Preliminary Physical Maps of the *Chironomus* Genome, with a Focus on Genes Potentially Involved in Response to Heavy Metals.

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ABSTRACT: Larvae of the genus *Chironomus* are often able to survive high levels of pollution. This makes them of value as potential indicators of pollution and for studies of the genetic mechanisms that permit such survival. Identification of genes that are up-regulated in the presence of pollutants, such as heavy metals, provides the potential to detect increasing levels of pollution, and so take appropriate remedial action before pollution levels become high enough to cause irreparable damage. This paper reports preliminary studies using the Australian species, *C. duplex* Skuse, to identify and map genes that are known to be involved in the response to heavy metals in other species of animals and plants. One of these genes, HSP70, was shown to contain a deleted copy in *C. duplex* and its close relative *C. occidentalis* Skuse. The physical location of the heavy metal related genes, and other *Chironomus* genes already in the literature, has been determined by *in situ* hybridization to the polytene chromosomes of *C. duplex* and of the North American species *C. dilutus* Shobanov et al., which is commonly used in bioassays for pollution. The results demonstrate that a map constructed for one species can be extrapolated to other species of interest in the genus, because of the good knowledge of the phylogenetic relationships of the banding patterns of the polytene chromosomes. Some phylogenetic implications of the results are discussed.

Human activities have resulted in pollution of many aquatic ecosystems with pesticides and toxic wastes, including heavy metals. Such pollutants can disrupt the stability of ecosystems, resulting in irreversible changes and serious biotic loss. There is an increasing awareness of the

need for methods by which levels of pollution can be monitored so that remedial action can be taken before there is irreparable damage to the ecosystem.

Chironomus are ideal candidates for investigation, because of their abundance in lentic ecosystems and the important position they occupy in the food chain as major food sources for fish, other vertebrates, and invertebrates. There have been many studies demonstrating that larvae of the genus *Chironomus* are able to survive and adapt to high levels of pollution and may be the only insects present (Armitage et al., 1995). They have been extensively studied for their ecological response to pollutants (eg. Janssens de Bisthoven et al., 1992; Govinda et al., 2000; Groenendijk et al., 2002), and are EPA-approved test organisms in the U.S.A (USEPA, 1996). As yet little is known of the genetic mechanisms by which adapted chironomids are able to tolerate higher pollution levels, although studies have begun to identify some of the genes that may be involved (Mattingly et al., 2001; Yoshimi et al., 2002, Karouna-Renier and Zehr, 2003). The genes isolated were shown to be upregulated in the presence of heavy metals or insecticides. However it is not known whether the genes obtained in these studies are involved in adaptation to pollutants as there are no linkage data available for genes involved in adaptation at this time. Rather, the initial impetus for such studies is the possibility that the level of upregulation will be correlated with the level of pollutants in the system, and that an increase in activity of such genes can be detected at lower levels of pollutants than is possible by other means (Beaty et al., 1998; Mattingly et al., 2001). These studies are still at an early stage, and much more sophisticated analyses will be necessary before it becomes clear whether such an approach will be viable (Beaty et al., 1998). In order to provide some of the necessary background information that will be required, this paper reports on the physical mapping of genes possibly involved in heavy metal metabolism or response to pollution, as well as other anchor genes, to the polytene chromosomes. Since the phylogenetic relationships of the

polytene chromosomes of *Chironomus* species are well known, the results for one species can be extrapolated to other species of *Chironomus*. However, in some cases, the species commonly used in pollution studies such as *C. riparius* Meigen (= *C. thummi* Goetghebuer) in Europe and *C. dilutus* (formerly included in *C. tentans* Fabricius) in North America, use different schemes for identification of the bands on the polytene chromosomes. The results for genes obtained from other species can still be used for creation of physical maps by the use of comparative *in situ* hybridization. In this study labelled segments of DNA from genes derived from various species of *Chironomus* have been used to compare the physical maps obtained for the Australian species *C. duplex*, and the North American species *C. dilutus*. The results indicate how such studies can resolve phylogenetic problems when the appearance of the bands alone is not conclusive. On a broader phylogenetic scale, some attempt is made to relate the *Chironomus* linkage groups to those of the well studied dipteran species *Anopheles gambiae* Giles and *Drosophila melanogaster* Meigen (Bolshakov et al., 2002).

Materials and Methods

Stocks of *Chironomus dilutus,* originally from Winnipeg, Manitoba, Canada, and *C. duplex*, originally from Werribee, Victoria, Australia, are maintained in the laboratory (Martin et al., 1980; Martin and Lee, 1988). Polytene chromosomes were prepared and utilized for *in situ* hybridization using the protocols for Biotin-labelling (Phillips et al., 1994) or DIG-labelling (Phillips et al., 1999). Gene segments for use as probes were obtained by PCR using degenerate primers (Table 1), and the amplified products were cloned into pGEM-T Easy (Promega). Other genes were obtained as purified DNA from cloned genes or gene segments, kindly provided by colleagues in other laboratories, and were labelled in the same manner. Gene sequences

obtained in this study have been lodged in GenBank with Accession numbers: AY490744-AY490756 and AY494854-AY494855.

The physical maps were prepared using digital images of the chromosome arms of each species. The banding patterns of the *C. duplex* arms were labelled according to the schemes established by Keyl (1961) for arms A, E, and F, and by Devai et al. (1989) for arms B, C, and D. In the case of *C. dilutus*, they were labelled according to the scheme of Beermann (1952), as applied by Kiknadze et al. (1996).

Results

Genes that have been implicated in response to heavy metals in species of *Chironomus* or other organisms and that have been successfully amplified in this study are listed in Table 1, along with the primers used to amplify them by PCR.

