



Three decades of studies on chromosomal polymorphism of *Drosophila willistoni* and description of fifty different rearrangements

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Abstract

Drosophila willistoni (Insecta, Diptera) is considered a paradigm for evolutionary studies. Their chromosomes are characterized by multiple paracentric inversions that make it hard to identify and describe chromosomal polymorphisms. In the present report we attempted to systematize the description of all the 50 inversions found in the last three decades, since we have been studying the chromosomes of several individuals of 30 different populations, including the one used in the genome sequencing project (*Gd-H4-1*). We present the photographic register of 11 arrangements in the left arm of the X chromosome (XL), eight in the right arm (XR), 10 in the left arm of chromosome II (IIL), eight in its right arm (IIR) and 13 in chromosome III. This information also includes their breakpoints on the reference photomap. A clear geographic difference was detected in XL and XR, with different fixed arrangements depending on the origin of the population studied. Through the comparison of all X arrangements it was possible to infer the putative ancestral arrangements, *i.e.*, those related to all the remaining arrangements through the small number of inversions that occurred in the past, which we will call XL-A and XR-A. In the autosomes (IIL/IIR and III), fixed inversions were detected, but most are segregating in different frequencies along the geographical distribution of the *D. willistoni* populations.

Keywords: cytogenetic, polytene chromosomes, inversions, *Drosophila willistoni*.

Introduction

Sixty years after Dobzhansky (1950) first described the polytene chromosomes of *Drosophila willistoni*, the large number of chromosomal inversions found in populations of this species still attracts the attention of geneticists. When Dobzhansky first published his work, the focus was on evolution from a population genetics viewpoint (Dobzhansky and Powell, 1975; Ehrman and Powell, 1982; Krimbas and Powell, 1992). Now, *D. willistoni* is part of a bigger scenario of evolutionary studies on *Drosophila* that began with the publication of the comparative genome sequences of 12 closely related fruit fly species (*Drosophila* 12 Genomes Consortium, 2007). Ten species were sequenced for the first time (*D. sechellia*, *D. simulans*, *D. yakuba*, *D. erecta*, *D. ananassae*, *D. persimilis*, *D. mojavensis*, *D. virilis*, *D. grimshawi* and *D. willistoni*) and two were genomes of *Drosophila melanogaster*, which had

previously been sequenced in 2000 (Adam *et al.*, 2000), and *D. pseudoobscura*, published in 2005 (Richards *et al.*, 2005). With these results, a rich source of genomic data is now available and can be used in comparisons to other important organisms, such as humans, as well as for identifying novel genes and other functional elements in insect genomes.

The results of the *Drosophila* 12 Genomes Consortium (2007) revealed that, despite the overall similarities, several unexpected differences occur among the species studied, especially in aspects related to ecology and behavior, reflecting adjustments to the vastly different environments in which they evolved. Among the sequenced species, *Drosophila willistoni* was the only Neotropical one, and proved to be exceptional in several aspects of its genome: its large genome size, unusually skewed codon usage, high content of transposable elements (15%, only lower than that of *D. ananassae*: ~25%) and an apparent lack of selenoproteins (Vicario *et al.*, 2007).

This major genome sequencing and analysis effort has brought about the need to reevaluate the available physical maps, in part to aid the mapping of assembled scaffolds (Schaeffer *et al.*, 2008). In Schaeffer *et al.* (2008) we presented a definitive and individual photomap of the polytene chromosomes of *Drosophila willistoni* used to anchor the

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assembled scaffolds of the *Drosophila* 12 Genomes Consortium (2007). The main results of this study are in agreement with Muller's idea that the chromosomes of different *Drosophila* species are syntenic with respect to their genic content, although several rearrangements occurred over time, including inversions and fusions (Muller, 1940). So, as a rule, even when an extreme rearrangement of the chromosome elements has occurred, synteny relationships usually remain relatively conserved (Bhutkar *et al.*, 2008).

Drosophila willistoni has a wide distribution, extending from Mexico and Florida to southern Brazil, Argentina and Uruguay, and from the Atlantic to the Pacific Ocean (Spassky *et al.*, 1971; Ehnman and Powell, 1982). It is the most common drosophilid species inhabiting South American forests. The chromosomes of natural populations of *D. willistoni* are characterized by multiple chromosome inversions (Sperlich and Pfriem, 1986; Krimbas and Powell, 1992; Powell, 1997) and in more than three decades working with this organism, we have never collected stable monomorphic populations in the field. This is the case for the southern borders of its geographical distribution, such as Uruguay, where the natural populations of *D. willistoni* still have a high level of polymorphism (Valente *et al.*, 2001, 2003). This said, the vast chromosomal polymorphism for inversions is one of the paramount characteristics of this species (Burla *et al.*, 1949; Da Cunha *et al.*, 1950, 1959; Townsend, 1952; Da Cunha and Dobzhansky, 1954; Valente and Araújo, 1985, 1986; Valente *et al.*, 1993; 2003; Rohde *et al.*, 2005), and it appears that being chromosomally polymorphic is the rule for *D. willistoni*. Faced with this, it was a special challenge to choose one recent collected isofemale line of *Drosophila willistoni* to represent the species in the genome sequencing project (*Drosophila* 12 Genomes Consortium, 2007). GD-H4-1 was finally chosen for this purpose due to its lack of inversion heterozygosity. The strain is the result of various laboratory generations of single brother-sister mating descending from an isofemale line collected in the Caribbean island of Guadeloupe.

In common with several other species that belong to the *Sophophora* subgenus of *Drosophila*, *D. willistoni* has a high polymorphism for paracentric inversions. It is hard to explain why there are more inversions in the subgenus *Sophophora* than in *Drosophila* as a whole, but according to Sperlich and Pfriem (1986) this may be due to several factors, including genetics (more fragile chromosomes), ecology (these flies inhabit diversified environments) and history (phylogenetically old species may accumulate more inversions).

