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FISH mapping of microsatellite loci from *Drosophila subobscura* and its comparison to related species

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Abstract Microsatellites are highly polymorphic markers that are distributed through all the genome being more abundant in non-coding regions. Whether they are neutral or under selection, these markers if localized can be used as co-dominant molecular markers to explore the dynamics of the evolutionary processes. Their cytological localization can allow identifying genes under selection, inferring recombination from a genomic point of view, or screening for the genomic reorganizations occurring during the evolution of a lineage, among others. In this paper, we report for the first time the localization of microsatellite loci by fluorescent in situ hybridization on *Drosophila* polytene chromosomes. In *Drosophila subobscura*, 72 dinucleotide microsatellite loci were localized by fluorescent in situ hybridization yielding unique hybridization signals. In the sex chromosome, microsatellite distribution was not uniform and its density was higher than in autosomes. We identified homologous segments to the sequence flanking the microsatellite loci by browsing the genome sequence of *Drosophila pseudoobscura* and *Drosophila melanogaster*. Their localization supports the conservation of Muller's chromosomal elements among *Drosophila* species and the existence of multiple intrachromosomal rearrangements within each evolutionary lineage. Finally, the lack of microsatellite repeats in the homologous *D. melanogaster* sequences suggests convergent evolution for high microsatellite density in the distal part of the X chromosome.

Keywords cytogenetic map · *Drosophila* · fluorescent in situ hybridization · genome rearrangement · microsatellite loci · repetitive DNA · genome comparison

Abbreviations

DAPI	4'-6-Diamidino-2-phenylindole
dsub	ID names of the clones
FISH	fluorescent in-situ hybridization
HP1	Heterochromatin Protein 1
NTP	nucleotides triphosphate
PCR	polymerase chain reaction
rDNA	ribosomal DNA
SSC	Sodium Chloride-Sodium Citrate buffer
SSR	simple sequence repeats
UCSC	University of California Santa Cruz

Introduction

Microsatellites are highly polymorphic markers constituted by simple sequence tandem repeats (SSR) of one to six nucleotides per unit (Bachtrog et al. 1999). They are ubiquitous among eukaryotes and have been described from a great variety of *taxa* (Wilder et al. 2002), being generally considered neutral unless linked to *loci* under strong selection. Although SSRs typically

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represent selectively neutral DNA markers, random expansions or contractions appear to be selected against for at least part of SSR loci, presumably because of their effect on chromatin organization, regulation of gene activity, recombination, DNA replication, cell cycle, mismatch repair system, etc. (Li et al. 2002). Some satellite and microsatellite DNA are organized in clusters of 100–10,000 tandem repeats and have been mainly associated with constitutive heterochromatin (Lohe et al. 1993; Cuadrado and Jouve 2007a). However, some dinucleotide and trinucleotide repeats are also distributed through most euchromatic regions (Pardue et al. 1987; Cuadrado and Jouve 2007b). Furthermore, these two microsatellite classes are the most frequent repeats and have been widely used in forensics, population and conservation genetics, and for inferring selection in a wide range of species due to their high polymorphism and easy amplification. Some of those studies use microsatellite loci isolated from the same species from genomic libraries with clone inserts ranging from 200 to 800 bp depending on the methodology used (Pascual et al. 2000; Carreras-Carbonell et al. 2004); however, others involve microsatellite cross-species amplification (Carreras et al. 2006). It has been shown that amplification success is correlated with divergence between the source and the focal species in birds and fish (Primmer et al. 2005; Carreras-Carbonell et al. 2008), and 50% of loci are polymorphic between species presenting around 6% divergence for the 16S rDNA (Carreras-Carbonell et al. 2008). However, the conservation of flanking sequences for particular microsatellite motifs can span to 470 million years, suggesting that these sequences, although generally considered neutral, may play an important role in eukaryotic genomes and could be under strong selective constraints (Rico et al. 1996). Whether they are neutral or under selection, these markers if localized could be used for exploring the dynamics of the evolutionary process. Although the chromosomal distribution of different repeat types has been assessed in several species (Cuadrado and Jouve 2007b; Chang et al. 2008), only a few microsatellite loci have been physically localized until now by fluorescent in situ hybridization using as hybridization probes bacterial artificial chromosome inserts containing the loci of interest (Samollow et al. 2007; Di Meo et al. 2008) Furthermore, the physical localization of microsatellites and its comparison to other related species can give insight on the speciation process, on the genomic reorganization during the evolution of a lineage, or the microsatellite conservation through time. *Drosophila* is a very interesting genus for studies addressing genome evolution because there are 12 different species that have been fully sequenced (*Drosophila* 12 Genomes Consortium 2007). The ancestral karyotype within the *Drosophila* genus consists of one dot and five acrocentric chromosome pairs. It is well known that the gene content of these six different elements is highly conserved, although there is extensive gene reshuffling within elements, referred to as Muller's elements (Muller 1940; *Drosophila* 12 Genomes Consortium 2007). Unfortunately, there is still a great gap of information on most *Drosophila* species, but comparative studies with available genomic sequences can be a very useful evolutionary tool for screening homologies and evaluating chromosomal synteny.

