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Testing Chromosomal Phylogenies and Inversion Breakpoint Reuse in Drosophila

Josefa González,[1] Ferran Casals[2] and Alfredo Ruiz[3]

Departament de Genètica i de Microbiologia, Universitat Autònoma de Barcelona, 08193 Bellaterra (Barcelona), Spain

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ABSTRACT

A combination of cytogenetic and bioinformatic procedures was used to test the chromosomal phylogeny relating *Drosophila buzzatii* with *D. repleta*. Chromosomes X and 2, harboring most of the inversions fixed between these two species, were analyzed. First, chromosomal segments conserved during the divergence of the two species were identified by comparative *in situ* hybridization to the *D. repleta* chromosomes of 180 BAC clones from a BAC-based physical map of the *D. buzzatii* genome. These conserved segments were precisely delimited with the aid of clones containing inversion breakpoints. Then GRIMM software was used to estimate the minimum number of rearrangements necessary to transform one genome into the other and identify all possible rearrangement scenarios. Finally, the most plausible inversion trajectory was tested by hybridizing 12 breakpoint-bearing BAC clones to the chromosomes of seven other species in the *repleta* group. The results show that chromosomes X and 2 of *D. buzzatii* and *D. repleta* differ by 12 paracentric inversions. Nine of them are fixed in chromosome 2 and entail two breakpoint reuses. Our results also show that the cytological relationship between *D. repleta* and *D. mercatorum* is closer than that between *D. repleta* and *D. peninsularis*, and we propose that the phylogenetic relationships in this lineage of the *repleta* group be reconsidered. We also estimated the rate of rearrangement between *D. repleta* and *D. buzzatii* and conclude that rates within the genus Drosophila vary substantially between lineages, even within a single species group.

THE cytological analysis of animal and plant chromosomes has a long tradition in the study of evolution (White 1973). Recently, the availability of physical maps and whole-genome sequences from many species has provided an unparalleled opportunity to investigate further the structural changes in the eukaryotic genome. The studies carried out so far have led to three main conclusions about genome evolution (Eichler and Sankoff 2003; Sankoff 2003; Kazazian 2004; Coghlan *et al.* 2005; Shapiro 2005): (1) the eukaryotic genome is exceptionally malleable, (2) rates and patterns of chromosomal rearrangement vary significantly between different evolutionary lineages, and (3) repetitive DNA sequences are ubiquitous in eukaryotic genomes and responsible for most of the structural dynamism.

Paracentric inversions are the most frequent type of rearrangement within the Drosophila and Anopheles genera with a similar average rate of rearrangement fixation, ~0.05 disruptions/Mb/MY (González *et al.* 2002; Sharakhov *et al.* 2002). However, in the more distant comparison between Drosophila and Anopheles, extensive interchromosomal exchange has been observed (Zdobnov *et al.* 2002). In nematodes, the rearrangement rate is four times that in Drosophila and the ratio of translocations to inversions to transpositions is 1:1:2 (Coghlan and Wolfe 2002). Whole-genome duplications as well as various types of rearrangements characterize plant genome evolution, the evolution rate of the most dynamic plant genomes (~0.03 disruptions/Mb/MY) being half the rate reported for insects (Lagercrantz 1998; Yogeeswaran *et al.* 2005). In mammals, the X chromosome shows extensive syntenic conservation whereas the autosomes show a variety of intra- and interchromosomal rearrangements and the Y chromosome is an example of rapid and unconstrained evolution. In addition, rates of mammalian chromosome evolution vary radically among lineages (Murphy *et al.* 2005).

In Drosophila, one of the most striking features of chromosomal evolution is the nonuniform distribution of inversions (Sperlich and Pfriem 1986; Powell 1997). Some species have dozens of inversions segregating in all chromosomes, other species carry them in only one or two chromosomes, and there are species with no polymorphic inversions. Another remarkable observation is the nonrandom distribution of inversion breakpoints within a given chromosome, with many sites

[1]*Present address:* Department of Biological Sciences, Stanford University, Stanford, CA 94305.

[2]*Present address:* Unitat de Biología Evolutiva, Facultat de Ciències de la Salut i de la Vida, Universitat Pompeu Fabra, 08003 Barcelona, Spain.

[3]*Corresponding author:* Departament de Genètica i de Microbiologia, Facultat de Biociències, Universitat Autònoma de Barcelona, 08193 Bellaterra (Barcelona), Spain. E-mail: alfredo.ruiz@uab.es