Literature reports are unclear regarding the real number of variants segregating in natural populations of *D. willistoni*. The criteria available for descriptions of polytene chromosome inversions are somewhat confusing, not only due to the more fragile structural nature of the chromosomes, but also due to imprecise descriptions of inversion

breakpoints. The *camera lucida* drawings made by Dobzhansky (1950), Da Cunha and Dobzhansky (1954) and Da Cunha *et al.* (1950, 1959) show large numbers of inversions present in the same chromosome, making the identification of each particular inversion and respective breakpoints very difficult. This difficulty in accurately identifying inversions is also a consequence of the small size of several *D. willistoni* inversions, which hinders the recognition of several of these in homozygosis. Furthermore, the chromosomes of *D. willistoni* also present several ectopic pairings that make accurate analyses difficult. As a consequence, the estimates available for this high polymorphism in the literature consider only the arrangements in heterozygosis (Burla *et al.*, 1949; Da Cunha *et al.*, 1950; Townsend, 1952; Da Cunha and Dobzhansky, 1954; Dobzhansky, 1957; Valente and Araújo, 1985; 1986; Valente *et al.*, 1993; 2003), resulting in an underestimation of the true number of variants segregating in the natural populations of *D. willistoni*.

Over the last three decades our group has worked towards producing a good photomap of *D. willistoni* polytene chromosomes. At first, Valente and Morales (1985) redrew the reference map of Dobzhansky (1950), improving the identification of landmark bands that are useful to understand complex inversion configurations. Subsequently, Regner *et al.* (1996) presented the first photomap of *D. willistoni* polytene chromosomes, considering the arrangements present in natural populations of southern Brazil as a base to map insertion sites of the *P* transposable element. The value of this photomap, however, has proven to be restricted to populations inhabiting the southern reaches of the American continent. Further observations by Rohde (2000, Ph.D. dissertation, Federal University of Rio Grande do Sul, Porto Alegre), taking into consideration 22 American populations from Florida to Uruguay, concluded that different "standard" arrangements of the X chromosome occur along the geographical distribution of the species.

Herein we revised all these descriptions of inversions found in the salivary gland chromosomes, presenting new results in an attempt to systematize the knowledge of the *D. willistoni* chromosomal polymorphism based on 30 Neotropical populations. This approach led to the detection of 50 different arrangements in all five chromosomal arms and the description of different geographical patterns of chromosome evolution in autosomes and the sex chromosome (X).

Materials and Methods

We studied 30 *Drosophila willistoni* populations collected at different times and from several areas (Table 1) of the Neotropical distribution range of this species (Spassky *et al.*, 1971). The flies, maintained in the laboratory by mass crosses or as isofemale lines, were reared on corn flour culture medium (Marques *et al.*, 1966) and kept at

Table 1 - Description of 30 different *Drosophila willistoni* populations studied for chromosomal polymorphism.

Population code	Place of collection	Latitude/Longitude	Collection Year (Collector)
FLO	Florida, United States of America	25°00'N/81°20'W	Stock Center (-)
MEX	Apazapan, Veracruz, Mexico	19°15'N/96°45'W	1998 (1)
MON	Monkey Hill, Saint Kitts Island, Caribbean Sea	17°19'N/62°43'W	Stock Center (2)
GUA	Guadeloupe Island, Caribbean Sea	16°15'N/61°35'W	Stock Center (3)
ECU	Jatun Sacha, Tena, Napo, Ecuador	01°06'S/77°61'W	1998 (4)
TRA	Santa Maria de Tracuateua, Moju, Pará, Brazil	01°04'S/46°53'W	1990 (5)
PAR	Caxiuanã National Forest, Melgaço, Para, Brazil	01°42'S/51°31'W	1997 (5)
MAN	Manaus, Amazonas, Brazil	03°00'S/60°00'W	1986 (5)
ZOO	Zoo, Dois Irmãos Estadual Park, Recife, Brazil	08°00'S/34°56'W	2008 (6)
CAT	Catimbau National Park, Buíque, Pernambuco, Brazil	08°24'S/37°09'W	2008 (6)
WIP	Ipitanga, Lauro de Freitas, Bahia, Brazil	12°54'S/38°19'W	1965 (7)
BRA	Brasília, Distrito Federal, Brazil	15°47'S/47°55'W	1999 (8)
PNE	National Park of Emas, Goiás, Brazil	18°60'S/52°00'W	1993 (-)
CIP ₁	Cipó Hill, Santana do Riacho, Minas Gerais, Brazil	19°20'S/43°40'W	1995 (9)
CIP ₂	Cipó Hill, Santana do Riacho, Minas Gerais, Brazil	19°20'S/43°40'W	1996 (9)
RIB	Ribeirão Preto, São Paulo, Brazil	21°10'S/47°50'W	1995 (10)
MEL	Mel Island, Paraná, Brazil	25°32'S/48°18'W	1994 (6)
TAB	Tabuleiro Hill, Santa Catarina, Brazil	27°42'S/48°34'W	1997 (11)
RAT	Ratones Grande Island, Santa Catarina, Brazil	27°29'S/48°36'W	1997 (11)
ISC	Santa Catarina Island, Santa Catarina, Brazil	27°42'S/48°30'W	1997 (11)
TUR	Turvo Estadual Park, Rio Grande do Sul, Brazil	27°20'S/53°10'W	1994 (12)
DLA	Dois Lajeados, Rio Grande do Sul, Brazil	29°10'S/51°54'W	1995 (6)
POA	Porto Alegre (18J), Rio Grande do Sul, Brazil	30°02'S/51°14'W	1989 (13)
MSA	Santana Hill, Porto Alegre, Rio Grande do Sul, Brazil	30°04'S/51°08'W	1995 (12)
ITA ₁	Itapuã Estadual Park, Viamão, Rio Grande do Sul, Brazil	30°17'S/51°01'W	1995 (14)
ITA ₂	Itapuã Estadual Park, Viamão, Rio Grande do Sul, Brazil	30°17'S/51°01'W	1997 (14)
COR	La Coronilla, Rocha, Uruguay	33°56'S/53°85'W	1994 (15)
TER	Santa Teresa National Park, Castillos, Rocha, Uruguay	33°58'S/53°32'W	2003 (6)
MVD	Montevideo, Montevideo, Uruguay	34°83'S/56°16'W	2000 (15)
PIR	Piriapolis, Maldonado, Uruguay	34°86'S/55°27'W	1994 (15)

Numbers in the last column refers to the name of flies' collectors: (1) Joana Carneiro da Silva, (2) David Bruck, (3) Peter Chabora, Lee Ehrman and Jeffrey R. Powell, (4) Patrick O'Grady, (5) Marlúcia Martins, (6) Claudia Rohde, (7) Antônio Rodrigues Cordeiro and Helga Winge, (8) Rosana Tidon, (9) Carlos R. Vilela and L. Mori, (10) Fabio de Melo Sene, (11) Daniela Cristina De Toni and Paulo Roberto Petersen Hofmann, (12) Luciano Basso da Silva, (13) Luciana Pereira Regner, (14) Luciano Basso da Silva and Victor Hugo Valiati, (15) Beatriz Goñi.