Drosophila subobscura is a Palearctic species with a vast distribution area that in the last decades has invaded the west coast of South and North America (Prevosti et al. 1988; Ayala et al. 1989). This species has the ancestral *Drosophila* karyotype presenting five major acrocentric chromosomes denominated A, the sexual one, and J, U, E, and O, the autosomes, as well as one punctiform called the dot chromosome. There is high polymorphism of paracentric inversions in this species which has been shown to be adaptive and respond to environmental factors such as global warming (Balanyà et al. 2006). It has been suggested that inversions are conserving allelic combinations which have been favored by selection (Dobzhansky 1950). However, since this species has not been sequenced yet, markers with wide distribution along the genome can be very useful to track the basis of chromosomal inversion adaptation. Microsatellites are good candidate markers since they are expected to be evenly distributed along chromosomes. Furthermore, in *D. subobscura*, microsatellite markers have been shown to be a good tool for inferring colonization processes (Pascual et al. 2001, 2007) and for studying the evolutionary dynamics of local adaptation (Simões et al. 2008). Nevertheless, their localization is fundamental to analyze the dynamics of inversion polymorphism and track the genetic basis of genome-wide recombination and adaptation.

In the present study, we report the localization on polytene chromosomes of 72 dinucleotide microsatellites previously isolated from a *D. subobscura* DNA library (Pascual et al. 2000). As localization technique,

we used fluorescent in situ hybridization (FISH) since previous essays with non-fluorescence probes failed in the detection. Moreover, we analyzed microsatellite loci distribution and density throughout the genome, contrasting results among the sex chromosome and autosomes with implications on microsatellite function and generation. Finally, we searched in silico the complete genomes of *Drosophila melanogaster* and *Drosophila pseudoobscura* for the presence of the *D. subobscura* 72 microsatellite loci to assess the conservation of this repetitive, presumably non-coding DNA and the synteny between and within chromosomal elements.

Materials and methods

Fly stock and preparation of polytene chromosome slides

A stock of the homokaryotypic strain *cherry-curled* (*chcu*) of *D. subobscura* was used to obtain polytene chromosomes slides to which the microsatellite probes were hybridized. The *chcu* strain is standard for all chromosomes (A_{st}, E_{st}, J_{st}, U_{st}) except for chromosome O which is O_{3+4}. Third-instar larvae had to be grown under density-controlled conditions to obtain readable slides. Approximately 150 eggs were collected periodically, transferred to 150-ml bottles with *Drosophila* medium, and grown at 18°C. Polytene chromosome slides were obtained as described in Lim (1993) with some modifications. Briefly, third-instar larvae were dissected on 45% acetic acid. Their clean salivary glands were transferred to a non-siliconized coverslip, fixed for 4 min on 1:2:3 solution (1 volume 86% lactic acid/2 volumes water/3 volumes glacial acetic acid), and gently squashed. The slides were kept at 4°C between 6 and 24 h, dived in liquid nitrogen to pry off the coverslip with a razor blade, and submerged in a 95% ethanol bath chilled at −80°C. The bath was kept at room temperature for about 3 h until slides slowly reached this temperature, after which they were air-dried, scored on a contrast phase microscope, and stored until used for hybridization for <2 weeks at 4°C.