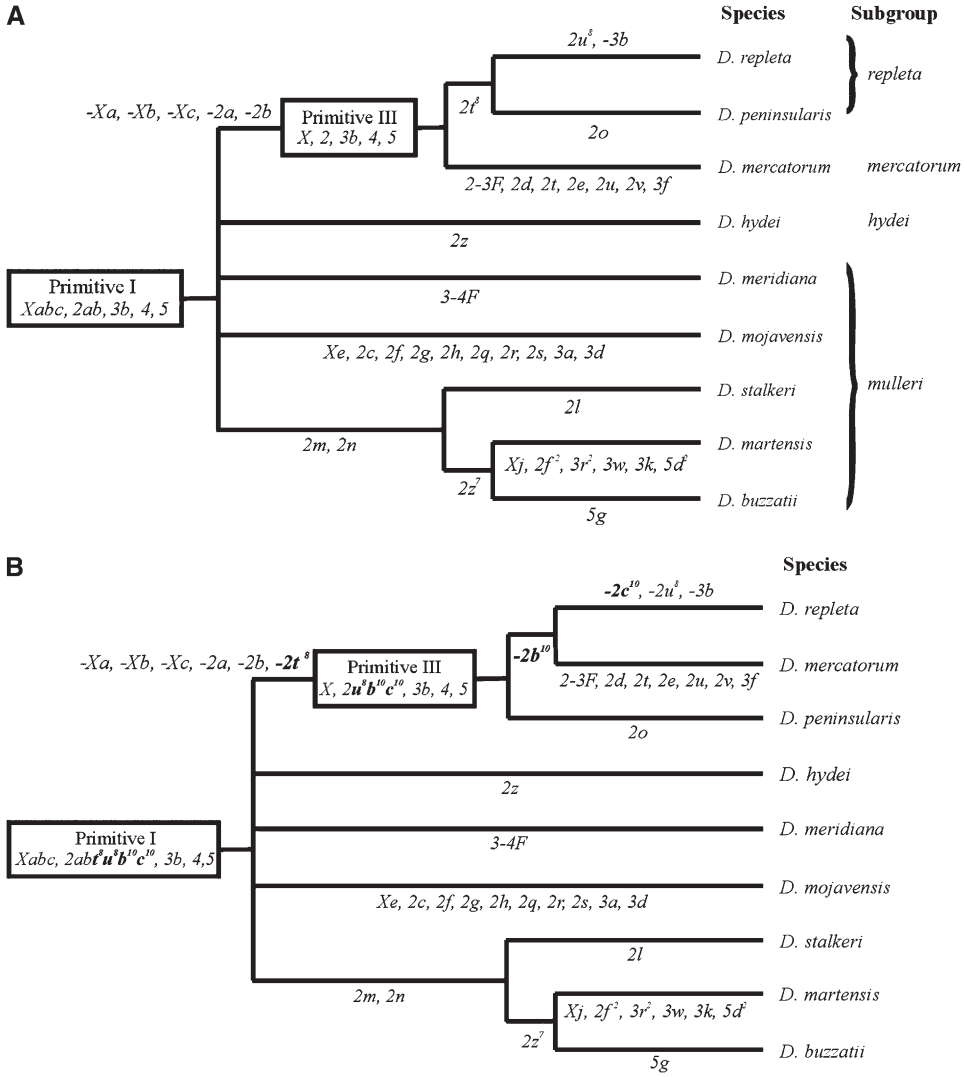

FIGURE 1.—Phylogenetic relationships between the species analyzed in this study. Chromosomal inversions fixed in each branch of the tree are indicated. Inversions fixed in the lineage from Primitive I to *D. repleta* are represented with the minus sign to indicate that they must be subtracted from the formula of Primitive I to get the formula for *D. repleta*. (A) Current phylogeny based on comparisons of polytene chromosome-banding patterns (WASSERMAN 1992; RUIZ AND WASSERMAN 1993). (B) Phylogeny inferred from the results of *in situ* hybridizations of DNA probes to the polytene chromosomes (RANZ *et al.* 2003; this work). Inversion changes in the phylogenetic tree are shown in boldface type.

where two or more inversion breakpoints seem to coincide (TONZETICH *et al.* 1988; WASSERMAN 1992; CÁCERES *et al.* 1997). Similar observations have been made in the mosquito Anopheles (COLUZZI *et al.* 2002). These results are based on the cytological comparison of polytene chromosomes and may suffer from the limited resolution of this method. Breakpoint clustering has also been described as an outstanding feature of mammal genome evolution (PEVZNER and TESLER 2003; BOURQUE *et al.* 2004; MURPHY *et al.* 2005). PEVZNER and TESLER (2003) coined the word "reuse" to describe this phenomenon. We use this term here as a shorthand for "close clustering" but emphasize, following their proponents, that it does not imply the use of exactly the same genomic position (nucleotide) as an endpoint of different rearrangements.

The *repleta* species group belongs to the Drosophila subgenus, which diverged from the Sophophora subgenus, the other main lineage in the Drosophila genus, 40–62 MYA (POWELL 1997; TAMURA *et al.* 2004). This species group is one of the largest in the genus and includes >100 species, many of them cactophilic species living in the deserts and arid zones of the American continent. In an outstanding cytological effort, WASSERMAN (1982, 1992) determined the inversion relationships between 70 species and divided the *repleta* group into five subgroups: *repleta*, *mercatorum*, *hydei*, *mulleri*, and *fasciola*. He used *Drosophila repleta* chromosomes (WHARTON 1942) as a reference and inferred that the chromosomal arrangement of the ancestor of the *repleta* species group, named Primitive I, was one differing from that of *D. repleta* by six inversions and that can be represented as *Xabc*, *2ab*, *3b*, *4*, *5* (Figure 1A).

The chromosomes of *D. buzzatii*, a member of the *mulleri* subgroup that diverged from *D. repleta* 15–22 MYA (SPICER 1988; RUSSO *et al.* 1995), have been particularly difficult to disentangle. This was one of the first *repleta* group species to be cytologically analyzed (WASSERMAN 1954) and in the subsequent 50 years its inversion phylogeny has suffered many alterations (WASSERMAN 1962, 1982, 1992; RUIZ *et al.* 1982). RUIZ and WASSERMAN (1993) proposed that the *D. buzzatii* chromosomes derive from the putative ancestral karyotype of the *repleta* group, Primitive I, by the fixation

of four inversions: *2m*, *2n*, $2z^7$, and *5g* (Figure 1A). However, they also discovered that the *D. buzzatii* chromosome 2 differs from that of *D. repleta* by two small additional inversions, $2t^8$ and $2u^8$. Being fixed in the lineage leading from Primitive I to *D. repleta* (Figure 1A), these two small inversions were not incorporated to the *D. buzzatii* chromosome maps (Ruiz and Wasserman 1993).