17 ± 1 °C and 60% relative humidity. Three populations (FLO, WIP and PNE) were old stocks from UFRGS Department of Genetics. Besides FLO, two others (MON and GUA) were also obtained from the Stock Center at Tucson/Arizona and represent inbred lines originally collected from Central America islands. All five stocks had been maintained for many years in the laboratory and thus a substantial reduction in genetic variability was to be expected. The other 25 populations were recently collected by us or kindly sent in by collaborators (Table 1). They represent isofemale lines of at least 10 inseminated females per population.

Squash mounts of polytene chromosomes present in salivary gland cells of third instar larvae were made using a

modification of the technique described by Ashburner (1967), fixed with acetic acid (45%), transferred to a drop of acetic acid, lactic acid and water (3:2:1) and stained with acetic orcein (2%). For each larvae, inversion polymorphism characterization was made by photomicrographic analysis of the five long chromosomal arms (XL, XR, IIL, IIR and III) of the chromosome set using a Zeiss photomicroscope and phase contrast at 1000X magnification. The photomicrographs were edited using Adobe Photoshop 5.0 and CorelDraw X3. The usual procedure adopted to study chromosomal inversions in *Drosophila* (analysis of the chromosomes of larvae produced by crossing males of unknown karyotype with homokaryotypic females of

known karyotype) was not employed due to the virtual absence of homokaryotypic viable stocks of *D. willistoni*.

Chromosomal polymorphism of *D. willistoni* was studied according to the reference photomap published in Schaeffer *et al.* (2008) for each chromosomal arm. This photomap is being presented here in only one standard picture, with all chromosomal arms together (Supplementary Material, Figure S1). Both heterozygous and homozygous inversions were analyzed and counted for frequency analysis. For the calculation of inversion frequencies we considered each inversion in the heterozygous state once, and each inversion in the homozygous state (representing each chromosome homologue) twice. The total number of chromosomal arms analyzed was twice the number of individuals analyzed.

The similarity index was calculated based on the number and frequency of paracentric inversions among 30 geographical populations. The resulting dendrogram was generated with Past version 2.04 (Hammer *et al.*, 2001) and the Morisita index (Morisita, 1959).

Results

Figures 1 to 5 present the configuration of different arrangements of XL, XR, IIL, IIR and III chromosome of *D. willistoni*, with their own inversions. Table 2 presents the frequency of 50 different arrangements found in 30 populations studied. Inversions IIL-D and IIL-E are difficult to tell apart, and for this reason, are grouped for frequency analysis in Table 2.

All the photomaps and photomicrographs (Figures 1 to 5) are oriented the same way, with the proximal (centromeric) regions of the chromosomes to the left and the distal (free) regions to the right. The distal regions of the chromosomes are usually well polytenized and allow the different chromosomes to be recognized. Since there are different fixed configurations for the XL and XR chromosomal arms, we use capital letters to distinguish between them, like XL-A or XR-A, both presented in the standard photomap of the species (Figure S1). In total, we observed at least 10 different chromosomal arrangements segregating on the XL arm and eight on the XR arm. Two XL arrangements, XL-C and XL-H, were found only in the homozygous state (data not shown) and two inversions (XL-J and XL-I, Figure 1) were only found by Regner and Valente (unpublished data) for other Southern Brazilian populations. Rohde *et al.* (2005) reported the unique pericentric inversion found until now in *D. willistoni* (XL/XR-p), both in homo and heterozygous states. In the autosomes, we were able to detect 10 different arrangements on the IIL arm, eight on the IIR arm and 13 on the III chromosome. As shown in Figures 1 to 5, the precise identification of each breakpoint of the 50 different arrangements was possible, or facilitated, because many inversions were detected in the homozygous state. Although heterozygous inversions are easily detected in cytological preparations the breakpoints

are very often concealed above the loop, especially in very small inversions that are common in *D. willistoni*. This is probably one of the reasons why there has been, until now, a virtual absence in the literature of descriptions of homozygous inversions in *D. willistoni*.

Figures 1 and 2 also present an estimated evolutionary relationship between the arrangements of XL and those of XR. For example, the inversions (*In*) (XL)_{F₁+F₂} occurred over the fixed inversion (XL)_D, which in turn occurred over the XL-A standard. One possible evolutionary relationship (that will be presented in detail in another paper, with descriptions and comparisons of their homozygous forms) is that different arrangements of XL and also of XR can have occurred and were selected along the wide geographic distribution of *D. willistoni*. We propose that XL-A is the ancestral configuration, and XL-D, XL-E and XL-B are descendant forms with their own inversions, respectively: *In*(XL)_{F₁+F₂}, *In*(XL)_{B,G}, and *In*(XL)_{J,K,I}. As shown in Table 2, the inversion frequency of all arrangements of XL, and also XR segregate in different frequencies with a clear latitudinal cline, from North to South America. Compared to the XR chromosomal arm, the XL one presents a more fixed arrangement (4 to 3) among populations. On the other hand, all the autosomes (IIL, IIR and III) have a basic arrangement, within which may occur different inversions, but with a clear latitudinal cline of the respect frequencies, similar to what was seen for the X chromosome. By combining all inversion frequencies, a dendrogram was generated (Figure 6) showing that geographically proximate populations are much more closely related.