Microsatellite probes

A total of 72 previously isolated microsatellite loci were used as probes (Pascual et al. 2000). The mean length of the perfect repeat was14.48±7.09 bp, and the mean size of their flanking region was 475.83±98.90 bp (see Table 1). The amplifications of the probes for 64 loci were carried out using the forward (M13F) and reverse (M13Rmut) primers present in the pUC18 plasmid in which the loci were cloned. The probes for the other eight loci, since clones were not available, were amplified from *chcu* genomic DNA with primers designed to maximize probe length using the sequence of the clones of the microsatellite library (Genbank accession numbers GU732209-80). The amplified probes ranged between 300 and 700 bp and were labeled by PCR with a dNTP mix including 0.35 mM digoxigenin-11-dUTP, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, and 0.65 mM dTTP. Amplification was conducted in a 25-μL reaction volume with 10× buffer, 3 μL dNTP mix, 0.2 μM of each primer, 1 U Taq polymerase (Amersham Pharmacia Biotech), and 1 μL of clone or genomic DNA. The amplification reaction was carried out using an Applied Biosystems 2700 machine, beginning with an initial denaturation of 5 min at 94°C, followed by 30 cycles of denaturation for 30 s at 92°C, primer annealing for 30 s at 50°C and extension for 1.5 min at 65°C, and a final step of 5.5 min at 65°C. PCR product precipitation was carried out as in Lim (1993) with some modifications. In a final volume of 100 μL, about 600–800 ng of amplified DNA was mixed with 6 μg of salmon sperm. Precipitation was accomplished by adding 0.1 volume of 2.5 M sodium acetate and 2 volumes of 100% ethanol, mixed by inversion and incubated at −70°C for 20 min. DNA was recovered by centrifugation, air-dried, and frozen until used. The pellet was resuspended in 10 μL of hybridization solution containing 5 μL of deionized formamide, 2 μL of 50% dextran sulfate, and 1 μL of 20X SSC.

Hybridization and detection

In situ hybridizations were carried out following the fluorescent hybridization protocol for mitotic chromosomes (Pimpinelli et al. 2000). Probes were detected with an anti-digoxigenin-rhodamine antibody (Roche) adding 200 μL of a detection solution (40 μL of 20× SSC, 2 mg of bovine serum albumin, and 2 μL of 10% Tween20) containing 260 ng of the antibody and incubating the slides in an opaque container humidified with 2× SSC for 45 min at 37°C. To eliminate excess of fluorescence, slides were dipped at 42°C in three consecutive baths of 4× SSC with 0.1% Tween20, for 5 min each; afterwards, they were rinsed first with 2× SSC and secondly with distilled

Table 1 Localization of the 72 microsatellite markers by *in situ* fluorescent hybridization on the polytene chromosomes of *D. subobscura,* ordered by chromosome and cytological band, and localized by BLAST on the genomes of *D. pseudoobscura* and *D. melanogaster*