Inversion phylogenies are usually considered very reliable and have been used as a benchmark for comparison with allozyme and DNA sequence phylogenies and assessment of congruence (MacIntyre and Collier 1986; O'Grady *et al.* 2001). There are, however, several sources of error, one of them being observational mistakes (Wasserman 1963, 1992). As a way to overcome the limitations of cytological studies and get a deeper insight into the molecular organization and evolution of *D. buzzatii* chromosomes, we undertook a decade ago a project to map DNA clones to its salivary gland chromosomes by *in situ* hybridization (Ranz *et al.* 1997). The map location of nearly 300 molecular markers from *D. melanogaster* was compared between *D. buzzatii* and *D. repleta* (Ranz *et al.* 2003). The results were consistent with some of the previous cytological results but uncovered also another two inversions fixed in chromosome 2, $2b^{10}$ and $2c^{10}$, overlooked in previous studies. These two inversions are apparently fixed in the lineage leading from Primitive I to *D. repleta*, yet no attempt was made to determine their distribution in the phylogenetic tree (Ranz *et al.* 2003). In a further effort to determine the precise number and extent of structural changes between *D. buzzatii* and *D. repleta*, we have turned to clones from the recently produced BAC library and BAC-based physical map of the *D. buzzatii* genome (González *et al.* 2005). We have mapped to *D. repleta* chromosomes 180 BAC clones from *D. buzzatii* chromosomes X and 2 that harbor most of the rearrangements fixed between these two species (Figure 1A). Specifically the aims of this study are: (i) to determine the number and orientation of conserved chromosomal segments between *D. buzzatii* and *D. repleta*, (ii) to test the inversion phylogeny proposed for *D. buzzatii*, (iii) to ascertain whether the coincidence of inversion breakpoints described by cytological inspection of chromosomes still holds at an enhanced resolution level, and (iv) to estimate the rate of chromosomal rearrangement between *D. repleta* and *D. buzzatii* for comparison with that of other lineages.

MATERIALS AND METHODS

Flies: Stocks of nine Drosophila species representing four of the five subgroups in the *repleta* species group were used (see Figure 1A). All stocks are homokaryotypic for the standard arrangement in all chromosomes except the *D. mercatorum* stock that is homokaryotypic for the polymorphic inversion $2v^3$. The stocks of *D. repleta* (15084-1611.06), *D. peninsularis* (15081-1401.00), *D. meridiana* (15081-1341.03), *D. stalkeri* (15081-1451.00), and *D. mojavensis* (15081-1352.10) come from the Tucson Drosophila Stock Center; the remaining stocks belong to the collection of the Departament de Genètica i de Microbiologia (Universitat Autònoma de Barcelona).

Probes: A total of 180 clones from the *D. buzzatii* BAC library CHORI-225 (available from BACPAC Resources at http://bacpac.chori.org) were successfully hybridized to the polytene chromosomes of *D. repleta*. Most clones were hybridized individually but 42 clones were hybridized as pools of 2 or 3 nearby clones and each pool was considered a single marker. Pools were designed to close the gaps between clones already hybridized. BAC clones were chosen according to their localization in the *D. buzzatii* genome map (González *et al.* 2005) to optimize coverage of chromosomes X (57 clones) and 2 (123 clones). Particular attention was paid to those regions that have been rearranged during the divergence between *D. repleta* and *D. buzzatii*. When two clones mapping relatively close in *D. buzzatii* hybridized to two different chromosomal sites in *D. repleta*, clones spanning the region between them were also hybridized. This allowed us to determine accurately the boundaries of chromosomal segments conserved throughout evolutionary time and also to infer the minimum number of inversion breakpoints fixed in that particular genomic region. Clones giving one signal in *D. buzzatii* and two signals in *D. repleta* contain an inversion breakpoint fixed between the two species. When this result was obtained with a pool of clones, further hybridizations were carried out with each clone separately until we identified the clone containing the breakpoint. Twelve clones containing inversion breakpoints were then hybridized to the polytene chromosomes of other species of the *repleta* group to test for the presence of the inversions fixed between *D. buzzatii* and *D. repleta* in the other species.

***In situ* hybridization and chromosomal maps:** Only female larvae were used for hybridizing the BAC clones mapping on the X chromosome because the single X of the male shows a somewhat reduced level of hybridization while the efficiency of hybridization on the female X is equivalent to that on the autosomes (Pardue *et al.* 1987). Polytene chromosome squashes, hybridization, and detection were carried out as in Montgomery *et al.* (1987). When possible, the same DNA previously used for fingerprinting and *in situ* hybridization of BAC clones in *D. buzzatii* (González *et al.* 2005) was used as a probe to hybridize to *D. repleta* chromosomes. Otherwise DNA from BAC clones was extracted following the alkaline lysis miniprep protocol available from http://bacpac.chori.org/protocols.htm. Probes were labeled with biotin-16-dUTP by random primer. Hybridization results were recorded as digital images captured with a phase contrast Nikon Optiphot-2 microscope at 600× magnification and a Nikon Coolpix 4500 camera. Hybridization signals were localized using the cytological map of *D. repleta* (Wharton 1942). For the other species of the *repleta* group cut-and-paste reconstructions of chromosome 2 according to the inversions putatively fixed during their divergence were produced and used to locate the signals (Wasserman 1992; Ruiz and Wasserman 1993).

Bioinformatic analysis: GRIMM software (Tesler 2002) implements the Hannenhalli and Pevzner algorithms for computing unichromosomal and multichromosomal genomic distances and was used here to calculate the minimum number of rearrangement events required to transform one genome into another. This algorithm also finds optimal scenarios for the transformation of one genome into another via these rearrangement events. The program was run online at the publicly available server http://www-cse.ucsd.edu/groups/bioinformatics/GRIMM/, using the linear unichromosomal genome and signed conserved segments options. Each chromosome (X or 2) or chromosomal region (distal

or proximal half of chromosome 2) was separately analyzed. The program gives one of the multiple optimal scenarios to transform one genome into the other. To find out all the possible scenarios we ran the program several times, introducing each time one of the inversions in the source genome until all the possible combinations of inversions were found.