ALPHA-TUBULIN (AT): Mattingly et al. (2001) cloned the cDNA of a metal responsive alpha-Tubulin 1 gene from *Chironomus dilutus*, but did not attempt to localize it to the polytene chromosomes. A portion of an homologous gene was amplified and cloned from *C. duplex*. When DIG-labelled and used for *in-situ* hybridization, this probe labelled two sites on the chromosomes of both *C. duplex* and *C. dilutus*. One site was on arm E, near the junction of arms E and G in *C. duplex* and about 13B1 in *C. dilutus* (Fig. 2a). Comparison with the site of binding in the related Australian species *C. oppositus* Walker and *C. 'februarius'* (provisional name), indicates that the band in *C. duplex* is 4e. The other was on arm G, about one quarter of the length of the arm from the distal end in *C. duplex*, and about 1C1 in *C. dilutus* (Fig. 2c).

GLUTATHIONE SYNTHASE (GS): This gene has not been cloned from any *Chironomus* species, so degenerate primers were designed based on sequence of this gene from various organisms in GenBank. These amplified a fragment from *C. duplex* that showed homology to GS of these

other organisms. When DIG-labelled and used for *in situ* hybridization, this fragment bound to a single site on arm A of both species; at about A8f in *C. duplex*, and about 20A1 in *C. dilutus* (Fig. 1a).

GLUTAMYL-CYSTEINE SYNTHASE (GCS): This gene again has not been cloned from any *Chironomus* species. In mammals it is a heterodimer comprising a catalytically active heavy subunit (GCS-CAT) and a light subunit (GCS-REG) that regulates the affinity of GCS-CAT for substrates and inhibitors (Griffith, 1999). The primers were designed from conserved regions of GCS-CAT sequences in GenBank. They were used successfully to amplify a fragment that showed homology to GCS-CAT of other organisms. When DIG-labelled and used for *in situ* hybridization, this fragment bound to a single site on arm B: at about 27e in *C. duplex*, and about 11C1 in *C. dilutus* (Fig. 1b).

HSP70 FAMILY: This family includes both the inducible heat-shock genes, HSP70 (P70), and the constitutively expressed cognate genes, HSC70 (C70) (Rubin et al., 1993). These genes are very well conserved in the 5' region of the gene, but differ at the 3' end. The primers of Yoshimi et al. (2002) are for the 5' region, but have amplified only C70 fragments homologous to HSC70-4 of *D. melanogaster*, in *C. yoshimatsui* (Yoshimi et al., 2002), *C. dilutus* (Karouna-Renier et al., 2003) and when used with *C. duplex* in this study. The DIG-labelled fragment binds to sites on all chromosome arms, which probably includes most of the C70 genes, as well as P70. Some sites are not clear, possibly because of low homology between the probe and the sequence of that member of the family. In *C. duplex*, clear binding was observed at about 3i and 7a on arm A (Figs. 1a and 2d); at about 21b on arm B (Figs. 1b and 2d); at about 4i on arm C (Fig. 1c); at about 16b and 15b on arm D (Fig. 1d); at about 14f on arm F (Fig. 2a); and at two sites on either arm E or G, one near the distal end of arm G (Figs. 2c and d) and the other at about 4a on arm E (Fig. 2b). For reasons given below, the more distal site is considered to be

band 7b of arm E. In *C. dilutus*, clear binding was obtained at about 18A1 and 20A1 on arm A (Fig. 1a); at about 6A1 of arm B (Fig. 1b); at about 7C2 on arm D (Fig. 1d); at about 12C1 on arm E (Fig. 2a); at about13B4 on arm F (Fig. 2b); and at about 1B1 on arm G (Fig. 2c).

In order to obtain sequence from the HSP70 members of the family, new primers (Table 1) were designed for the 3' end of the gene, where there was homology between the HSP70 genes of other Diptera, but the sequence differed from that of the HSC70 genes. These primers were found to amplify two fragments from DNA of *C. duplex*, which differed in size by 98 bp (Fig. 3). Sequencing of the two fragments indicated the presence of a 29 amino acid (AA) in-frame deletion, followed by a further 11 nucleotide deletion that caused a frame-shift and generates a new stop-codon four AA before the usual site. The same primers were therefore used on genomic DNA of the close relatives of *C. duplex* (*C. australis* Macquart, *C. occidentalis* Skuse, and *C. oppositus* Walker) as well as *C. dilutus*. A single PCR product corresponding to the longer sequence was obtained in *C. dilutus, C. australis* and *C. oppositus*, but the second, deleted sequence, was also present in *C. occidentalis* (Fig. 3). Only the longer fragment was DIG-labelled and used for *in situ* hybridization, and bound to a single site in each species. This site corresponds to one of the sites labelled by the HSC70 probe (Fig. 2d), but appears to be on arm G in *C. duplex*, while it is at about 12C1 on arm E in *C. dilutus* (Fig. 2c).

CU,ZN SUPEROXIDE DISMUTASE: Another gene that has not previously been cloned from any *Chironomus* species, so degenerate primers were designed from conserved regions of exon 2, as indicated by sequences in GenBank. These primers amplified a region with apparent homology to Cu,Zn SOD from other organisms. However, there is an additional 626 bp sequence in the amplified region of the *Chironomus* gene that is not present in other insects. The presence of a consensus gt/ag boundary to this extra sequence suggested it was an intron and this was confirmed by sequencing a cDNA copy of the gene, where it had been excised. An intron of

sporadic phylogenetic occurrence has been recorded in exon 2 of a few other Diptera (Kwiatowski et al., 1994), but this occurs about 40 bp upstream of the intron position in *C. duplex*. When DIG-labelled and used for *in situ* hybridization, the SOD fragment bound to a single site on arm D: at about 4b in *C. duplex*, and about 2B5 in *C. dilutus*.

