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Chironomid Midges (Diptera, Chironomidae) Show Extremely Small Genome Sizes

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Chironomid midges (Diptera; Chironomidae) are found in various environments from the high Arctic to the Antarctic, including temperate and tropical regions. In many freshwater habitats, members of this family are among the most abundant invertebrates. In the present study, the genome sizes of 25 chironomid species were determined by flow cytometry and the resulting C-values ranged from 0.07 to 0.20 pg DNA (i.e. from about 68 to 195 Mbp). These genome sizes were uniformly very small and included, to our knowledge, the smallest genome sizes recorded to date among insects. Small proportion of transposable elements and short intron sizes were suggested to contribute to the reduction of genome sizes in chironomids. We discuss about the possible developmental and physiological advantages of having a small genome size and about putative implications for the ecological success of the family Chironomidae.

Key words: genome size, C-value, insects, chironomids, ecological adaptation

INTRODUCTION

Chironomids, or non-biting midges, often constitute the most abundant group of insects in freshwater, and also include terrestrial and marine species. The family Chironomidae is estimated to comprise about 10,000 species in the world (Cranston, 1995a) and is widely distributed since these midges are found in a large variety of habitats (Fig. 1). Chironomids are especially remarkable as many species show a wide range of tolerance to harsh environmental conditions, such as hypoxia, low temperatures, high salinity, low pH or desiccation (Pinder, 1986). Indeed, many species are tolerant to poorly oxygenated conditions, because most Chironomids possess a large quantity of hemoglobins, proposed to act in oxygen storage (Pinder, 1986; Burmester and Hankeln, 2007). Chironomids are thus widely distributed and ecologically adapted to a great variety of environments. Since chironomid larvae are found in most of the types of freshwater ecosystems, they constitute good bioindicators to assess the water quality and the impact of chemicals from human activities on aquatic environments (Madden et al., 1995). The adults form sometimes huge mating swarms, which may represent a sanitary threat for human populations, especially causing allergies in hypersentitized people (Cranston, 1995b). Chironomid midges are also economically important, as their larvae are reared in mass to produce live or lyophilized food for fish farms and aquarium fish keepers around the world.

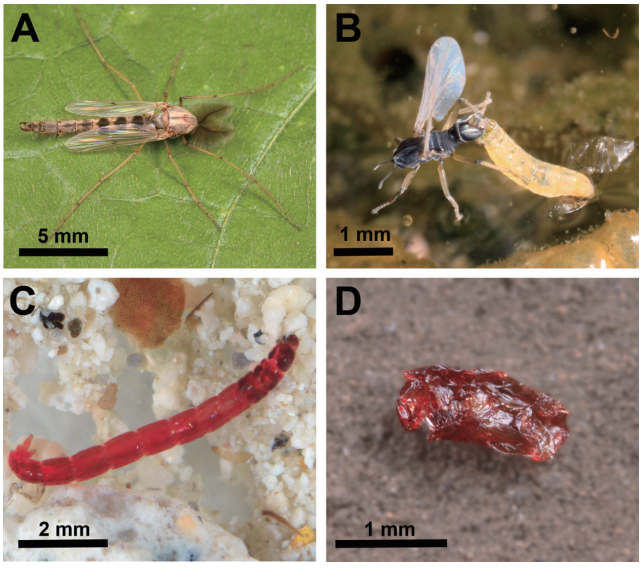

Fig. 1. Pictures of some Chironomid species, showing larval or adult stages. **(A)** Male adult of *Chironomus plumosus*, the biggest of all Chironomids. Note the fluffy antennae, characteristic of males in most of the species in this family. **(B)** Mating of *Clunio tsushimensis* on a seashore. This marine species shows a conspicuous sexual dimorphism with an apterous worm-like female. **(C)** *Polypedilum tamanigrum* larva found in an acidic stream. Most Chironomid larvae are red, since their body fluid contains hemoglobins, and they are thus commonly called bloodworms. **(D)** Larva of the anhydrobiotic species *Polypedilum vanderplanki* in the dry state. Upon rehydration, this larva can revive as fast as within 20 min.