RESULTS

Identification of conserved segments: Detailed results for the 180 *D. buzzatii* BAC clones hybridized to the polytene chromosomes of *D. repleta* are given in supplemental Tables S1 and S2 (at http://www.genetics.org/supplemental/). The X chromosome clones amount to 53 markers and represent 23 contigs of the *D. buzzatii* physical map of chromosome X (González *et al.* 2005). Another 69 markers (gene clones, cosmids, and P1 phages) previously mapped to the X chromosome of both species (Ranz *et al.* 2003) were also included in our analysis, raising the total number of available markers to 122. The chromosome 2 clones amount to 106 markers, representing 22 contigs of the *D. buzzatii* physical map of chromosome 2 (González *et al.* 2005). We included in the analysis another 143 markers previously mapped to this chromosome in the two species (Ranz *et al.* 2003) for a total number of 249 markers. The markers are distributed all along chromosomes X and 2, effectively covering the entire chromosome length in both cases.

The chromosomal localization of all markers was compared in the two species to identify the number and orientation of conserved segments. A conserved segment is defined here as a set of markers that are consecutive (show the same relative order) in *D. buzzatii* and *D. repleta*. Seven conserved segments were identified in chromosome X and 17 in chromosome 2 (Figures 2 and 3). Most conserved segments are quite big, made up of at least 10 markers (supplemental Tables S1 and S2 at http://www.genetics.org/supplemental/). However, a few smaller conserved segments were also identified.

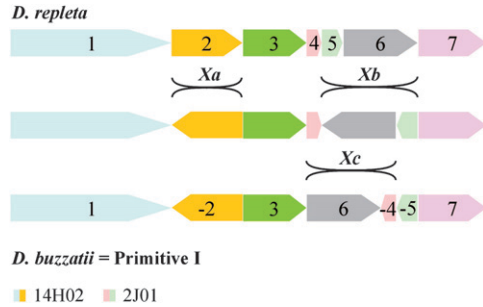

Figure 2.—Relative position and orientation of the seven chromosome X segments conserved during the divergence between *D. repleta* and *D. buzzatii*. Conserved segments are numbered consecutively (from telomere to centromere) in *D. repleta*. The three rearrangements necessary to transform the X chromosome of one species into that of the other are indicated.

Despite their small size, these conserved segments span at least three chromosomal bands with different markers hybridizing to different bands, allowing us to ascertain their relative orientation in both species. Most segments were precisely delimited with the aid of BAC clones that yield two hybridization signals in *D. repleta* and thus contain an inversion breakpoint fixed between the two species. Two such clones were identified in chromosome X, whereas 15 such clones were found in chromosome 2 (Table 1).

Estimation of the number of rearrangements: GRIMM software (Tesler 2002) was used to estimate the minimum number of rearrangement events and find optimal scenarios for the transformation of one genome into another. A minimum of three rearrangement events are needed to transform the X chromosome of one species into the other. The extension and relative position of these three rearrangements (Figure 2) agree well with the three inversions, *Xa*, *Xb*, and *Xc*, fixed in the lineage from Primitive I to *D. repleta*. However, the breakpoints of these inversions (given in Table 1) differ slightly from those previously reported

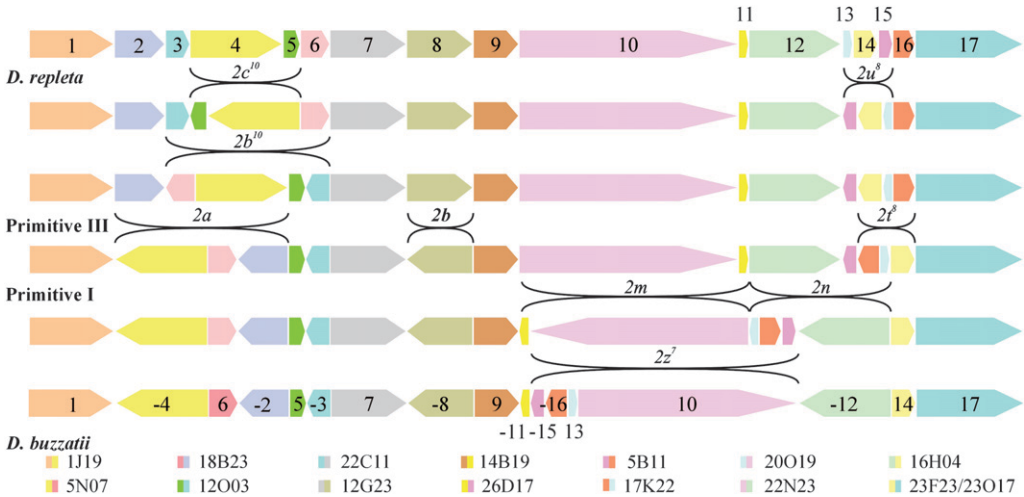

Figure 3.—Relative position and orientation of the 17 chromosome 2 segments conserved during the divergence between *D. repleta* and *D. buzzatii*. Conserved segments are numbered consecutively (from telomere to centromere) in *D. repleta*. Nine rearrangements are required to transform chromosome 2 of one species into that of the other. The particular inversion trajectory depicted here is the one subject to testing and corroborated by further evidence.

TABLE 1

Paracentric inversions fixed between *D. repleta* and *D. buzzatii* on chromosomes X and 2

		Distal breakpoint		Proximal breakpoint	
Chromosome	Inversion	Band[a]	Clone	Band[a]	Clone
X	*Xa*	C4d	14H02-D	D4d	14H02-D
	Xb	F1d	—	G1g	—
	Xc	E4d	2J01-P	F2h	2J01-P
2	*2a*	A3c	1J19-D	C1e	1J19-D
	$2b^{10}$	B1b	18B23-D 22C11-P	C3a	18B23-D 22C11-P
	$2c^{10}$	B1i	12O03-D 5N07-P	C1k	12O03-D 5N07-P
	2b	C6a	12G23-D	D1g	12G23-D
	$2t^{8}$	G1g	5B11-D 23F23-P 23O17-P	G2g	5B11-D 23F23-P 23O17-P
	$2u^{8}$	F6e	17K22-P 22N23-D	G2a	17K22-P
	2m	D3e	14B19-D 20O19-P	F2a	14B19-D
	2n	F2a	16H04-P	F6h	16H04-P 20O19-D
	$2z^{7}$	F1h	26D17-D 22N23-P	G2a	26D17-D

D. buzzatii BAC clones giving two hybridization signals in the *D. repleta* chromosomes and containing inversion breakpoints are also indicated (P or D after clone name indicates that the clone contains the proximal or distal inversion breakpoint in the *D. buzzatii* chromosome).