OTHER AVAILABLE CLONES: Genes which are not involved in the heavy metal pathway, for which cloned sequence was obtained, are listed in Table 2, along with the source of the material. In some cases these genes had already been cytologically located by *in situ* hybridization, but in other cases this has been done for the first time in this study. The Balbiani ring genes (BR1-3), which code for genes involved in silk production, were known to be on arm G (Fig. 2c) and are named for their association with the BRs of *C. tentans*, and hence also of *C. dilutus* which has two sequences of arm G in common with *C. tentans* (Kiknadze et al., 1996). No clone of BR2 was available but, since the clones of BR1 and BR3 hybridized to other sites on what had previously been considered to be part of arm E (Martin, 1971, cf. Fig. 2c), it is assumed that the main BR of *C. duplex* is BR2. Clones of other genes associated with silk production, ssp160 and sp140, have also been localized previously. Ssp160 is associated with BR4 in *C. pallidivittatus* (sensu Edwards) (Hoffman et al., 1996), but is not present in *C. tentans* or *C. dilutus* (Martin et al. 2002). However the equivalent band is marked in Fig. 2c as '160'. This gene is immediately distal to the nucleolus in *C. duplex* (Fig. 2c). Sp140 was located to region 17 of arm F of *C. tentans* by Galli et al. (1990). The Biotin-labelled clone bound at about 17a on arm F of *C. duplex*, and about 17B3 on arm F of *C. dilutus* (Fig 2b).

Other genes previously mapped include histone and globin genes, and the gene for a sex influenced protein (SIP). Hankeln and Schmidt (1991) isolated a histone gene cluster and localized this cluster to five sites on arm D of *Chironomus thummi*. Subsequently, Hankeln et al. (1993) examined several European *Chironomus* species, including *C. tentans,* and showed

that most of these loci were conserved, although only four loci could be visualized in some species, including *C. tentans*. When Biotin-labelled, this cluster bound as expected to Arm D, with sites at about 15c, 10d, 10a, 19e and 22d in *C. duplex*, and at about 2A3, 3C2, 5C5 and 9A3 in *C. dilutus* (Fig. 1d), the latter corresponding to the map positions found in *C. tentans* by Hankeln et al. (1993). Hankeln et al. (1993) also used a cloned copy of an orphon histone, which has relatively little sequence homology to the main histone cluster. This orphon cluster hybridized to the telomere of chromosome IV (erroneously called arm E by Hankeln et al., 1993) of a number of species including *C. tentans*, and hence also *C. dilutus* (Fig. 2c). Schmidt et al. (1988) used *in situ* hybridization to localize the monomeric, Gb3, and the dimeric, Gb1, globin gene clusters in 13 *Chironomus* species including *C. tentans*. The monomeric locus was on arm E, at 1d according to the Keyl system, and at 16C1in *C. tentans*. This latter position is used here for *C. dilutus* (Fig. 2a), but the relative position in *C. duplex* cannot be inferred since the position of band group 1 of arm E has not been determined. The dimeric locus was determined to be on arm D, and at band 9A6 in *C. tentans*. Using Biotin-labelled probe, the location at 9A6 was confirmed in *C. dilutus* (Fig. 1d), and a location at 18A determined for *C. duplex* (Fig. 1d). The SIP was localized to about band 7C6 on arm D of *C. dilutus* (Chen et al., 1995), and in this study it has been localized to about band 8b on arm D of *C. duplex* (Fig. 1d).

The remaining seven genes have not previously been mapped. Only two of these are multicopy genes: Actin with four copies and Elongation Factor 1alpha (EF) with two copies. Two copies of the Actin were found to be on arm B, at about 21g and an unmapped band about one third from the distal end of the arm in *C. duplex*, and about 4a1 and 8A1 in *C. dilutus* (Fig. 1b). The third copy was on arm D, at about 24e in *C. duplex* and 10A3 in *C. dilutus* (Fig. 1d), and the fourth copy on arm F, at about 20a in *C. duplex*, and 12A2 in *C. dilutus* (Fig. 2b). The two copies of EF are on arms A and B, respectively at about 10e and 27d in *C. duplex*, and

20A2 and 11B7 in *C. dilutus* (Figs. 1a and b). For the single copy genes, 5S RNA mapped to arm B, at about 21f in *C. duplex* and about 2A3 in *C. dilutus* (Fig. 1b); Gart to arm C, at about 7c in *C. duplex*, and 1A5 in *C. dilutus* (Fig. 1c); Nanos to arm A, at about 16d in *C. duplex*, and 17A1 in *C. dilutus* (Fig. 1a); and the two proteins of the small ribosomal subunit, S8 and S26, to arms C and B, respectively. S8 bound at about band 8e in *C. duplex*, and 4C2 in *C. dilutus* (Fig. 1c), while S26 bound at about band 10b in *C. duplex*, and 6A2 in *C. dilutus* (Fig. 1b).

Discussion

Sequence with homology to six genes that may be associated with response to heavy metals has been obtained. These genes have been localized to specific bands of the polytene chromosomes of *C. duplex* and *C. dilutus*. While this is only a small number of genes, it must be borne in mind that the essential criterion for obtaining sequence in the manner utilized in this study is that that the genes must be relatively conserved so that degenerate primers can be devised based on sequences available in GenBank. This has excluded many well known genes, such as metallothionein (Lastowski-Perry et al., 1985), where only a specific arrangement of certain amino acids is conserved. Other techniques will be required to isolate such genes from species of *Chironomus*. In other cases the conserved regions are motifs that are found in broad families of genes, so that sequence of unknown origin was obtained in degenerate PCR amplifications. The heavy metal-related genes were therefore supplemented by a further 17 genes of diverse function, for which clones were available, to provide additional anchor points on these physical maps. Of the total number, sixteen genes were present as a single copy or cluster, while the others were present in multiple copies, with up to about ten different sites in the case of the HSC70 genes. The sites obtained cover all arms of the chromosomes, although there are only three genes located on arm C.