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Some important work has been conducted on the physiology and molecular biology of these insects. For example, the diversity of hemolymphatic hemoglobins, which constitutes a characteristic feature of chironomids, has been investigated in detail (Ruf et al., 1994; Hankeln et al., 1998), mainly within the genus *Chironomus* (Fig. 1A). More recently, the mechanism of tolerance to low temperatures in the Antarctic midge *Belgica antarctica* is well documented (Lee et al., 2006; Rinehart et al., 2006). In our research group, we have been studying for a decade extreme desiccation tolerance, known as anhydrobiosis, in the African sleeping chironomid, *Polypedilum vanderplanki* (Hinton, 1951, 1960a, 1960b; Watanabe, 2006). The expression of various chaperone proteins, antioxidants, and the ultimate vitrification of a trehalose matrix have been shown to be essential for successful anhydrobiosis (Kikawada et al., 2006; Sakurai et al., 2008; Cornette et al., 2010; Cornette and Kikawada, 2011). Recently, genomic DNA was shown to experience severe damage during anhydrobiosis and these damages were repaired slowly after rehydration (Gusev et al., 2010). Consequently, sequencing *P. vanderplanki* genome was needed for a better understanding of the mechanism of desiccation tolerance and of the organization of a chironomid genome directly interacting with a stressful environment. However, the genome size of *P. vanderplanki* remained unknown and data concerning the genome size of chironomids were restricted to a couple of species (Petitpierre, 1996; Schmidt-Ott et al., 2009). Furthermore, in various taxonomic groups, a correlation was found between genome size and some characters such as cell size, developmental complexity or metabolic rate (Gregory, 2005). Thus, a possible relation between genome size and specific traits in chironomids deserved to be investigated.

In the present work, we determined the genome sizes from an array of chironomids and data showed that the family Chironomidae comprises species with uniformly small genomes. The putative relationship between the small genome size of chironomids and their body size or adaptation to harsh environments is also discussed.

MATERIALS AND METHODS

Insects

Chironomid species were collected in various environments in Japan, Australia and Nigeria, or kindly provided by researchers. A Nigerian strain of the sleeping chironomid (*P. vanderplanki*, Fig. 1D), originally collected in temporary rock pools, is routinely reared in the laboratory on milk agar under a 13–11 h light:dark photoregime at 27–28°C (Watanabe et al., 2002). Chironomids collected in the field at the larval stage were reared in similar conditions, when possible, in order to obtain adults for species identification and flow cytometry analysis.

Female adults of the fruit fly, *Drosophila melanogaster* strain W1118, were used as a standard to calculate and express the C-values of chironomid species. Under our experimental conditions, the measured genome size of W1118 strain was not significantly different from that of Oregon-R and iso-1 strains and the generally accepted C-value for *D. melanogaster* strains Oregon-R and iso-1 is 0.18 pg (Rasch et al., 1971; Johnston et al., 1999; Bennett et al., 2003).

Flow cytometry

Heads of Chironomid larvae and adults, and *D. melanogaster* adults (3 individuals per sample) were chopped with microscissors in a solution of 0.5 % Triton X-100 in 1 ml PBS buffer and digested for about 2 h at room temperature. The resulting lysate was then stained with 5 μg/ml of propidium iodide (PI) for several minutes and filtered with CellTrics Filter using mesh diameter of 30 μm (Partec) in order to collect nuclei. The DNA contents of dissociated nuclei were measured by a Coulter Epics® Elite flow cytometry system (Beckman Coulter, Fullerton, CA) with the argon laser emitting 15 mW of exciting light at 488 nm. Fluorescent nuclei were detected using a high bandpass filter (610 nm). The C-value was calculated by multiplying the ratio of the mean fluorescence peak of the 2C sample/mean peak of the 2C standard with 0.18 pg, the haploid DNA content of *D. melanogaster* (Rasch et al., 1971). An example of the peaks obtained for *P. vanderplanki* and *D. melanogaster* nuclei is shown in Fig. 2.

RESULTS

Genome size estimates for the 25 species reported in this study are provided in Table 1. The obtained values range roughly threefold, from 0.07 pg in the marine Chironomid *Clunio tsushimensis* (Fig. 1B) to 0.20 pg in the Australian species *Paraborniella tonnoiri.* Thus, the family Chironomidae comprised uniformly very small genome sizes, even in comparison to their Dipteran relatives, whose genome sizes range up to 20-fold (Fig. 3).