[a] Breakpoint coordinates refer to the cytological map of *D. repleta* (Wharton 1942).

(Ranz *et al.* 2003). Inversions *Xb* and *Xc* are overlapping whereas inversion *Xa* is independent from the other two (Figure 2). GRIMM software provides three possible scenarios for the occurrence of these three inversions that differ in the temporal position of *Xa* relative to the other two inversions. The order of fixation of these three inversions was not investigated further as they appear to co-occur in all those species where they are present (Wasserman 1992). However, we can conclude that it must be *D. buzzatii* = Primitive I → *Xc* → *Xb* → *D. repleta* with inversion *Xa* taking place before *Xc*, after *Xc*, or after inversion *Xb*.

To explain the present organization of chromosome 2 in both species the minimum number of rearrangements is nine (Figure 3). Rearrangements taking place in the distal region of the chromosome, involving chromosomal segments 1–9, are independent of those occurring in the proximal region of the chromosome, segments 9–17. Thus, both regions can be separately analyzed. In the distal region, the minimum possible number of rearrangements is four (Figure 3) and there are eight different pathways transforming one chromosome into the other, implying the same four inversions and differing only in the order in which they took place. The extension and relative position of these four rearrangements agree well with the inversions *2a*, *2b*, $2b^{10}$, and $2c^{10}$ fixed in the lineage from Primitive I to *D. repleta* (Ranz *et al.* 2003), yet some of the breakpoint coordinates must be slightly modified (Table 1). In the proximal region of chromosome 2 at least five inversions are needed to transform the *D. repleta* chromosome into that of *D. buzzatii*. There are 18 possible scenarios with five inversions and different scenarios imply different inversions. This is so because there are only 9 conserved segments and eight breakpoints for five inversions, implying a minimum of two breakpoint reuses (see discussion). We have explored all possible scenarios and 2 of them approximately match the inversions proposed to have been fixed in this region between these species: *2m*, *2n*, $2z^{7}$, $2u^{8}$, and $2t^{8}$ (Figure 3). The 2 scenarios differ only in the order of occurrence of *2m* and *2n*, two tandemly arranged inversions that seem to share the middle breakpoint.

Test of the inversion phylogeny: Twelve clones containing inversion breakpoints were hybridized to seven other Drosophila species, besides *D. buzzatii* and *D. repleta*, to test for the presence of the inversions in these species. Each clone produces a single hybridization signal in *D. buzzatii* chromosome 2 (the source of the BAC clones) and two hybridization signals in *D. repleta* chromosome 2 (implying a breakpoint). If the clone produces a single hybridization signal in the chromosome of a third species, this implies a similar arrangement to that of *D. buzzatii*. If the clone yields two hybridization signals, then an arrangement similar to *D. repleta* can be inferred. The important point is that the two clones that represent the distal and proximal breakpoints of an inversion should behave similarly. That is, if the proposed inversion trajectory is correct, the results produced by the two breakpoints of an inversion should be congruent. This is not expected if the proposed inversion pathway is wrong.

Sixty-two out of 64 hybridizations attempted with the 12 breakpoint-bearing BAC clones were successful and the results were generally congruent (Table 2). For inversion $2u^{8}$ a clone containing the proximal breakpoint was available and this clone failed to produce a detectable hybridization signal in *D. hydei* and *D. meridiana*. In two other cases, one clone containing the breakpoint of an inversion gave one signal whereas a second clone containing the other breakpoint yielded two signals. This occurred with inversion *2m* in *D. meridiana* and inversion $2z^{7}$ in *D. peninsularis* (Table 2). These two exceptional cases were interpreted as meaning that the same arrangement as in *D. repleta* was present (but one of the hybridization signals could not be detected). The localization of the signals in chromosome 2 of these species (not shown) supports this interpretation.

Four clones containing the breakpoints of inversions $2b^{10}$ and $2c^{10}$ were hybridized to the chromosomes of

TABLE 2

Number of hybridization signals produced by 12 BAC clones containing inversion breakpoints in nine Drosophila species of the *repleta* group

Clone	18B23	22C11	12O03	5N07	17K22	23F23	5B11	14B19	20O19	16H04	22N23	26D17
Inversion	$2b^{10}$	$2b^{10}$	$2c^{10}$	$2c^{10}$	$2u^{8}$	$2t^{8}$	$2t^{8}$	*2m*	*2m*/ *2n*	*2n*	$2u^{8}/2z^{7}$	$2z^{7}$
Breakpoint	D	P	D	P	P	P	D	D	P/D	P	D/P	D
D. repleta	2	2	2	2	2	2	2	2	2	2	2	2
D. mercatorum	2	2	1	1	1	2	2	2	2	2	2	2
D. peninsularis	1	1	1	1	1	2	2	2	2	2	1	2
D. hydei	ND	ND	ND	ND	0	1	1	2	2	2	2	2
D. meridiana	ND	ND	ND	ND	0	1	1	1	2	2	2	2
D. mojavensis	ND	ND	ND	ND	1	1	1	2	2	2	2	2
D. stalkeri	ND	ND	ND	ND	1	1	1	1	1	1	2	2
D. martensis	ND	ND	ND	ND	1	1	1	1	1	1	1	1
D. buzzatii	1	1	1	1	1	1	1	1	1	1	1	1

D, distal breakpoint; P, proximal breakpoint; ND, not determined.