One unexpected result was the finding of a deleted copy of the HSP70 gene in both *C. duplex* and its allopatrically distributed sister species, *C. occidentalis*. *C. occidentalis* is cytologically distinct although it also has a tandem fusion of arms G and E (Martin et al., 1980). The presence of the same deletion of P70 in both species indicates that it occurred before the ancestors of the present day species became isolated on the west and east coasts of Australia respectively, at least since the last ice age about 20,000 years ago (Langford et al., 1995). Its retention in both species suggests that it is at least selectively neutral, but may even have some selective advantage. Deleted P70 copies have previously been reported in *D. mauritiana*, a member of the *D. melanogaster* subgroup, in which the number of copies of HSP70 has been increased from the ancestral two copies to at least four copies, and five in *D. melanogaster* itself (Bettencourt and Feder, 2001). In *D. mauritiana* the deleted copies are non-functional alleles, and two of the four copies of the gene appear to be pseudogenes. The deleted region in *C. duplex* partly coincides with the variable region affected in *D. mauritiana*, but also includes a more 5' region that is relatively conserved between both HSP and HSC genes. The function of this C-terminal end of the gene appears to be currently unknown (Nollen and Morimoto, 2002), so the consequences of these changes cannot be evaluated. It is not certain how many copies of P70 are present in *Chironomus*, but the presence of both bands in the examined individuals from four geographically dispersed populations of *C. duplex*, suggests that the deleted sequence is not an allele, but a separate gene. Further studies of HSP70 in *C. duplex* should be carried out to determine the number of genes involved, and their arrangement if more than one copy is present.

Some comparisons can be made between the present findings on the HSP70 family and findings from other studies. At least ten HSP70 sites were identified in the present study. A search of Ensembl Fruit Fly database (http://www.ensembl.org/Drosophila_melanogaster/)

indicates existence of about ten sites for the HSP70 family in *D. melanogaster*, and the equivalent Mosquito database (http://www.ensembl.org/Anopheles_gambiae/) located seven sites in *A. gambiae*. It would appear, then, that the number of members in *Chironomus* is similar to that in these other dipterans. Heat shock response has been studied in at least three other *Chironomus* species by investigating the development of puffs in the polytene chromosomes following heat shock. These show some similarities to, and some differences from, the sites identified by *in situ* hybridization. Thus Lezzi et al. (1981) found only four large puffs induced in *C. tentans,* none of which correspond to the sites of HSP70 family members in *C. dilutus*. However, the smaller puff sometimes observed in III-12B, probably corresponds to the site of HSP70. Similarly, only the HSP70 site at III-A3 on arm E of *C. riparius* (Morcillo et al., 1982) corresponds to the chromosomal locations identified in the present study. Nath and Lakhotia (1989) identified nine puffs spread across the four chromosomes in the tropical species *C. striatipennis*. The most heat shock responsive puff, at 6D on chromosome III, corresponds to the HSC70 site at about 7a on arm A in *C. duplex*, and the puff at III-2D to the HSP70 site. A similar comparison can be made for the AT genes, where it was found that the presence two loci in *Chironomus* is the same as in *A. gambiae*, while there are four AT loci in *D. melanogaster*.

PHYLOGENETIC CONSIDERATIONS: It has long been recognized that *in situ* hybridization is a means to confirm the accuracy of identifications based purely on the appearance of the bands of the polytene chromosomes (Schmidt et al., 1988). Some of the results in this study have necessitated a re-evaluation of the banding homologies of *C. duplex*, relative to those given by Martin (1971; 1979). Thus, the site for GCS was included in arm F in Martin (1971), whereas the presence of GCS on the AB chromosome of *C. dilutus* clearly indicates that the hybridizing band is on arm B. Re-examination of the banding pattern, which is often disrupted by the development of a nucleolus, showed that the centromere of arm F in *C. duplex* was actually at

the band labelled 4A7 in Fig. 5 of Martin (1971). The other case relates to the breakpoints of the tandem fusion between arms E and G (Martin, 1971). The bands of this region are not clear due to the presence of puffs that were interpreted to be position effects arising from the fusion. It was realized that some bands of arm E were unaccounted for, and it was assumed that these had been lost in the fusion process. However, the *in situ* hybridization results indicate that this most parsimonious interpretation was too simple. The puffs have been shown to be small nucleoli, in that they contain the genes of BR 1 and BR3 of *C. tentans*, consequently moving the site of the junction of between arms G and E further to the right from the point shown in Figs. 11 and 13 of Martin (1971) (Fig. 2c). The result for HSP70 further shows that not all the missing bands of arm E have been lost, but have been rearranged, possibly by subsequent inversions, so that some at least are now near the distal end of arm G (Fig. 2 c and d). This conclusion is supported by other data on the location of the HSP70 gene: besides the arm E location for *C. dilutus* (Fig. 2a), Barettino et al. (1988) identified HSP70 at III-A3b on arm E of *C. riparius*, and *in situ* hybridization to four Australasian *Chironomus* species, including *C. occidentalis*, also confirmed the location of this gene at E7b (data not shown). As noted above, the heat shock puff at III-2D of the Indian species *C. striatipennis* (Nath and Lakhotia, 1989), corresponds to this same band on arm E. Further examination of the polytene bands in the vicinity of the HSP70 locus of *C. duplex* confirms that it looks like group 7 from arm E, and it appears that at least part of group 6 may be immediately proximal to it (Fig. 2c).

There are phylogenetic aspects also to the linkage relationships, or synteny of the loci studied. Although *C. duplex* and *C. dilutus* are relatively distantly related, all genes studied have retained the same synteny, even the relatively rapidly evolving Histone clusters (Hankeln et al., 1993). This supports the contention that a map constructed for one *Chironomus* species can be transposed to any other species that has been mapped by one of the standards used in this