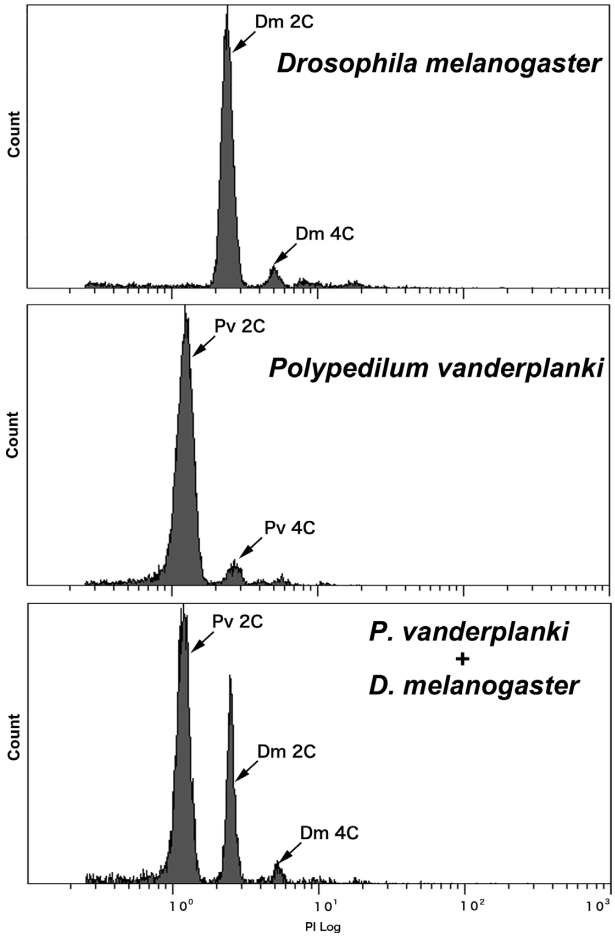

Fig. 2. Examples of nuclei count patterns obtained by PI-stained flow cytometry for *D. melanogaster*, *P. vanderplanki* and for a mixture of both species. The major peak was composed of 2C cells (G_0/G_1 phase).

Table 1. Summary of the estimated C-value data of Chironomids obtained in the present work, shown with the standard deviations (S.D.) and the number of individuals (*n*; M = male, F = female, L = larva).

Subfamily	Species name	Origin	Estimated C-values (pg)		
			Average	S.D.	*n*
Chironominae	*Polypedilum vanderplanki*	Nigerian strain	**0.10**	0.01	*7M, 12F, 6L*
	Polypedilum tamanigrum	Sukayu spa, Aomori, Japan	**0.11**	0.02	*8F*
	Polypedilum nubifer	Tsukuba, Japan	**0.10**	0.01	*8F, 6L*
	Paratanytarsus grimmii	Kisogawa river, Nagoya, Japan	**0.10**	0.00	*6L*
	Sergentia kizakiensis	Tsukuba, Japan	**0.10**	0.00	*5M*
	Tanytarsus takahashii	Tsukuba, Japan	**0.09**	0.02	*3M, 7F*
	Paraborniella tonnoiri	Merredin, Australia	**0.20**	0.03	*5M, 3F, 6L*
	Stictochironomus akizukii	Lake Biwa, Hikone, Japan	**0.13**	0.00	*5F*
	Chironomus nippodorsalis	Tsukuba, Japan	**0.12**	0.01	*3M, 3F*
	Chironomus sulfurosus	Ryuodani spa, Kirishima, Japan	**0.15**	0.01	*4F*
	Chironomus salinarius	Ichikawa, Japan	**0.16**	0.02	*12L*
	Chironomus plumosus	Lake Kasumigaura, Japan	**0.15**	0.01	*3M, 8F*
	Chironomus fusciceps	Unzen spa, Japan	**0.16**	0.02	*1M, 5F*
	Chironomus acerbiphilus	Lake Katanuma, Japan	**0.17**	0.01	*5F*
	Glyptotendipes tokunagai	Tsuchiura, Japan	**0.16**	0.00	*4F*
	Dicrotendipes pelochloris	Tsukuba, Japan	**0.12**	0.01	*6M, 9F*
Orthocladiinae	*Hydrobaenus tsukubalatus*	Tsukuba, Japan	**0.08**	0.01	*11M*
	Cricotopus sylvestris	Tsukuba, Japan	**0.11**	0.02	*4F*
	Cricotopus bicinctus	Tsukuba, Japan	**0.10**	0.00	*6M, 5F*
	Cricotopus sp.	Tsukuba, Japan	**0.15**	0.01	*5F*
	Clunio tsushimensis	Shirahama, Japan	**0.07**	0.00	*4M, 9F*
Diamesinae	*Diamesa japonica*	Mt. Nantai, Nikko, Japan	**0.08**	0.01	*4M, 14F*
Tanypodinae	*Ablabesmyia monilis*	Tsukuba, Japan	**0.16**	0.03	*8F*
	Tanypus punctipennis	Tsukuba, Japan	**0.16**	0.01	*4F*
Telmatogetoninae	*Telmatogeton japonicus*	Shirahama, Japan	**0.13**	0.01	*8F*