D. peninsularis and *D. mercatorum*. These clones were tested only in these two species because inversions $2b^{10}$ and $2c^{10}$ were seemingly fixed in the lineage leading from Primitive I to the *D. repleta* and *D. mercatorum* subgroups. In *D. peninsularis*, the four clones gave a single signal, indicating that this species does not contain any of the two inversions (Table 2). In *D. mercatorum*, clones containing the breakpoints of inversion $2c^{10}$ gave a single signal while clones containing the breakpoints of inversion $2b^{10}$ gave two signals (Table 2). Thus *D. mercatorum* contains inversion $2b^{10}$ but does not contain inversion $2c^{10}$. We cannot conclude when inversion *2b* took place but the order of the other inversions in the distal region of the chromosome is as follows: *D. buzzatii* = *Primitive* I → *2a* → $2b^{10}$ → $2c^{10}$ → *D. repleta*.

Eight clones containing the breakpoints of the five inversions fixed in the proximal half of chromosome 2 were hybridized to the chromosomes of seven other species of the *repleta* group (Table 2). The two clones from inversion $2t^{8}$ gave two signals only in *D. mercatorum* and *D. peninsularis*, implying that this inversion is fixed in both species (and absent in all the other species). In contrast, the clone containing the proximal breakpoint of inversion $2u^{8}$ produced a single signal in all species but *D. repleta*, implying that $2u^{8}$ is fixed only in the latter species. The three clones containing the breakpoints of inversions *2m* and *2n* gave a single signal in *D. stalkeri* and *D. martensis*, which means they are fixed in these two species (as in *D. buzzatii*), and two signals in the remaining species (that lack these two inversions). Finally, the two clones from inversion $2z^{7}$ gave a single signal in *D. martensis* that must be fixed for the inversion (as *D. buzzatii*) and two signals in the rest of the species (that must lack this inversion as in *D. repleta*). The order of inversions fixed in the proximal region of the chromosome is either *D. buzzatii* → $2z^{7}$ → *2m* → *2n* → $2t^{8}$ → $2u^{8}$ → *D. repleta* or *D. buzzatii* → $2z^{7}$ → *2n* → *2m* → $2t^{8}$ → $2u^{8}$ → *D. repleta*.

DISCUSSION

Inversion phylogeny of the *repleta* group: A number of bioinformatic approaches and methods are available for inferring the number and trajectory of rearrangements fixed during the divergence of two genomes from comparative mapping data (see Sankoff 2004 for a review). However, bioinformatic analyses of genome sequences often deviate from more traditional cytogenetic views of chromosomal evolution (Bourque *et al.* 2006; Froenicke *et al.* 2006). Here, a combination of bioinformatic and cytological approaches is used to determine the correct rearrangement phylogeny of *D. buzzatii*. We have used *in situ* hybridization of 180 clones from a *D. buzzatii* genomic BAC library to the chromosomes of *D. repleta* and previous mapping data for another 212 markers (Ranz *et al.* 2003) to determine the number and orientation of conserved segments in chromosomes X and 2 between *D. buzzatii* and *D. repleta*. The previous markers were spread approximately at random over chromosomes X and 2, thus leaving gaps or uncovered regions of variable size (Ranz *et al.* 2003). In contrast, we selected our BAC clones to optimize coverage and ascertain the extent and orientation of conserved segments. In most cases BAC clones were identified, encompassing the conserved segments boundaries (*i.e.*, bearing inversion breakpoints). Thus, we are reasonably confident that all conserved segments >150 kb have been identified. Seven conserved segments were found in chromosome X and 17 in chromosome 2. The extent and orientation of all conserved segments was determined (Figures 2 and 3). In addition we used GRIMM (Tesler 2002) to estimate the minimum number of rearrangements required to transform the chromosomes of *D. repleta* into those of *D. buzzatii* and to explore all possible scenarios or pathways.

The hybridization of 12 breakpoint-bearing clones representing seven different inversions in seven other

Drosophila species of the *repleta* group was used to ascertain the correct inversion phylogeny. The results are summarized in Figure 1B. *D. buzzatii* differs from *D. repleta* by 14 chromosomal inversions and its chromosomal arrangement can be represented by the formula: *Xabc*, $2abt^{8}u^{8}b^{10}c^{10}mnz^{7}$, *3b*, *4*, *5g*. The arrangement of the conservative chromosomes 3, 4, and 5 has not been analyzed here but inferred from previous work (Wasserman 1992; Ruiz and Wasserman 1993). The cytological map of *D. buzzatii* corresponding to its chromosomal arrangement can be seen in Figure 2 of González *et al.* (2005).

Ten inversions became fixed in the lineage from Primitive I to *D. repleta*: *Xa*, *Xb*, *Xc*, *2a*, *2b*, $2t^{8}$, $2u^{8}$, $2b^{10}$, $2c^{10}$, and *3b* (Figure 1B). Inversion $2t^{8}$ was found by Ruiz and Wasserman (1993) in *D. repleta* and *D. peninsularis* and led Wasserman (1992) to include the latter species in the *repleta* complex of the *repleta* subgroup. However, Ruiz and Wasserman (1993) did not analyze the chromosomes of *D. mercatorum*. The results presented here show that in fact this species also has $2t^{8}$ fixed and thus this inversion does not indicate a close relationship between *D. peninsularis* and *D. repleta*. Wasserman (1992) reported that only two inversions, *2a* and *2b*, have been fixed in the distal region of chromosome 2. Our results support the observations of Ranz *et al.* (2003), which proposed that besides *2a* and *2b* another two inversions, $2b^{10}$ and $2c^{10}$, have been fixed in this chromosomal region. These two inversions were overlooked in previous analyses because $2c^{10}$ is included within $2b^{10}$ and the combination of the two inversions nearly restores the original banding pattern. We also show that inversion $2b^{10}$ is found in *D. repleta* and *D. mercatorum* but not in *D. peninsularis* whereas $2c^{10}$ is exclusive of *D. repleta*. The closer relationship between *D. mercatorum* and *D. repleta* than between *D. peninsularis* and *D. repleta* contradicts the current inversion phylogeny (Figure 1A) but is consistent with diverse lines of evidence. Wharton (1944) classified *D. peninsularis* as a member of the *D. mercatorum* subgroup. Vilela (1983) noted that *D. peninsularis* is morphologically closer to *D. carcinophila* (a member of the *D. mercatorum* subgroup) than to any other described species and accordingly placed *D. peninsularis* within the *D. mercatorum* subgroup. Finally, Durando *et al.* (2000), using sequences from four mitochondrial genes and one nuclear gene, observed a close relationship between the *repleta* and the *mercatorum* subgroups. Their data and our cytological results suggest that the classification of these lineages as two separate subgroups is probably not warranted and should be reconsidered.