Discussion

In the present study, we tried to catalog the chromosomal inversions occurring in 30 *Drosophila willistoni* populations representative of almost its entire geographical distribution and to draw a general picture of the chromosomal inversion polymorphisms for this organism. We were able to analyze the chromosomes of 615 *D. willistoni* individuals, most of them being isofemale lines from natural populations. We found at least 50 different gene arrangements for various parts of the distribution range of the species, with evidence suggesting that northern and southern groups emerged gradually. With respect to the chromosomal arms XL and XL, our data are comparable with those reported by Levitan (1992), reviewing data of *D. robusta* and including his findings and those of Carson and Stalker (1947). The existence of inversion clines, with a tendency to lose heterokaryotypes toward the periphery of the species distribution (Carson, 1959) was partially found in *Drosophila willistoni*, especially with respect to the autosomes (Valente *et al.*, 2003). Our data on *D. willistoni* inversions from Uruguayan populations, however, are not indicative of less polymorphism in the marginal populations in the extreme south of the distribution of this species

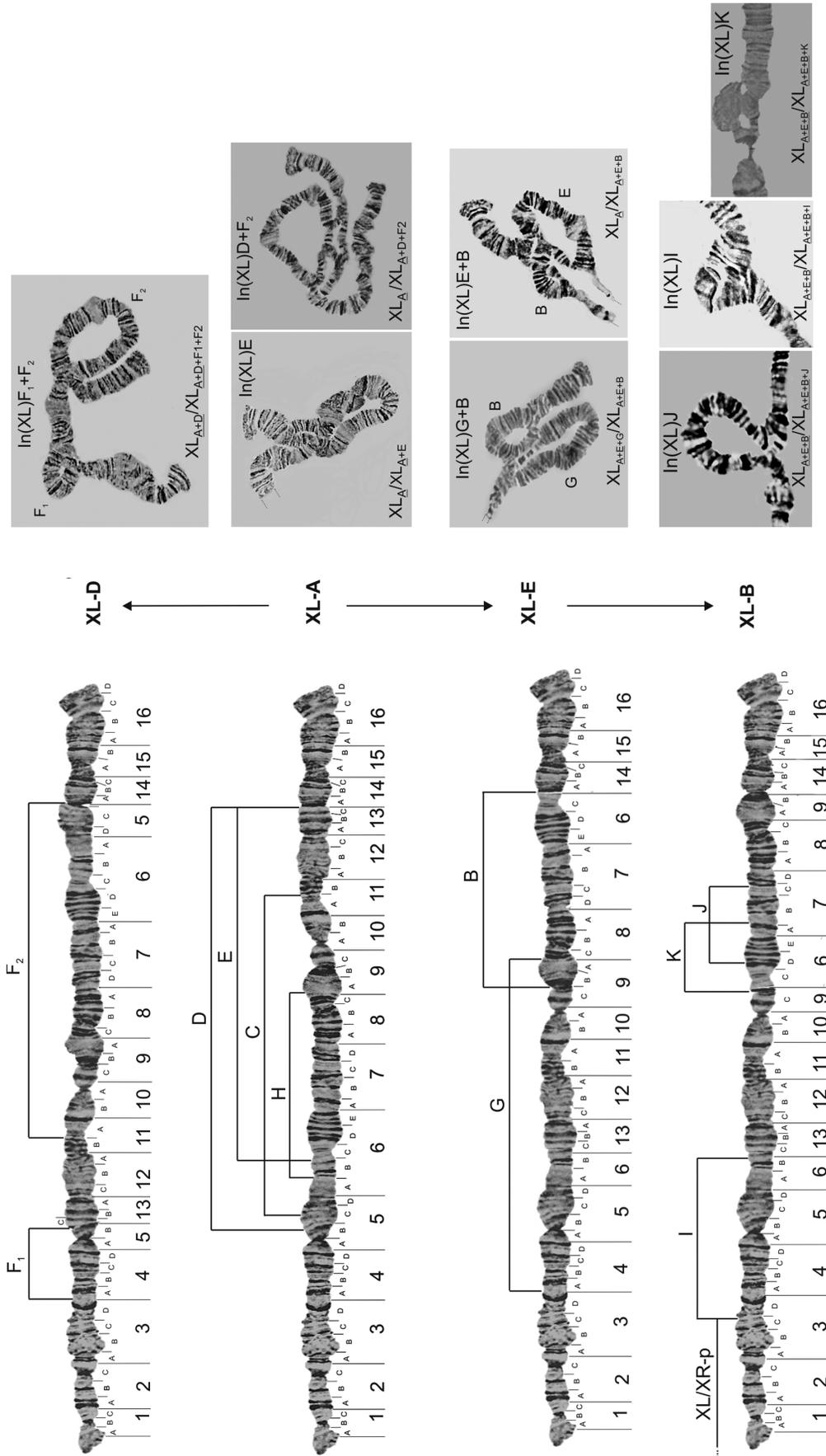


Figure 1 - Configuration of different basic arrangements of XL chromosomal arm (XL-A, XL-D, XL-E and XL-B) of *D. willistoni*. Lines represent inversion breakpoints. Each inversion (D, E, C, H, F₁, F₂, G, B, I, K, J and P₁) could be fixed or segregating in populations, depending of its geographical location. Inversions H and C were found only in homozygous state (data not shown). The pericentric inversion XL/XR-P₁ (in homo or heterozygous state) could be found in a previously publication of Rohde *et al.* (2005) and inversions I and J were previously found by Regner and Valente (unpublished data) for the populations in southern Brazil.