The present study allowed us to extend the Chironomid genome size dataset from three to 28 species, covering six of the 11 Chironomid subfamilies. Classified by subfamily, the mean C-values were 0.13 pg for Chironominae, 0.10 pg for Orthocladiinae, 0.08 pg for Diamesiinae, 0.13 pg for Prodiamesinae, 0.16 pg for Tanypodinae and 0.13 pg for Telmatogetoninae. The smallest genome sizes were observed in the subfamilies Orthocladiinae and Diamesinae. An apparent relationship was observed between genome sizes and the phylogeny within the family Chironomidae with uniform C-values around 0.10 pg for the genus *Polypedilum* and the relatively larger genome sizes around 0.15 pg in the genus *Chironomus* (Fig. 3). In addition, Orthocladiinae and Diamesinae apparently showed smaller genome sizes.

No clear correlation was found between the genome size of Chironomids and their body size (Figs. 4A, 4B).

DISCUSSION

We determined here the genome sizes of 25 chironomid species; these values were congruent with the genome sizes of the three species previously reportedin the literature (Petitpierre, 1996; Schmidt-Ott et al., 2009), that were comprised between 0.13 and 0.21 pg DNA. The results of this study showed that the genome sizes of chironomids were uniformly very small (Fig. 3, Table 1). The obtained mean C-value for the Chironomidae family is 0.13 ± 0.03 pg DNA, which is clearly smaller than *D. melanogaster* genome size. The smallest genome size among insects has been recorded in the Strepsiptera *Caenocholax fenyesi* with about 0.11 pg DNA (corresponding roughly to 108 Mb), although the Hessian fly *Mayetiola destructor* was also suggested to have an even smaller genome size of 0.09 pg (Johnston et al., 2004; Gregory, 2014). Here, we report at least three species with smaller genomes than any known insect: *C. tsushimensis*, *Diamesa japonica* and *Hydrobaenus tsukubalatus*, with C-values of 0.07 pg, 0,08 pg and 0.08 pg, respectively (Table 1). To our knowledge, *C. tsushimensis* (Fig. 1B) can be now considered as having the smallest genome size of any known insect, with a C-value of 0.07 pg DNA, corresponding roughly to 68 Mb. This value is congruent with the genome size of a related species, *Clunio marinus*, which was estimated once to 95 Mb and more recently to 87.2 Mb (Kaiser and Heckel, 2012; Tobias S. Kaiser, personal communication).

Recently, the genome of the Antarctic midge, *B. antarctica*, was sequenced (Kelley et al., 2014). The authors claimed that the genome size of this species, estimated to 89.5–105 Mb, was the smallest among all insects. However, we show here that this genome size is comprised within the normal range of chironomids values. The authors also suggested that the small genome size of *B. antarctica* was an adaptation to extreme cold environment. Our data may corroborate this hypothesis, since *D. japonica*, an alpine species from the subfamily Diamesinae also presents a tiny genome size. Another related Diamesinae, the Himalayan midge *Diamesa sp.* was actually found to be active at temperatures as low as –16°C (Kohshima, 1984). However, *Telmatogeton japonicus*, which is also able to develop at very low temperatures (Danks, 1971; Sunose and Fujisawa, 1982), shows a relatively large genome size, compared to the chironomid family average. A phylogenetic influence on the genome size should be taken into account here, since *B. antarctica* belongs to the Orthocladiinae subfamily, which presents small genome sizes on average. The species *C. tsushimensis* and *Hydrobaenus tsukubalatus*, showing the smallest genome sizes among chironomids, also belong to the Orthocladiinae subfamily. Thus, the small genome size of *B. antarctica* may simply constitute an ancestral trait, not the result of extreme cold adaptation.