Breakpoint reuse and genome evolution model: In the *repleta* species group the cytological coincidences between breakpoints of different inversions are quite common (Wasserman 1992). For instance, 96 breakpoint reuses can be inferred for the 208 inversions described in chromosome 2. This rate of coincidence still holds true within subgroups or even within complexes (Cáceres *et al.* 1997). Obviously, some of these apparent coincidences can be the consequence of the limited resolution of the cytological technique. The increased resolution provided by the *in situ* hybridization technique allows us to map more accurately inversion breakpoints and test for breakpoint reuses.

In the species analyzed here three breakpoint reuses have been described, one in chromosome X and two in chromosome 2. Wasserman (1992) stated that inversions *Xb* and *Xc* were arranged in tandem and shared the middle breakpoint. However, our results support the claim of Ranz *et al.* (2003) that these two inversions are in fact overlapping (Figure 2). In chromosome 2, Ruiz and Wasserman (1993) proposed that inversions *2m* and *2n* are also tandemly arranged and share the middle breakpoint and also that inversion $2z^{7}$ shares its distal breakpoint with that of inversion *2m*. The identification of clone 20O19 containing the proximal breakpoint of *2m* and the distal breakpoint of *2n* confirms the first coincidence. The fact that this clone produces two hybridization signals on the *D. repleta* chromosomes instead of three suggests that the two breakpoints must be very close to each other or even at the same molecular site. However, our results show that inversion $2z^{7}$ does not share its distal breakpoint with that of inversion *2m* although it is relatively close (see Figure 3). On the other hand, our results suggest that inversion $2z^{7}$ shares one breakpoint with inversion $2u^{8}$ (Figure 3). Again, the fact that clone 22N23 gives two hybridization signals instead of three suggests a close proximity of the two breakpoints. In summary, of three breakpoint reuses previously described (Ruiz and Wasserman 1993), only one still holds at the resolution level of the clone size used in this study (~150 kb), but a new one has been found, leaving the total at two. This coincidence of inversion breakpoints is intriguing and deserves further scrutiny at the DNA sequence level.

Breakpoint reuse is a quite common phenomenon in diverse organisms. Pevzner and Tesler (2003) compared the human and mouse genomes and observed clumps of closely located breakpoints that could not be explained by the "random breakage model" (Nadeau and Taylor 1984). They proposed an alternative model that envisages mammalian genomes as a mosaic of relatively short fragile regions with a high propensity for rearrangements and solid regions with a low propensity for rearrangements. These fragile regions may correspond to segmental duplications or regions with an unusually high concentration of transposable elements (TEs) or with a palindromic structure (Eichler and Sankoff 2003; Murphy *et al.* 2005). Segmental duplications represent ~5% of the human genome (Bailey *et al.* 2002) and ~2% of the mouse genome (Bailey *et al.* 2004a). They induce rearrangements by unequal crossing over (Shaffer and Lupski 2000) and are hotspots for mammalian chromosomal evolution (Bailey *et al.* 2004b; Zody *et al.* 2006).

TABLE 3

Rates of chromosomal rearrangement fixation (number of disruptions per megabase and per million years) in the genus Drosophila

		Muller's chromosomal element						
Subgenus	Species pair[a]	A	B	C	D	E	A–F	Reference[b]
Sophophora	*D. pseudoobscura–D. melanogaster*	0.088	0.144	0.153	—	—	0.128	1, 2
	D. subobscura–D. pseudoobscura		0.067	0.183			0.125	2
	D. subobscura–D. melanogaster		0.143	0.133			0.138	2
	D. pseudoobscura–D. melanogaster	0.087	0.055	0.092	0.055	0.073	0.072	3
	D. miranda–D. pseudoobscura	0.130–0.204	—	0.126–0.379	—	0.047–0.158	0.096–0.233	4
	D. miranda–D. melanogaster	0.011–0.026	—	0.021–0.048	—	0.007–0.032	0.012–0.030	4
	D. pseudoobscura–D. melanogaster	0.011–0.064	—	0.023–0.048	—	0.007–0.016	0.013–0.040	4
Drosophila	*D. virilis–D. novamexicana*	0.053	—	—	0.018	—	0.018	5
	D. virilis–D. montana	0.041	—	—	0.07	—	0.012	5
	D. montana–D. novamexicana	0.041	—	—	0.011	—	0.013	5
	D. virilis–D. buzzatii	—	—	—	—	0.021	0.021	6
	D. virilis–D. repleta	—	—	—	—	0.029	0.029	6
	D. repleta–D. buzzatii	0.005	0.001	0.001	0	0.006	0.004	7
Sophophora–Drosophila	*D. melanogaster–D. repleta*	0.087	0.021	—	0.045	0.079	0.058	8

[a] For all species pairs including *D. melanogaster*, we used the genome size and chromosome size in Celniker and Rubin (2003). For comparisons with *D. pseudoobscura*, the genome and chromosome sizes reported by Richards *et al.* (2005) were used. For comparisons between *repleta* group species, we used 154 Mb as the euchromatic genome size (Schulze and Lee 1986) and the relative sizes of each chromosome given by Wasserman (1992). Finally, for the *virilis* species group 150 Mb was used as the euchromatic genome size (Hartl and Lozovskaya 1995) and $\frac{1}{5}$ for each chromosome.

