a reduction in the size of the nucleolus is indicative of an overall decrease in RNA synthesis. They measured the diameter of the nucleolus (RND) using the method proposed by Bentivegna & Cooper (1993) and established a strong positive dose-response relationship between reductions in RND and level of contamination in the sediment used for rearing larvae of *Chironimus salinarius* ($P < 0.005$, $R^2 = 0.94$; $P < 0.01$, $R^2 = 0.91$).

Planello et al. (2007) record a decrease in NOR activity after Cd treatment using rDNA immunofluorescent probes. Similarly the NORs of other species of Chironomidae exposed to copper, Pb, dimethylnitrosamine and other chemicals show a decrease in activity (Aziz et al., 1991; Bentivegen & Cooper, 1993; Michailova et al., 2001a, b, 2006). In *M. pallidula* there was a significant change in the activity of the NOR located on chromosome EF from high to intermediate. However, the NOR on chromosome G always appeared to be highly active. This indicates an enhanced rRNA synthesis, which could denote an increase in protein synthesis. It is quite possible that larvae produce proteins that increase their tolerance to toxicants. Meregalli et al. (2002) report that the presence of mouthpart deformities in larvae of *Chironomus riparius* collected from polluted field sediments are associated with NOR activity. In *Chironomus bernensis* the activity of NOR_1 remained unchanged, while that of NOR_2 changed to intermediate or low in a trace metal polluted river (Petrova & Michailova, 2002).

These results for the species studied show that the regression in the activity of NORs is species-specific and was affected differently in each of the genomes studied: in *P. convictum*, the activity of the NOR on chromosome AB was reduced very little, yet, in *M. pallidula*, that of the NOR on chromosome EF was mainly at an intermediate level or in a state of collapse. However, future laboratory studies are required to test this hypothesis.

An interesting mechanism operates in *P. convictum*: in all the individuals studied, the telomere of chromosome G was in most cases very active in forming puff structures that resembled Balbiani rings, either in the homozygous or heterozygous state. This type of response was repeatedly confirmed by the different appearance of the centromere region of chromosome G and the expression of BR_1, whereas BR_2 was always active (Table 7). It is well known that telomeres are not active sites on chromosomes (e.g. telomeric DNA sequences activated by heat shock in *Chironomus piger* and *C. riparius*, Morcillo et al., 1988). In *Drosophila*, the telomeres represent "special" DNA/protein complexes that play a major role in chromosome stability and genome integrity. The telomeres of polytene chromosomes are composed of three domains: tandem *Het-A*, *TART*, and *TAHRE* transposons, TAS (telomere associated sequences) and distal euchromatic genes. Each domain has specific associated proteins (Zhimulev et al., 2004; Mason et al., 2008). On the other hand, Lezzi & Gilbert (1980) show that ions, such as K^+, Na^+, Mg^{2+} and Ca^{2+}, are the most important in influencing the gene activities at specific sites on the polytene chromosomes of *Chironomus tentans*, which is indicated by the presence of puffs. It is well known that puffs are morphological manifestations of gene activity and that their production is induced by various agents and chemical components in the environment (Zhimulev, 1996). This author cites many examples of the effect of different ions on the morphology of polytene chromosomes. The studies on isolated polytene chromosomes in vitro show that the chromosomes become more condensed than the chromosomes in vivo. When the concentrations of Ca^{2+} and Mg^{2+} are increased from 1 to 15 mM, the linear size of the chromosome decreases considerably, or specific puffs are induced, or other structures that resemble Balbiani rings are formed. These data support the idea presented here that the activity at sites near to and at the telomere might

be influenced by environmental conditions such as high concentrations of Ca^{2+} and HCO_3^- ions, originating from limestone, or by other ions, such as K^+, Na^+ and Mg^{2+}, found in the River Prądnik. However, the puff that resembles a Balbiani ring recorded on the telomere of chromosome G of *P. convictumin* is a novel structure that could be the basis of future molecular studies on the cell machinery involved in this response.