study. Within each chromosome arm, the linkage relationships vary, reflecting the chromosomal rearrangements that have occurred in the phylogeny of the species. Close to the centromeres, where inversion breakpoints are less frequent (Keyl, 1962), the relationships may be retained, while in the middle of the arms the relationships may be markedly different. This is seen, for example, in arm B, where GCS and EF are close together near the centromere in both species, but the closely linked group of an HSC70, an Actin and the 5S RNA gene seen in *C. duplex* is quite widely separated and in a different order in *C. dilutus* (Fig. 1b). On a broader scale, the question may be asked as to whether there is conservation of synteny with the chromosome arms of other well characterized dipterans like *D. melanogaster* and *A. gambiae.* Such information is of value for identifying genes that may be of interest for particular studies, eg. further genes involved in heavy metal tolerance, or to fill in gaps in the *Chironomus* map. It cannot be expected that there will be a one-to-one relationship between chromosome arms. For a start, the number of linkage groups differs from seven in *Chironomus*, to five in *A. gambiae* and *D. melanogaster*, ignoring the small fourth chromosome of the latter species which has no homologue in *A. gambiae* (Bolshakov et al., 2002). In addition, it is already known from the comparison of linkage groups between *Drosophila* and *Anopheles*, that while a majority of genes will be on the equivalent linkage group, many will have been moved to different linkage groups (Bolshakov et al., 2002). The example seen here with the Hsp70 gene in *C. duplex*, which is moved from the arm E to the arm G linkage group, clearly shows at least one way in which this may occur. Many of the genes used here have limitations for use in such an analysis: the histone genes may occur on different chromosome arms, even within *Chironomus* (Hankeln et al., 1993); the silk proteins have no currently-known orthologues in these species, and the elaboration of globins found in *Chironomus* is not seen in the other two dipterans. From the present data set it is possible that arm A of *Chironomus* may represent the same linkage group

as arm X of *A. gambiae*, although the linkage groups are not retained in their entirety. Thus while GS, Nanos and the isoenzyme MPI (J. Martin, unpubl.) are all on arm A of *Chironomus*, GS and MPI are on the *A. gambiae* X but Nanos is on arm 2L. A retinal degeneration gene on arm A of *Chironomus* also appears to have an ortholog on the X of *A. gambiae* (H. Chung, unpubl.) Other *Chironomus* chromosome arms presently show less apparent homology to those of *A. gambiae*; for example, GCS-CAT and the S26 and L10 (Galli and Wieslander, 1992) ribosomal proteins are on arm B in *Chironomus*, but while GCS-CAT and L10 are on 2R, S26 is on 3L in *A. gambiae*. Therefore more work needs to be done to determine the mosquito homologues of the *Chironomus* chromosome arms, and the extent of conservation of synteny between *Chironomus* and the other dipteran groups.

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Table 1. Investigated genes associated with response to heavy metals, the species from which they have been isolated, primers used for PCR and the chromosome arms on which they are located

Gene	Primers (5'-3')	Arm	Source	Reference
alpha-Tubulin (AT)	AT-F: GATTGTGCTTTCATGGTNGAYAAYGARGC AT-R: TACAACATACAACAAGCCATRTAYTTNCCRTG	E, G	*C. dilutus* *C. duplex*	Mattingly et al. 2001 This project
Glucose-6-Phospahate Dehydrogenase (G6)	G6pd56F2: GMGATCTGGCCAARAARAARATHTAYCC G6pd209R1: TCATCAGCTTYTGNACCATYTCYTTNCC	F	*C. tepperi*	This project (modified from Soto-Adames et al. 1994)
Glutathione synthase (GS)	GS-F1: TATTTCCGTGCTGGATATGARCCNGGNCAYTA GS-R2: ATTATTTCCTCCTCCTTCWCKYTGNGGYTT	A	*C. duplex*	This project
Glutamyl-cysteine synthase (GCS)	GCS-F: TTCATATGGAYGCTATGGGNTTYGGNATGGG GCS-R: AATTTGTTGATTGGATRTTYTCRAARTGRTC	B	*C. duplex*	This project
HSC70 (C70)	as Yoshimi et al. 2002	A, B, C, D, E, F, G	*C. yoshimatsui* *C. dilutus* *C. duplex*	Yoshimi et al. 2002 Karouna-Renier et al. 2003 This project
HSP70 (P70)	HSP70f3: GGHATYYTGAAYGTRTCRGCNAA HSP70r4: ARTCAACYTCCTCNACYGTNGG	E	*C. dilutus* *C. duplex*	Karouna-Renier &Rao 2003 This project
Cu,Zn Superoxide dismutase (SOD)	SOD-F: GCGAGCATGGATTCCAYRTNCAYGA SOD-R: GCCAATCGAGCTCCNGCRTTNCCNGT	D	*C. duplex*	This project

Table 2. Other mapped anchor genes, with the species from which they were isolated, the source from which they were obtained and the chromosome arm on which they are located.

Gene	Arm	Source	Reference
5S RNA	B	*D. melanogaster*	Bedo & Webb, 1990
Actin (A)	B(2), D, F	*C. samoensis*	Rebagliath & Kalthoff, unpubl.
Balbiani ring 1(BR1)	G	*C. tentans*	Wieslander et al. 1982
Balbiani ring (3)	G	*C. tentans*	Paulsson et al. 1990
Cla element (Cla)	F	*C. thummi*	Kraemer & Schmidt 1993
Elongation Factor 1alpha (EF)	A, B	*C. oppositus*	Corcoran, unpubl.
Gart (G)	C	*C. tentans*	Clark & Henikoff 1992
Globin cluster (dimeric) (Gb1)	D	*C. piger*	Hankeln et al. 1988
Globin cluster (monomeric) (Gb3)	E	*C. thummi*	Antoine & Niessing 1984
Histone cluster (H)	D(mult)	*C. thummi*	Hankeln & Schmidt 1991
Nanos (Na)	A	*C. samoensis*	Curtis et al. 1995
Ribosomal proteins, small subunit		*D. melanogaster*	M. Morgan (unpubl.)
S8	C	*C. duplex*	This project
S26	B	*C. duplex*	This project
Sex-influenced protein (SIP)	D	*C. dilutus*	Chen et al. 1995
sp140 (140)	F	*C. tentans*	Galli et al. 1990
ssp160 (160)	G	*C. thummi*	Hoffman et al. 1996