[b] References: (1) Segarra *et al.* (1995); (2) Papaceit *et al.* (2006); (3) Richards *et al.* (2005); (4) Bartolomé and Charlesworth (2006); (5) Vieira *et al.* (1997); (6) Ranz *et al.* (1999); (7) Wasserman (1992), Ruiz and Wasserman (1993), and this work; (8) Ranz *et al.* (2001), González *et al.* (2002).

The Drosophila genome is quite different from that of mammals: the amount of repetitive DNA is much lower (5 *vs.* 44%) and the fraction of segmental duplications is negligible (Lander *et al.* 2001; Celniker and Rubin 2003). Accordingly, inversion breakpoint reuse in Drosophila is likely to have a different cause. Molecular studies of breakpoint regions in natural Drosophila inversions have revealed the presence of TEs in some cases (Cáceres *et al.* 1999, 2001; Casals *et al.* 2003). By contrast no clear evidence for the implication of TEs was found in other studies (Wesley and Eanes 1994; Cirera *et al.* 1995; Andolfatto *et al.* 1999; Matzkin *et al.* 2005; Richards *et al.* 2005). The conclusion is that at least in some species or species groups, TEs are responsible for the origin of chromosomal inversions.

A plausible hypothesis for breakpoint reuse in Drosophila can be set forth. When an inversion has been generated by a TE, copies of this element will be flanking the inverted segment in the chromosome with the inversion (Lim and Simmons 1994; Gray 2000). If the inversion succeeds and goes to fixation, these TE insertions will be brought to fixation as well. These fixed TE insertions will have a much higher probability to be involved in further chromosome breakages than the rest of the TE insertions (that usually have a rather low population frequency because of the equilibrium between transposition and selection; Charlesworth *et al.* 1994). Although one expects that these insertions will be removed by deletion in the long run (Petrov *et al.* 1996; Singh and Petrov 2004), they may last in the genome several million years (average time to loss of 50% nonfunctional DNA is ~12 MY). Furthermore, because of the reduction of recombination in the heterokaryotypes, inversion breakpoint regions often accumulate additional TE insertions besides the one that originated the inversion (Cáceres *et al.* 2001, 2003; Sharakhov *et al.* 2006). When the inversion goes to fixation, some of these TE insertions may become fixed with the inversion while other may remain polymorphic. In any case, the unusual high density of TE insertions at inversion breakpoints will increase the chances of further chromosome breakages at these sites, *i.e.*, the chances of breakpoint reuse. One prediction of this model is that inversions sharing breakpoints are expected to arise in a temporal succession within the same lineage, something that seems to be frequent in the *repleta* species group (Wasserman 1992). In our study, the model would apply to the breakpoint coincidence between inversions *2m* and *2n* (which occurred in the same lineage) but not to that between inversions $2z^7$ and $2u^8$ (which occurred in different lineages).

Rates of chromosomal rearrangement fixation in the genus Drosophila: Our results show that three paracentric inversions in chromosome X and nine in

chromosome 2 have been fixed during the divergence between *D. repleta* and *D. buzzatii*. These numbers agree with the general pattern in the *repleta* group where chromosome 2 has been found to be the most dynamic, harboring ~70% of all inversions (Wasserman 1992). The size of the genome of the *repleta* group species is ~220 Mb with 70% in the euchromatin (Schulze and Lee 1986). The total number of inversions fixed between these two species is 14 and thus we can estimate an average rate of rearrangement fixation of 0.004 disruptions/Mb/MY.

Rates of rearrangement fixation have been estimated in the genus Drosophila using different species pairs. We have normalized these estimates as the number of disruptions per megabase and per million years to make them comparable (Table 3). These estimates must be taken with caution because different estimation methods have been used for different comparisons and some of the studies did not include all chromosomal elements. However, they are likely to be accurate enough for a broad overview of chromosomal evolution in the genus. Two conclusions can be drawn. First, rates of rearrangement vary between chromosomal elements as proposed by González *et al.* (2002), although the element exhibiting the highest rate can vary. Second, rates of rearrangement differ between lineages, the rate within the Sophophora subgenus being generally higher that that within the Drosophila subgenus (Papaceit *et al.* 2006). This agrees well with the distribution of polymorphic inversions in these subgenera (Sperlich and Pfriem 1986; Powell 1997). The lowest rate for the entire genus is that observed here for the comparison *D. buzzatii–D. repleta*. The highest rate is probably that corresponding to the comparison *D. miranda–D. pseudoobscura* (Bartolomé and Charlesworth 2006) that is 24–58 times higher. Twenty-fold differences in rearrangement rate have been reported between different vertebrate lineages (Coghlan *et al.* 2005; Murphy *et al.* 2005). It is remarkable that similar differences can be found within a single genus of flies. Substantial variation in rearrangement rate is also evident even within the *repleta* group (Figure 1B). Some lineages, such as those leading to *D. mercatorum* or *D. mojavensis*, are highly dynamic, whereas other lineages, such as that of *D. hydei*, are much more conservative. Four factors may help to explain the great variation in rearrangement rates between Drosophila lineages: generation time, population size, mutation rate, and environmental conditions (*i.e.*, selection regime). While all of them probably make a contribution, we believe that mutation rate may play a crucial role. If chromosomal inversions are generated by TEs, their generation rate is likely to vary greatly between lineages because so does the activity of TEs. It is intriguing, however, that the rate of rearrangement seems to be highest in the subgenus Sophophora where until now clear evidence for the implication of TEs in the generation of chromosomal inversions has not been found.

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