P. vanderplanki is the only insect species known to achieve anhydrobiosis in a completely dehydrated state (Fig. 1D) and recover after rehydration (Hinton, 1951,

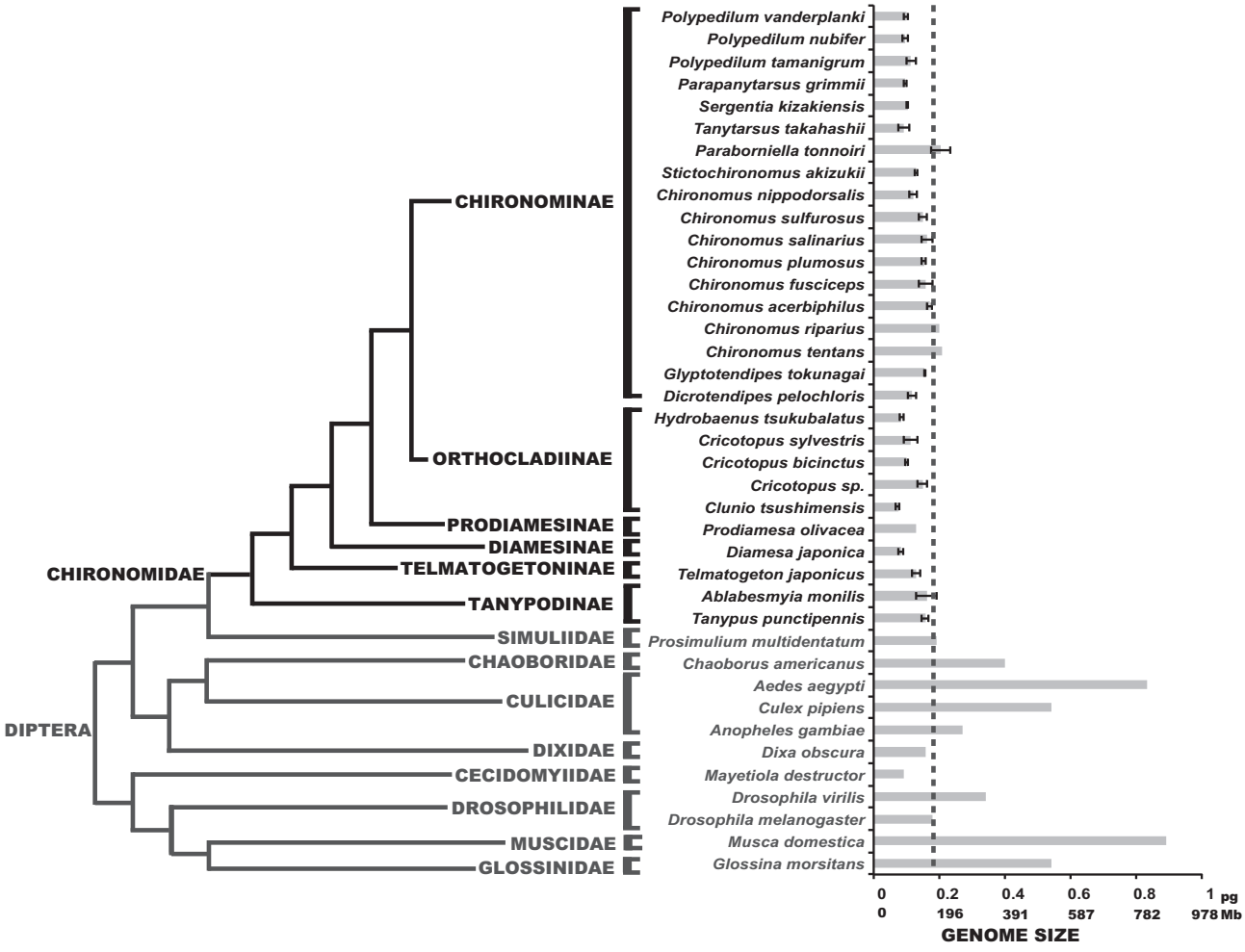

Fig. 3. Phylogeny of Diptera showing estimated C-values. The Chironomid family is shown in black. The dash line shows the genome size of *D. melanogaster*. The phylogenetic cladogram is a consensus from different Chironomid and Dipteran trees (Yeates and Wiegmann, 1999, 2005; Saether, 2000; Cranston et al., 2012). Errors bars show standard deviation. Genome sizes without error bars were obtained from previous studies listed in the Animal Genome Size Database (Gregory, 2014).