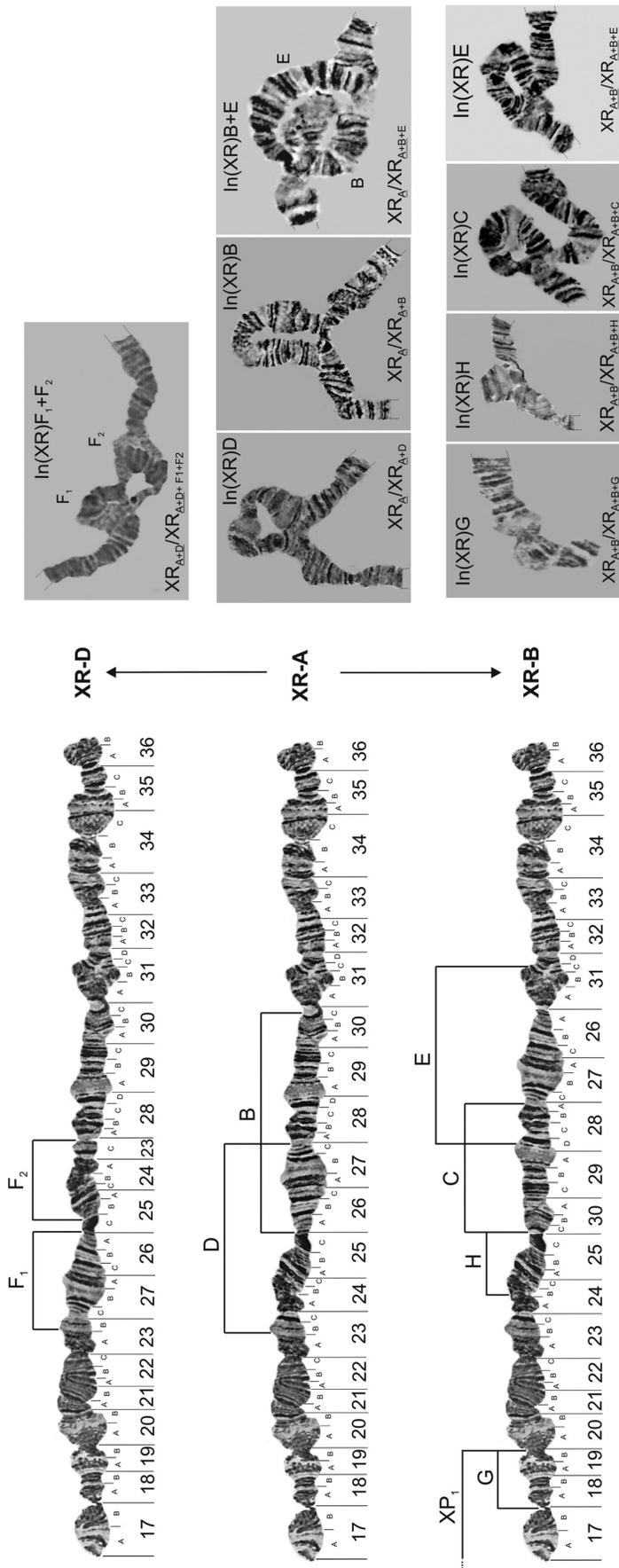


Figure 2 - Configuration of different basic arrangements of XR chromosomal arm (XR-A, XR-B and XR-D, XR-E) of *D. willistoni*. Lines represent inversion breakpoints. Each inversion (D, B, F₁, F₂, E, C, H, G and P₁) could be fixed or segregating in populations, depending of its geographical location. The pericentric inversion XL/XR-P₁ (in homo or heterozygous state) could be found in a previous publication of Rohde *et al.* (2005).

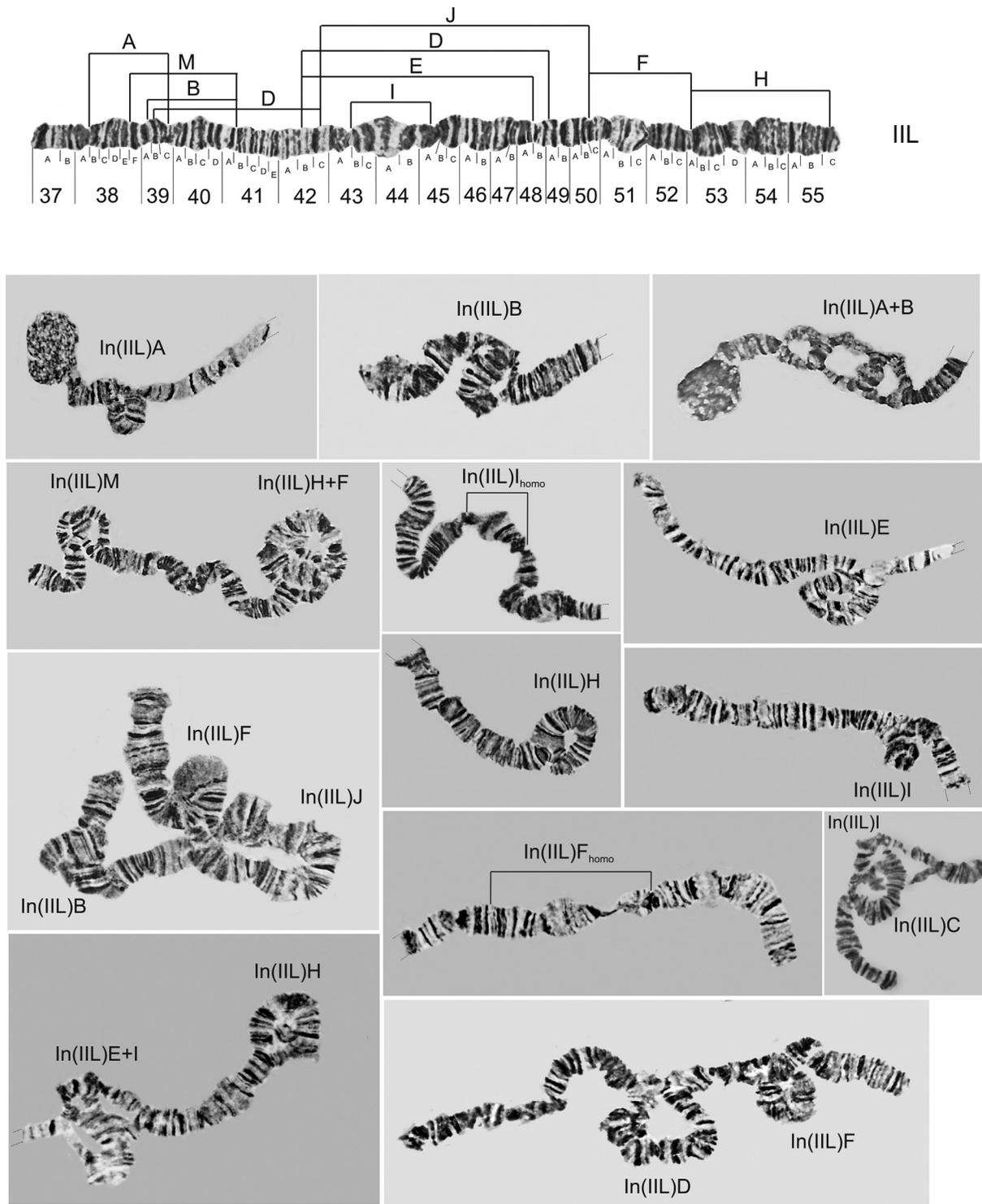


Figure 3 - Distribution of inversion breakpoints observed on chromosomal arm IIL of *D. willistoni* and photomicrographs of inversions (in homo and heterozygous state) detected.

(Valente *et al.*, 2003). Chromosomal variation found in *D. willistoni* was mainly due to inversions and, for the first time, homozygous inversions were identified and included in the chromosomal polymorphism analysis. This was due to careful maintenance of isofemale lines, which allowed us to obtain chromosomal preparations of high resolution

quality. The precise analysis of each inversion was done through the use of several different photomicrographs representing all possible configurations. This was also done for homozygous chromosomal arms.