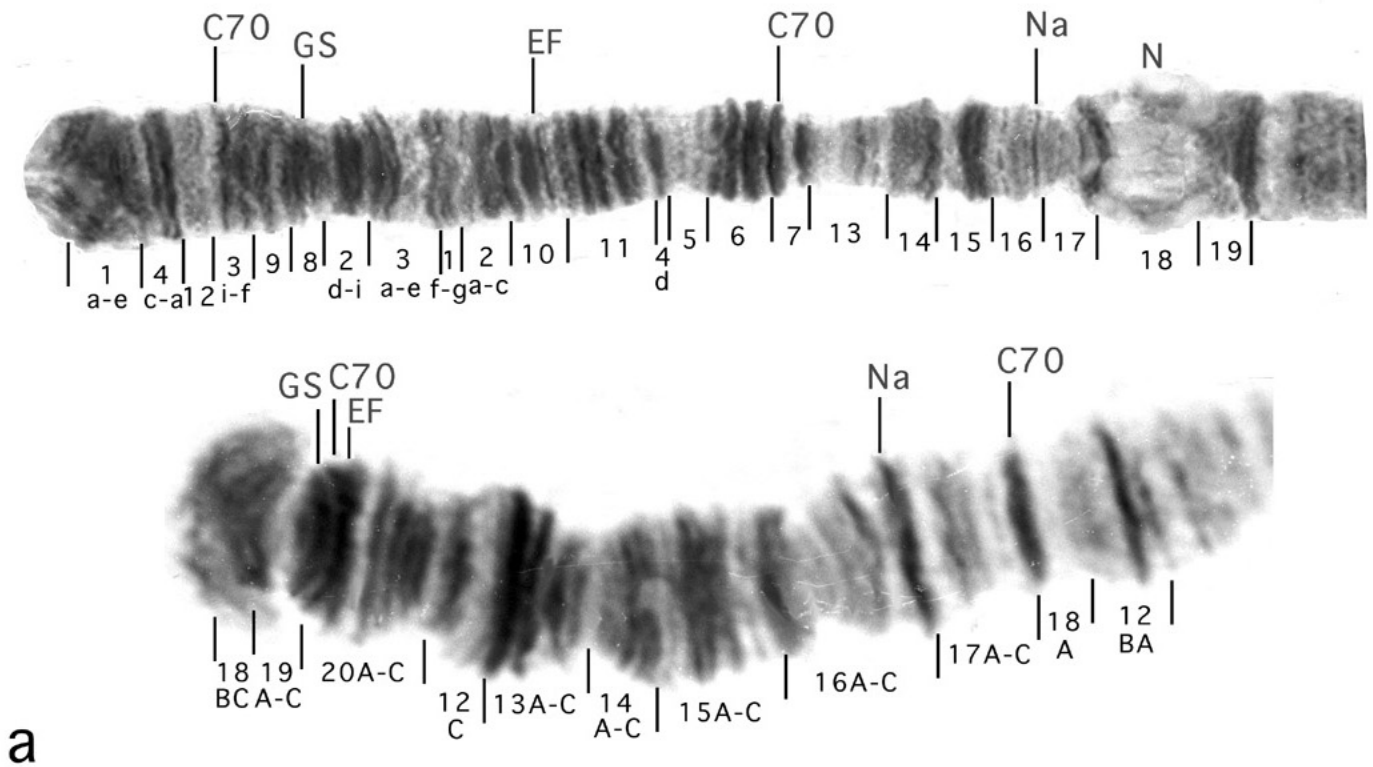
Captions to Figures:

Fig. 1. Comparison of arms A, B, C and D of *C. duplex* (upper member of each pair) and *C. dilutus* (lower member of each pair). *C. duplex* mapped by Keyl pattern, *C. dilutus* by Beermann pattern Arms arranged with the centromere at the right end. a. Arms A1.1 of *C. duplex* and nA2.2 of *C. dilutus*; b. Arms B1.1 of *C. duplex* and nB1.1 of *C. dilutus*; c. Arms C1.1 of *C. duplex* and nC1.1 of *C. dilutus*; d. Arms D1.1 of *C. duplex* and nD2.2 of *C. dilutus*. Abbreviations as in Tables 1 and 2.

Fig. 2. Comparison of arms E, F, and G of *C. duplex* and *C. dilutus*, mapped as in Fig. 1. a. Arms E1.1 of *C. duplex* and nE3.3 of *C. dilutus*; b. Arms F1.1 of *C. duplex* and nF3.3 of *C. dilutus*; c. Arms G1.1 of *C. duplex* and hG1.1 of *C. dilutus* (centromere at left end). The vertical line underneath the arm of *C. duplex* indicates the presumed junction of arms G and E in Martin (1971); d. Chromosomes GEA and BF of *C. duplex* probed with DIG-labelled HSC70 (above) and GEA of *C. duplex* probed with DIG-labelled HSP70 (below). Hybridization bands indicated by arrows.

Fig. 3. 1.2% agarose gel, stained with ethidium bromide, showing the amplified Hsp70 fragments in some *Chironomus* species. Lane 1. pGEM DNA markers (Promega); 2. *C. duplex*, French Island, Victoria; 3. *C. duplex*, Flinders island, Tasmania; 4. *C. duplex*, Nhill, Victoria; 5. *C. duplex*, Werribee, Victoria; 6. *C. occidentalis*, Albany, Western Australia; 7. *C. australis*, Hepburn, Victoria; 8. *C. oppositus*, Moggs Creek, Victoria; 9. *C. dilutus*, Winnipeg, Manitoba; 10. No DNA control.