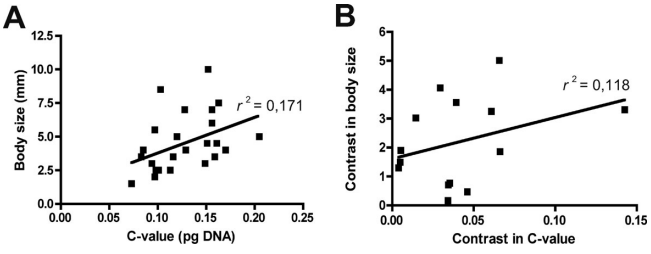

Fig. 4. Relationship between body size and genome size among the chironomid species investigated in the present study. **(A)** For each species, the mean total body length is shown in millimeters and the mean C-value is expressed in pg DNA. Only a weak positive correlation (r^2 = 0.171, n = 25), materialized by a solid line, was observed between the two variables. **(B)** At the genus level, means of the same values were transformed by the method of phylogenetically independent contrasts, based on the phylogeny of Cranston et al. (2012). The correlation between these transformed contrasts, materialized by a solid line, was not significant (r^2 = 0.118, *n* = 13). Data analysis was performed on Prism 4.0 software for Macintosh (GraphPad Software, Inc.).

1960a; Watanabe, 2006; Cornette and Kikawada, 2011). Our research group recently published a comparative analysis of the genome sequences of the desiccation-tolerant *Polypedilum vanderplanki* and the desiccation-sensitive congeneric *Polypedilum nubifer* (Gusev et al., 2014). The comparison showed that both species had similar genome sizes and that only a limited set of duplicated gene clusters was related to anhydrobiosis in *P. vanderplanki* (Gusev et al., 2014). The genome sizes inferred from genome assembly were 104 Mb for *P. vanderplanki* and 107 Mb for *P. nubifer* and these values did not differ markedly from the genome sizes measured for these species in the present study, which corresponded to approximately 98 Mb. *P. vanderplanki* and *P. nubifer* genomes showed a similar low proportion of DNA repeats and only a small number of transposable elements were found, compared to other dipteran species. This reduction of the proportion of transposable elements was also observed in the Antarctic midge, *B. antarctica* (Kelley et al., 2014). The average intron length was also considerably reduced in the Antarctic midge and in both *P. vanderplanki* and *P. nubifer* as well (Gusev et al., 2014). Since the des-

iccation sensitive and freeze intolerant *P. nubifer* shares these features with the Antarctic midge and the anhydrobiotic midge, a low proportion of transposable elements and short intron length may constitute a characteristic shared by all chironomids and may be not directly related to the tolerance to extreme environments, even if an adaptive effect could not be excluded.

What could be the adaptive traits potentially associated with the tiny genome sizes among the Chironomidae family? In fact, several developmental and ecological factors have been suggested to correlate to genome sizes. First, a positive correlation between genome size and body size has been found in many taxa and this correlation appears as a relatively general phenomenon (Gregory, 2005). This correlation has been reported also in mosquitoes (Ferrari and Rai, 1989), but it is not always clear in other insect taxa (Gregory and Hebert, 2003; Ardila-Garcia and Gregory, 2009). Within the chironomids, we found only a weak positive correlation between genome size and body size (Fig. 4A). The mean C-value for the genus *Chironomus* (0.15 pg) was higher than that for the genus *Polypedilum* (0.10 pg), and this difference may be attributable to the generally larger body size of *Chironomus* species. However, the phylogenetic distance between both genera could also explain this difference of genome sizes. Consequently, we corrected our data at the genus level with phylogenetically independent contrasts analysis (Garland and Adolph, 1994; Garland et al., 2005) and as a result, the correlation between genome size and body size turned out to be non significant (Fig. 4B). The narrow range of Chironomid genome sizes (only two–threefold) and the average small body size of these species (most do not exceed a few millimeters) probably make difficult to find any correlation between both traits within the Chironomidae family.