In addition to the above mentioned functional alterations it is interesting to note that in both of the species studied new, unknown chromosome rearrangements were observed. The species studied showed several levels of karyological changes. In both species the lowest level was rare and consisted of somatic aberrations that occurred in chromosomes in few cells in the salivary gland with a frequency less that 1%. These rearrangements are small and often associated with anthropogenic factors (Dobzhansky, 1970; Caceres et al., 1997). As somatic chromosome rearrangements can be caused by stress agents (Lagadic & Caquet, 1998; Sella et al., 2004; Michailova et al., 2012a) we suggest that the somatic alterations observed in both species indicate the existence of pollution induced stress, which might be due to heavy metals (Cd, Pb and Zn) that occurred at higher concentrations in the sediment in the River Prądnik than in unpolluted water bodies (Förstner & Salomons, 1980; Szarek-Gwiazda & Mazurkiewicz-Boroń, 2006). Based on the geoaccumulation index, the sediment in the River Prądnik was moderately polluted with Pb and Zn, and highly polluted with Cd. Taking into consideration the adverse effect of heavy metals in sediment on the biota that live there, the concentration of Zn in the sediment in the River Prądnik was higher than the probable effect level (PEL, Smith et al., 1996). Above this concentration, Zn is often toxic for organisms. Additionally, the results indicate that considerable amounts of the metals that occur in the sediment are potentially mobile and available to biota (Förstner, 1986). Contamination of the sediment by Cd, Pb and Zn was probably caused by atmospheric emissions, human activity in the catchment basin and scientific activity, e.g. flushing of sediment from caves near the study site. Cave sediment in this area contains higher concentrations of Ni, Pb, Zn, Fe (about two-fold higher), Cu and Zn (three-fold higher) than in the sediment in the River Prądnik (Helios-Rybicka et al., 1991). Therefore, the wide spectrum of somatic rearrangements recorded in the genome of both species might be affected by Cd, Pb and Zn occurring in the sediment in the River Prądnik. There is evidence indicating that zinc generally has a beneficial effect on the genome by reducing the toxicity of cadmium (Coogan et al., 1992). The studies of Michailova et al. (2009a, b) show that trace metals are able to induce different types of somatic chromosomal rearrangements, which can be used as biomarkers of stress agents in aquatic ecosystem. The concentrations of other metals (Cu, Ni and Cr) in the sediment were low (Förstner & Salomons, 1980; Szarek-Gwiazda & Mazurkiewicz-Boroń, 2006). However, it is difficult to monitor all toxicants in the environment. Individual chemicals rapidly change their characteristics in an aquatic environment. Integration among pollutants is more often present than absent. Individual chemicals are modified and integrated by physical, chemical and biological processes. The level of environmental contamination, therefore, is determined by a multitude of chemicals that interact simultaneously and synergistically (Baršiene & Bucinskience, 2001).

Some other aberrations may reflect local adaptations and occur at a higher frequency (White, 1977). In *M. pallidula* they accounted for between 5.26% and 57.14% and in *P. convictum* between 5.55% and 27.8%. They are associated with adaptations for living in specific environmental conditions such as at the low temperature of streams that receive cold water (7.2–8.5°C) from many springs (Galas, 2005). In this area the climate is characterized by low temperature, heavy rainfall and a long period with snow cover (Brzeźniak & Partyka, 2008). The aberrations recorded may also have an important role in the process by which species adapt to high concentrations of nutrients (NO_3^-, NH_4^+ and PO_4^{3-}) and chlorides in the water caused by human activity, e.g. small villages with poor sewage systems and tourism (Miernik & Walega, 2008). Also, some inversions with a local adaptive value are recorded in other species of chironomids. For instance, in *Chironomus plumosus* there are heterokaryotypes that are better able to survive periods of anoxia (Vest Peterson, 1984). McCreadie & Colbo (1992) report specific aberrations in Simuliidae that vary in relation to water quality and characteristics of aquatic basins.

A third level of difference is the differentiation of karyotypes. These are fixed homozygous inversions that occurred in 100% of the individuals of *M. pallidulla* from the River Prądnik but not in the individuals from the Bulgarian population. The population in Bulgaria occurred in fish pools at Raslog at the foot of Mt. Pirin, where the species is monomorphous (Michailova, 1989). The fixed homozygous inversions recorded indicate differences in their karyotype, which are the main markers of the early stages of species divergence (Keyl, 1962) and might be related to adaptive process in the early stages of speciation. Similarly, fixed sequences are found in the Holarctic midge *Glyptotendipes barbipes* (Martin & Porter, 1973). Also, Butler et al. (1999) record cytogenetic differentiation between Palearctic and Nearctic populations of *C. plumosus.* So, the karyotype divergence between Bulgarian and Polish populations is likely to be associated with adaptive processes in the early stage of speciation. However, in the future it may be possible, using a detailed analysis of the morphology of all the developmental stages plus a DNA analysis, to clarify the taxonomic status of Polish populations.

The results obtained in this study confirm the idea of Michailova et al. (1996), Logadic & Caquet (1998) and Steinberg et al. (2008) that the genome is very sensitive to changes in the environment and demonstrate that changes in the genome can be used to detect pollutants in aquatic ecosystems. Moreover, the results also show that the response of species at the cytogenetic level, i.e. alterations in the structure and function of the salivary gland

chromosomes, are the result of the influence of environmental genotoxic agents and changes in physicochemical parameters of the water environment.

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