The upper band is 468 bp and the lower band is 370 bp.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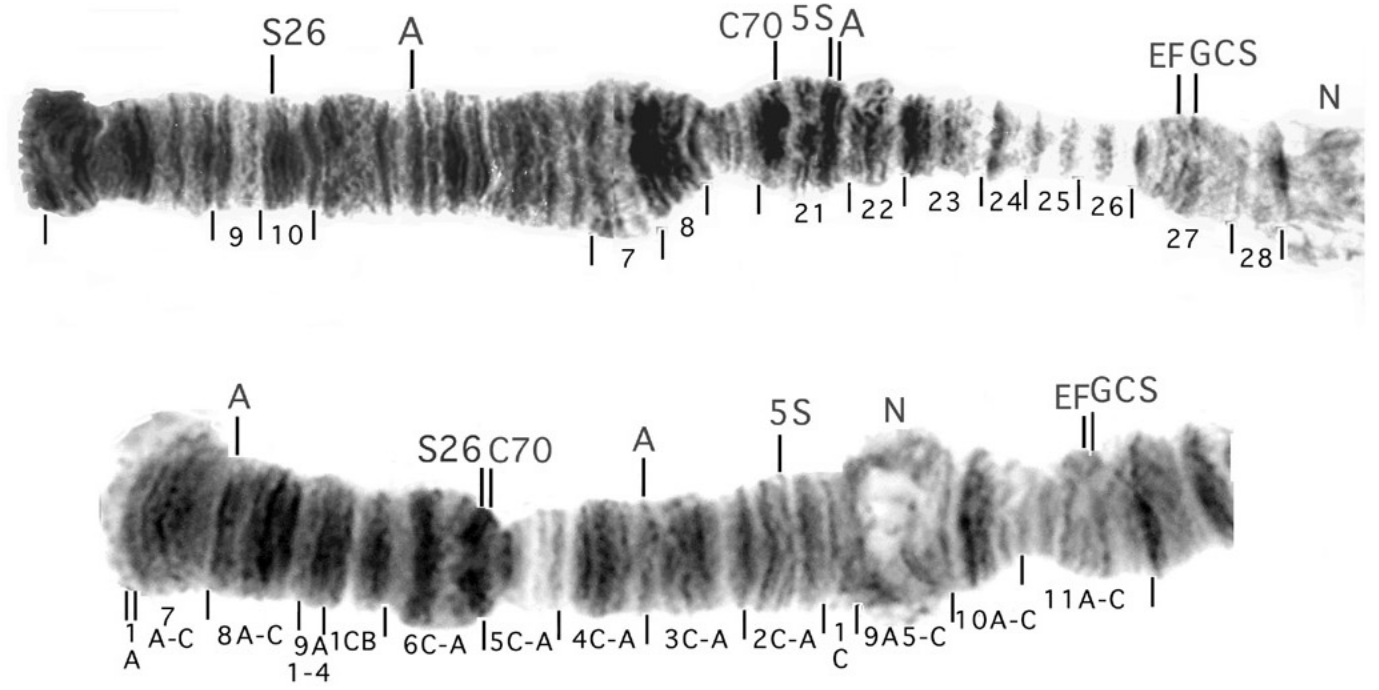
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3C-A
2C-A
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10A-C
11A-C

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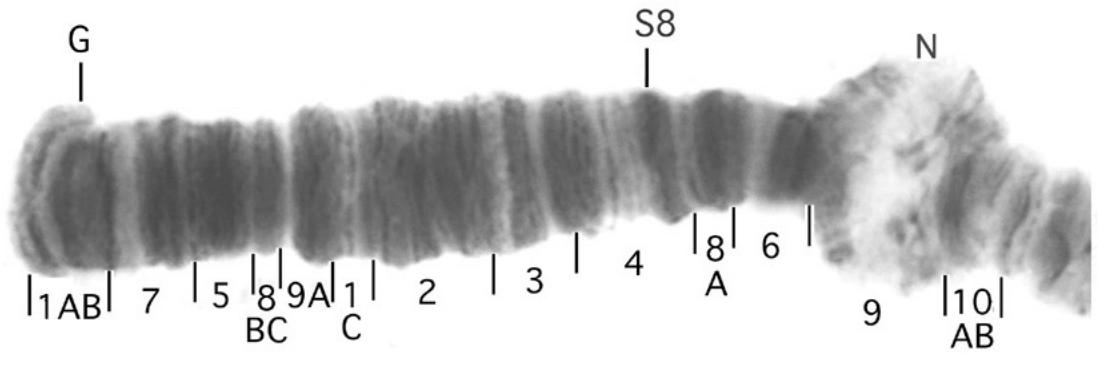
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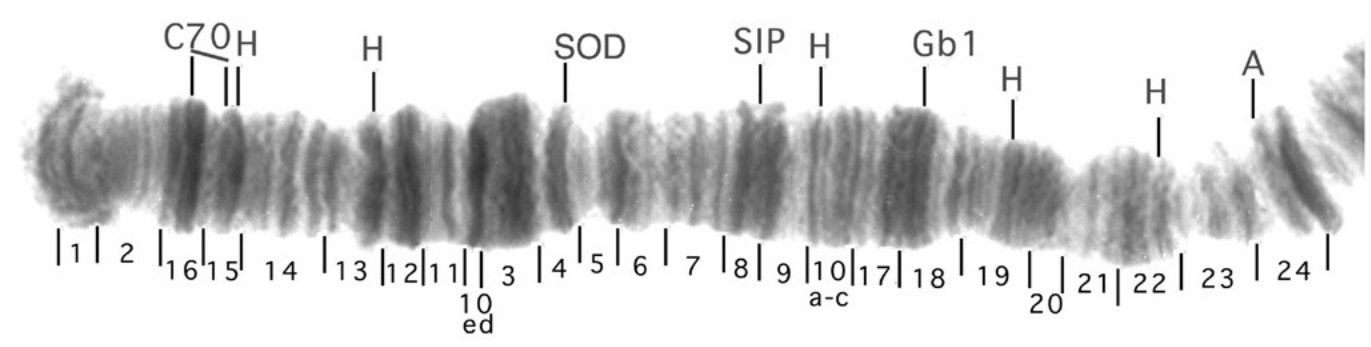
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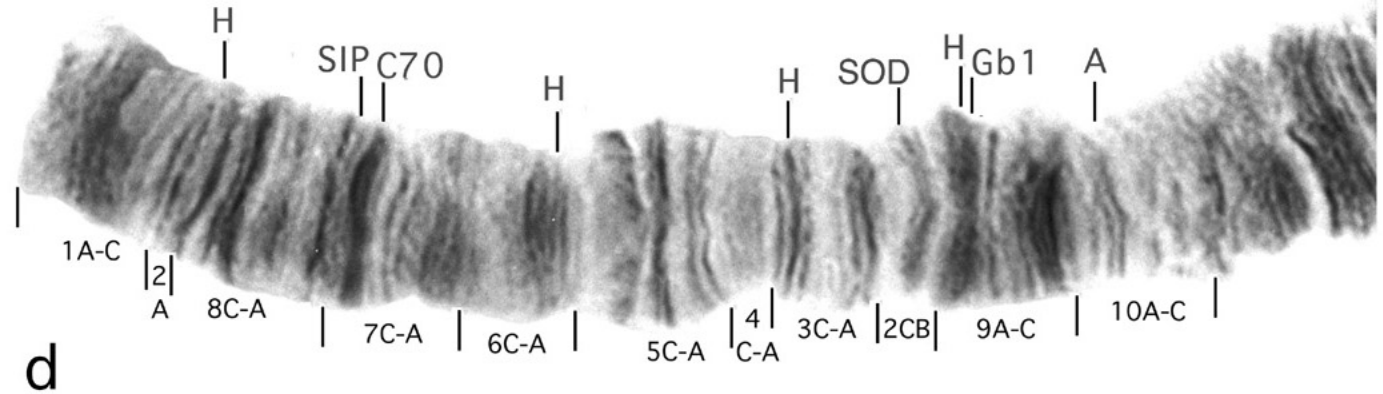
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3C-A
2CB
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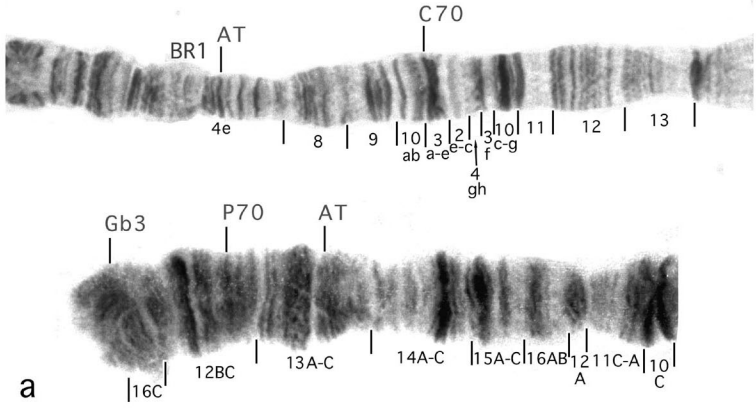
10A-C