Due to the large number of inversions and different types of combination of arrangements, a definitive discus-

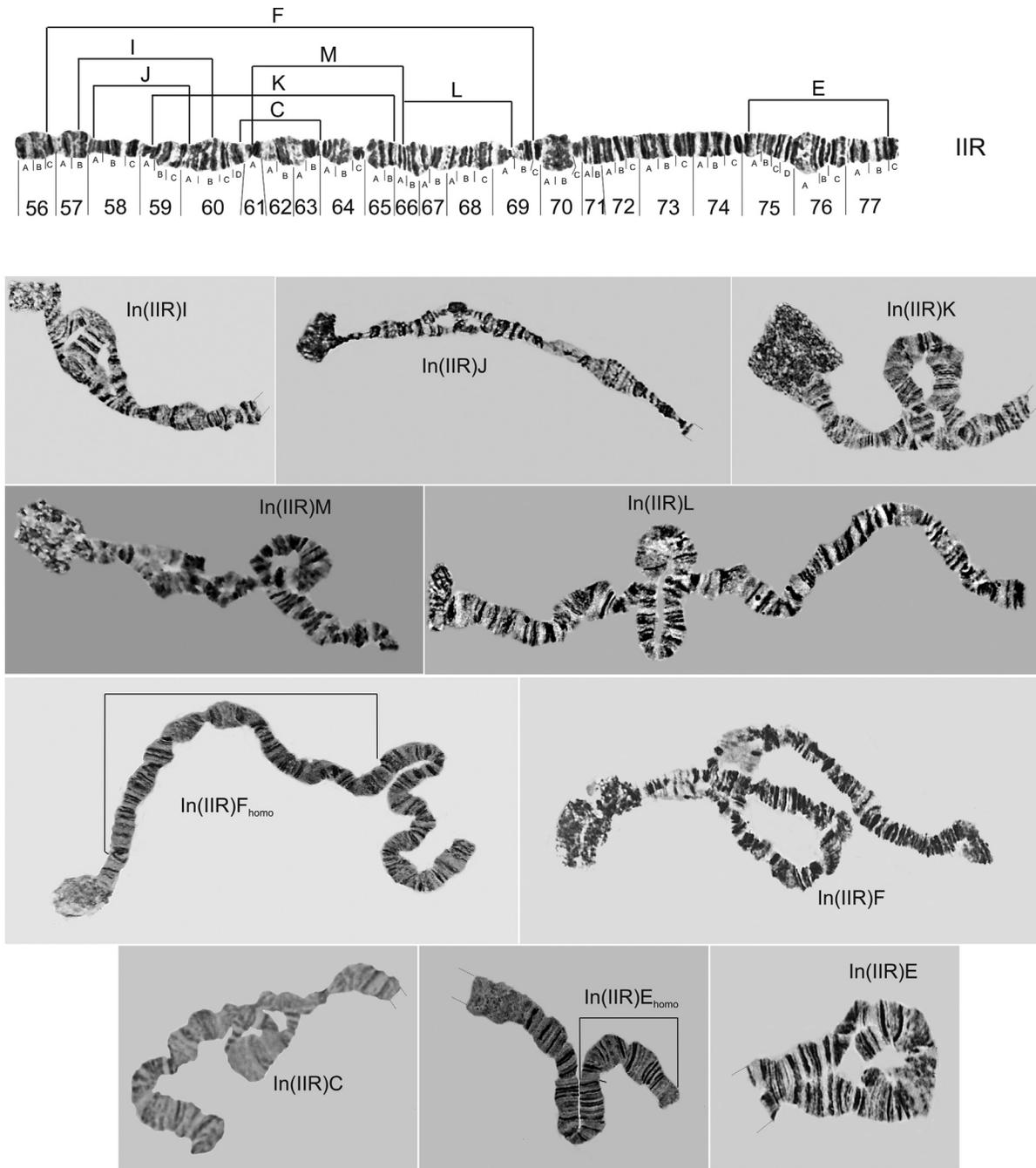


Figure 4 - Distribution of inversion breakpoints observed on chromosomal arm IIR of *D. willistoni* and photomicrographs of inversions (in homo and heterozygous state) detected.

sion on their macro-geographic distribution will be dealt with in upcoming paper. Nonetheless, some generalizations can be made. None of the sampled natural population was totally chromosomally monomorphic for all chromosomal arms. With respect to the X chromosome, a clear discontinuity of arrangements among populations was observed (Table 2). The 18 central-south Brazilian and Uruguayan populations (BRA to PIR) mainly presented the XL-B or XL-E, and XR-B or XR-E arrangements. Three northern Brazilian populations (ZOO, CAT and WIP) presented

both standard arrangements as fixed: XL-A and XR-A. The other north Brazilian populations (MAN, TRA, PAR) and ECU, MEX and FLO mainly presented the XL-D or XL-F₁+F₂, and XR-F or XR-D. Finally, two Caribbean island populations (MON and GUA) presented the arrangements XL-A and XR-C as fixed. The XL-A arrangement, considered as the standard arrangement in *D. willistoni* (Schaeffer *et al.*, 2008), was found in samples as far away as MEX (close to the north limit of the species range) and ITA (close to south limit of the species range), with fre-