Among insects, another correlation was suggested between genome size and developmental complexity. Whereas hemimetabolous insects, with gradual nymphal molts only, show a wide range of genome sizes (C-values from 0.18 to 16.93 pg), holometabolous insects with their complex metamorphosis have C-values restricted within a putative 2 pg threshold, for most of them (Gregory, 2002, 2005). The influence of developmental complexity on genome size is here obvious because holometabolous insects undergo intensive morphological remodeling during the limited time of metamorphosis and a small genome size presents advantages for accomplishing cell divisions at a high rate. Chironomids are holometabolous insects and their metamorphosis is extremely rapid. Their pupal stage may last only one day, or even just a few hours for certain species (Cranston, 1995c). For example, *C. tsushimensis* shows a complex metamorphosis with pronounced sexual dimorphism (Fig. 1B).

The relation between genome size and the rate of cell division also influences the developmental rate. For example, the rapid life cycle of aphids was suggested to be related to their small genome sizes (Ma et al., 1992). Thus, small genome sizes should constitute an advantage for chironomid species rapidly developing in temporary environments. Chironomids from the genus *Clunio* can develop into temporary tide pools and also show synchronized emergence of the adults in relation to the moon cycle and during the short window of the low tide (Kaiser and Heckel, 2012). A high developmental rate should be needed to achieve this. Note that small polychaete annelids developing rapidly in similar interstitial environments show smaller genome sizes than macrobenthic species (Gambi et al., 1997). For Antarctic midges, such as *B. antarctica* or *Eretmoptera murphyi* (Lee et al., 2006; Worland, 2010), larval development lasts two years, mostly arrested in a frozen state, but metamorphosis and reproduction must occur during the very short summer period. In this case, a high developmental rate is needed and this may be facilitated by *B. antarctica* small genome. This phenomenon was illustrated by a study on angiosperm plants, showing that the species able to complete their entire life cycle during the short Antarctic summer presented the smallest genome sizes (Bennett et al., 1982). Another extreme, the anhydrobiotic midge *P. vanderplanki* is adapted to ephemeral rock pools. Here again, high developmental rate in a temporary habitat may be linked with the small genome size of this species. However, the Australian species *P. tonnoiri*, which is also exposed to desiccation on similar rock pools (Jones, 1975; Adams, 1985; Frouz et al., 2003), shows a relatively large genome size for a chironomid (Fig. 3 and Table 1).

Abiotic stresses are also likely to influence DNA integrity and as a consequence, genome size. For example, larvae of *P. vanderplanki* experience massive DNA damage after anhydrobiosis and efficient DNA repair occurs during the few days following rehydration (Gusev et al., 2010). In plants, a negative correlation was found between genome size and radiation tolerance (Bennett and Leitch, 2005) and mutation rate was also lower in species with smaller genome size (Abrahamson et al., 1973). Consequently, the small genome size of *P. vanderplanki* may represent an advantage to avoid the accumulation of deleterious mutations during the DNA repair events associated with cycles of desiccation and rehydration. As a marine species, *C. tsushimensis* is also exposed to high salinity stress, which is lethal for most insect species. High salinity and other abiotic stresses are known to generate intracellular reactive oxygen species (ROS), which were suggested as a main source of DNA damage (Franca et al., 2007; Gill and Tuteja, 2010). This might also be related to small genome size. However, other marine and high salinity tolerant species, such as *T. japonicus* or *Chironomus salinarius* do not show genome sizes as small as *C. tsushimensis*. Acid-tolerant species, such as *Chironomus sulfurosus*, *Chironomus acerbiphilus* or *Polypedilum tamanigrum* (Fig. 1C) can survive in hot springs with a pH as low as 1.4 (Doi et al., 2004; Takagi et al., 2005). Here again, no clear correlation between tolerance to acidic stress and small genome size could be observed and the phylogenetic influence appeared more important, with smaller genome size for the genus *Polypedilum* and larger genome sizes in the genus *Chironomus*.

To conclude, the tiny genome sizes observed in chironomids are probably the result of an ancestral reduction of the number of transposable elements and of the length of introns in their genome structure. Among the Chironomidae family, genome sizes differed according to phylogeny at the subfamily level and at the genus level. As inferred from correlations in various taxa, the small genome size of chironomids theoretically could have constituted a preadaptation to

unstable and extreme environments through high developmental rate and low mutation rate. We hope that this study will encourage future Chironomid genome projects and that comparative genomics within this taxon will help to understand how Chironomid genomes are interacting with their environment.

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