d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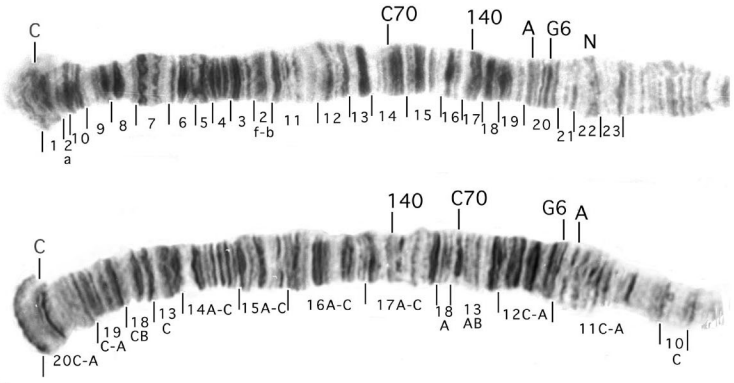
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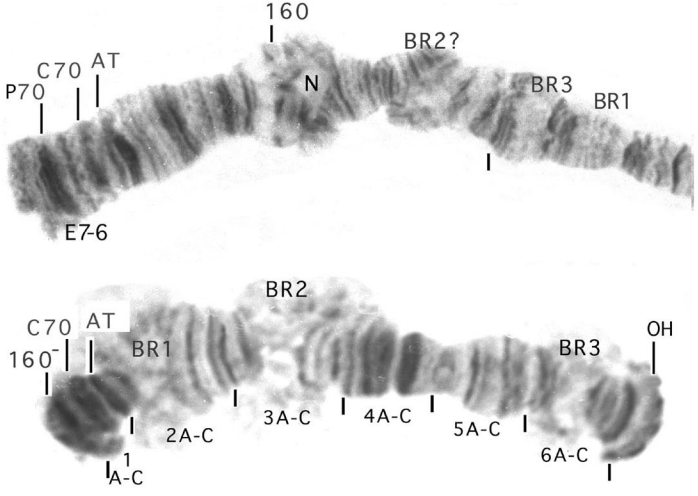
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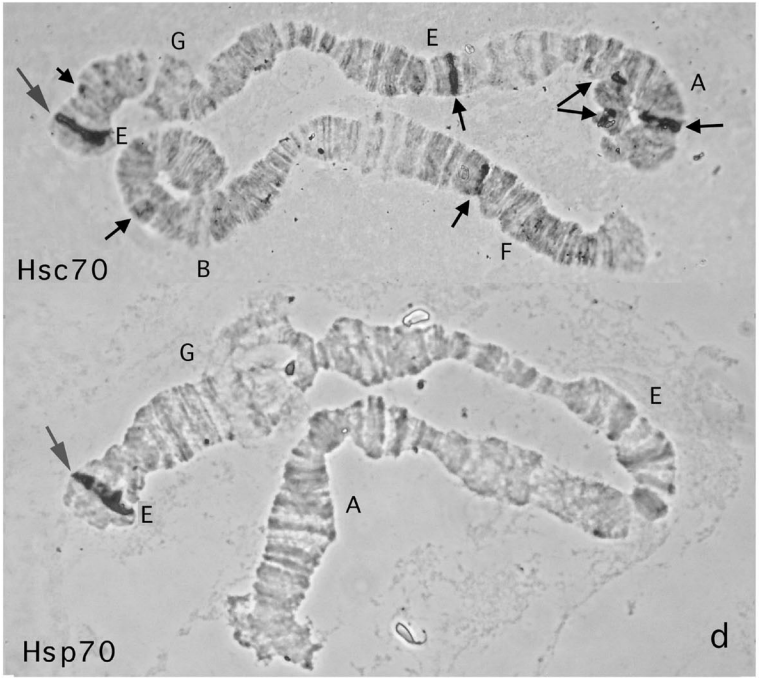
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