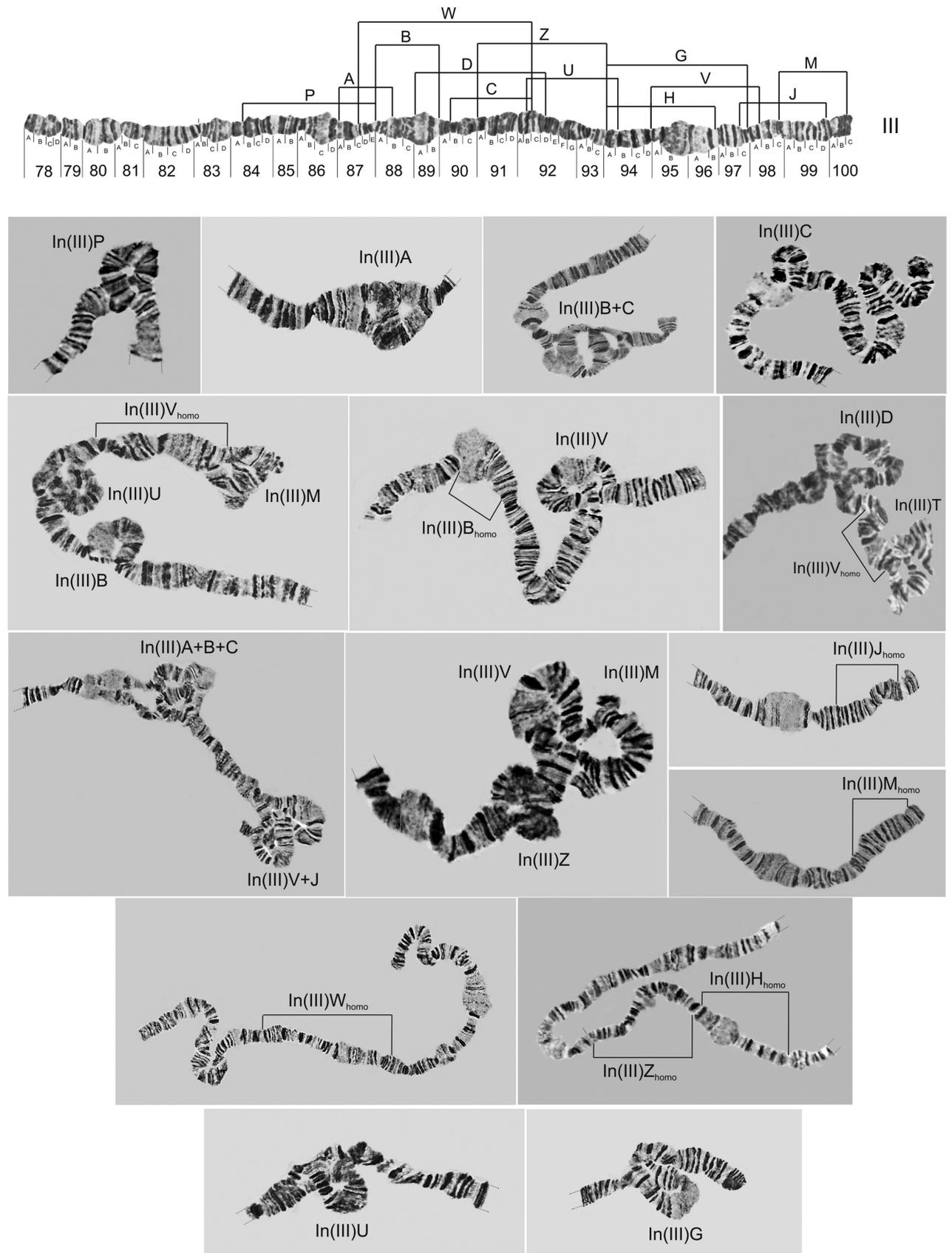


Figure 5 - Distribution of inversion breakpoints observed on chromosome III of *D. willistoni* and photomicrographs of inversions (in homo and heterozygous state) detected.

Table 2 - Frequency of different arrangements of X chromosome (XL and XR arms), II (IIL and IIR) and III in populations of *Drosophila willistoni*. *N* means the number of individuals analyzed.

Arrangements	Populations																														
	FLO	MEX	MON	GUA	ECU	TRA	PAR	MAN	ZOO	CAT	WIP	BRA	PNE	CIP ₁	CIP ₂	RIB	MEL	TAB	RAT	ISC	TUR	DLA	POA	MSA	ITA ₁	ITA ₂	COR	TER	MVD	PIR	
XL-F ₁ +F ₂	1.0	0.875			0.833	1.0	0.312	0.476				0.100																			
XL-C			1.0	1.0																											
XL-D							0.625	0.523																							
XL-A		0.125			0.167		0.061		1.0	1.0	1.0				0.078	0.075		0.030	0.179							0.009					
XL-G												0.100				0.050															
XL-K																					0.033										
XL-E												0.400		0.356	0.438	0.375	0.010	0.120	0.074	0.100	0.033	0.352				0.047			0.100		
XL-B											0.400		1.0	0.644	0.484	0.500	0.990	0.850	0.747	0.900	0.934	0.648	1.0	1.0	1.0	0.944	1.0	0.900	1.0	1.0	
XR-D		0.554																													
XR-F						1.0		0.944	0.405			0.150																			
XR-A	1.0	0.446	1.0	1.0			0.547		1.0	1.0	1.0			0.192	0.391	0.150															
XR-C																					0.001	0.016									
XR-H																															
XR-G																															
XR-E																															
XR-B																															
XR-P																															
XL/XR-P																															
IIL-I	0.529	0.107	1.0	0.650	0.273	0.765	0.305	0.405	0.272	0.650	1.0	0.040	0.421	0.269	0.422	0.350	0.459	0.324	0.357	0.167	0.100	0.463	0.100	0.652	0.033	0.472	0.278	0.050			
IIL-F	0.441	0.946	1.0	1.0	0.722	0.412	0.278	0.864	0.950	1.0	0.100	0.100	0.065	0.516	0.425	0.286	0.441	0.571	0.467	0.150	0.467	0.150	0.250	0.163	0.033	0.259	0.222	0.225	0.150	0.125	
IIL-A	0.147	0.179			0.278	0.177	0.194	0.095	0.050		0.050	0.050	0.184	0.077	0.008	0.075	0.010	0.059			0.017										
IIL-D/E						0.029																									
IIL-B					0.222																										
IIL-H																															
IIL-C																															
IIL-M																															
IIL-J	0.500																														
IIR-E	0.441	0.143			0.100	0.389	0.941	1.0	0.167	0.409	0.350	0.250	0.210	0.154	0.078	0.100	0.031														
IIR-K	0.059																														
IIL-L																															
IIR-J		0.125																													
IIR-I		0.018																													
IIR-F																															
IIR-C																															
IIR-M																															
IIL-J																															
IIL-B	0.441	0.167	0.100		0.055	0.794	0.111	0.333	0.545	0.350	0.214	0.100	0.105	0.231	0.125	0.125	0.133	0.176	0.321	0.267	0.267	0.500	0.350	0.283	0.300	0.380	0.417	0.150	0.250	0.125	
IIL-C	0.412				0.555		0.333	0.214				0.200	0.053	0.115	0.062	0.075	0.163	0.176					0.200	0.098		0.065	0.025	0.150	0.125		
IIL-A							0.028					0.200	0.210	0.077	0.094	0.075							0.033	0.050	0.148	0.050	0.098	0.025			
IIL-V												0.750	0.300	0.269	0.141	0.200	0.010	0.029				0.033	0.133	0.241	0.150	0.163	0.233	0.167			
IIL-U																															
IIL-M		0.241			0.333		0.028	0.095					0.105	0.154	0.094	0.275															
IIL-G							0.222	0.286					0.158	0.192	0.016	0.200															
IIL-W	0.206	0.055			0.100	0.176	0.055		0.136																						
IIL-Z					0.278																										
IIL-H																															
IIL-D																															
IIL-P																															
N	17	28	10	10	10	10	17	18	21	11	10	28	10	19	13	32	20	49	17	14	15	30	27	10	46	15	54	18	20	10	16

quencies varying between 0.009 and 0.179, being exclusive to northern populations of Brazil (Table 2). With respect to XR-A, found in samples from FLO (in the north limit of the species range) to RIB (central Brazil) their frequencies ranged from 0.150 to 0.548, being fixed in six different populations, as presented in Table 2.

It is interesting to note in Table 2 that in each population analyzed just one or two possible arrangement of the XL or XR chromosomal arms were observed at frequencies above 0.1 (or 10%). Wallace (1953 a, b) hypothesized that the simultaneous presence in a population of three successively overlapping inversions creates difficulties in maintaining a co-adaptive system (*i.e.*, co-adaptation for the three possible pairs of inversions), since crossovers create a linkage of alleles between the two remote members of the triad. Because of this, a co-adaptive system would not be maintained. Our data regarding the arrangements of the X chromosome found that the populations of *D. willistoni* studied (except population BRA) are in accordance with Wallace's predictions, but Krimbas and Loukas (1980) found that there were 46 exceptions (11.7%) to Wallace's rule among of a total of 394 cases of polymorphism in one of the chromosomes of a *D. subobscura* population. The authors suggested that in a chromosome triad, a frequency of 0.0005 (or 0.05%) was the minimum possible for any of the three coexisting members. If we accept that this is also true for the X-chromosomes of our *D. willistoni* populations, then some populations for the XL arm (PAR, CIP₂, BRA, RIB, TAB, RAT, TUR and ITA₂) and some for the XR arm (MAN, BRA, CIP₂, RAT, ISC and TUR) would also be exceptions to Wallace's rule. Table 2 shows that not just three, but four arrangements exist (in populations BRA, RIB and TUR), produced by successive overlapping inversions, if the frequency limit of the less frequent inversion is raised to 0.001 (or 0.10%).

In the autosomes of *D. willistoni*, we observed regional characteristics regarding inversion frequencies, with some arrangements appearing to be fixed in some populations (IIL-I and IIL-F in the WIP population and IIR-E in the PAR population, for example) while other arrangements occurred at very high frequencies (IIL-I in the TRA population, IIL-F in MEX and ECU populations, IIR-E in the TRA population, III-W in the TRA population and III-J in TRA and PIR populations). However, it is possible that at least the inversions referred to by Da Cunha *et al.* (1950) as the most common ones (IIL-F, IIR-E, III-J, III-B) are the same inversions described here. In Figure 5, one can see that chromosome III, the most polymorphic among the autosomes, has many inversions that are separated from each other by only a few sections. So it is possible that we may have made mistakes when trying to correlate photomicrographs of this chromosome with the drawings made by previous authors, although we tried to minimize such errors by naming the inversions as they were found and compar-

ing them with the map of Valente and Morales (1985), preserving the original descriptions.

From the dendrogram presented in Figure 6, the conclusion is that the analysis of heterozygous and homozygous frequency inversions in *D. willistoni* chromosomes is a good method for grouping populations. It can be also seen that geographically isolated populations, such as ECU and BRA, form two unique clusters, with the BRA population equally distant from the two biggest clusters, the southern and northern ones. Our results also support the theory that X chromosomes and autosomes are under different selective pressures and shed light on the particular chromosomal phylogeny of the X chromosome of *D. willistoni*, which will be published in more detail elsewhere. Due to the presence of fixed rearrangements in the X and other chromosomes, we do not recommend that only heterozygous inversions be used in inversion polymorphism analyses of this species.

Unfortunately, it was very difficult to compare our results with other previous publications, since few of them included photomicrographs of the inversions in the heterozygous state or their breakpoints in a photomap. This is the case with two recent interesting chromosomal studies on *D. willistoni* populations from Mexico (Salceda, 2006, 2010). The author found two frequent arrangements segregating in the XL chromosomal arm (XL "A" and "D") plus the standard "ST", and two arrangements in XR (XL "D" and "E") plus the standard "ST". Considering the frequencies of XL and XR presented by the author, it could be suggested that XL "ST" is our XL-F, being an arrangement resulting from two homozygous inversions occurring at the same time, In(XL)F₁+F₂ (as presented in Figure 1). When both inversions (F₁ and F₂) occur together in both chromosomal arms XL of the same female (so, in homozygosis) they originate the arrangement XL-F, present in our Mexican populations at a frequency of 0.875 (or 87.5%) (data not shown). With respect to XR "ST", it's not possible to suggest correlations with our data because we found two XR homozygous arrangements at similar frequencies: XL-A (0.357) and XL-D (0.464). Besides XL "ST", Salceda (2010) found seven more XL inversions, and besides XR "ST", six more inversions in XR.

Due to the presence of paracentric inversions, the rate of genome rearrangement in *Drosophila* is one of the highest among all eukaryotes (Ranz *et al.*, 2001; González *et al.*, 2007; Bhutkar *et al.*, 2008). Ranz *et al.* (2001) estimated that between the *Drosophila* and *Sophophora* subgenera, for example, the evolution rate was 0.9-1.4 chromosomal inversions fixed per million years, based on the divergence time between the subgenus (Spicer, 1988; Russo *et al.*, 1995). According to Richard *et al.* (2005), selection may favor inversions because they maintain epistatic combinations within the inverted segment (Dobzhansky 1949; Charlesworth and Charlesworth 1973; Wu and Beckenbach, 1983; Otto and Barton, 2001),

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Supplementary Material

The following online material is available for this article:

- Figure S1 - Reference photomap of *Drosophila willistoni*.

This material is available as part of the online article from <http://www.scielo.br/gmb>.

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