Drosophila subobscura				*D. pseudoobscura*			*D. melanogaster*		
Marker	Repeat motif	Flanking	Band	Chr	Id(bp)	Band[a)]	Chr	Id(bp)	Band
Chromosome A (Muller element A)									
dsub11	$(CA)_{13}$	423	03B	XL	183	15A	X	39	03D3
dsub17	$(GT)_{18}$	472	03D	XL	66	07A	X	30	12D2
dsub37	$(TG)_{17}T(TG)_4TG(TG)_4$	565	06C	XL	116	12C	X	72	02B1
dsub76	$(GT)_3GC(GT)_4GC(GT)_4GCC(GT)_4CC(GT)_9$	343	08B	XL	185	05B	X	22	10B3[b]
dsub05	$(AC)_{26}TCAC$	393	08E	XL	126	11A	X	52	18B6
dsub45	$(CA)_2TG(CA)_{15}$	398	08E	XL	91	10C	X	82	18A4
dsub66	$(GT)_{18}$	637	09B	XL	46	08B	X/2L[c]	19	_[b]
dsub52	$(GT)_{14}$	535	09C	XL	287	06A	X	32	12F5
dsub30	$(CA)_{11}$	368	10C	XL	158	08A	X	43	08A2
dsub21	$(AG)_{18}$	504	11A	XL	103	16B	X	93	09A3
dsub78	$(CA)_2CT(CA)_{21}$	391	11A	XL	53	16B	X	35	09A3
dsub40	$(TG)_{18}$	567	11A	XL	123	16B	X	54	09A2
dsub54	$(GA)_3GG(GA)_{13}$	610	11B	XL	227	04C	X	65	16A5
dsub33	$(GT)_{17}$	457	11D	XL	323	07D	X	242	03B3
dsub32	$(TG)_{20}CC(GT)_4$	592	12A	XL	55	01C	X	32	20A4
dsub39	$(CA)_{15}$	597	12B	XL	185	06B	X	169	05E1
dsub70	$(TG)_{14}$	375	13A	XL	64	16A	X	50	11E1
dsub58	$(CA)_{31}$	391	13B-C	XL	111	16A	X	36	16B7
dsub44	$(GA)_{14}$	775	13C	XL	84	08B	X	62	03F2
dsub43	$(CA)_{19}$	362	13E	XL	103	03B	X	76	01C3
dsub50	$(CA)_{17}$	343	15A-B	XL	61	07C	X	52	19B1
dsub67	$(TG)_4TC(TG)TC(TG)_2AG(TG)_{13}$	456	15B	XL	50	07C	3L	22	77B2[b]
dsub19	$(GA)_{27}$	329	15B-C	XL	61	07D	3L	22	64A3[b]
dsub77	$(CA)_{13}$	533	15D-E	XL	104	14A	X	33	09F5
dsub61	$(GT)_{14}$	414	16C	XL	72	05A	3R	23	92B2[b]
dsub57	$(CA)_{13}(CAGA)_3$	487	16D	XL	91	02B	3L	20	64F5[b]
Chromosome U (Muller element B)									
dsub10	$(TG)_{27}GG(TG)_8$	469	36A	4	114	89A	2L	86	40D2
dsub03	$(CA)_5TACAA(CA)_{13}$	482	37B	4	249	89B	2L	197	37B13
dsub31	$(GT)_{12}$	485	42B	4	195	86E	2L	129	25E2
dsub42	$(CT)_{20}TG(CT)$	531	44B	4	114	86D	2L	67	35A3
dsub64	$(GA)_{28}GG(GA)_3$	381	48B	4	85	98A	2L	51	21D2
dsub15	$(AC)_4AA(AC)_{11}$	531	52A	4	250	96D-E	2L	193	33D2
marker	repeat motif	flanking	Loc	Chr	Id(bp)	Loc[a]	Chr	Id(bp)	Loc
Chromosome E (Muller element C)									
dsub46	$(GA)_{21}$	527	54D-E	3	373	73C	2R	71	55E10
dsub68	$(AC)_{18}$	477	54D-E	3	144	73C	2R	88	55E10
dsub35	$(TG)_{19}TT(TG)4$	496	55D	3	183	72C	2R	72	44B1
dsub79	$(GA)_{19}$	339	58A	3	161	77A	2R	114	58B1
dsub36	$(AC)_{23}$	366	59B	3	132	69C	3R	25	92C3[b]
dsub20	$(GA)_{24}$	499	62B	3	38	79D	2R	76	51B11

Table 1 (continued)

Drosophila subobscura				*D. pseudoobscura*			*D. melanogaster*		
dsub53	$(GT)_{11}TG(GT)_5$	744	70A-B	3	87	65D	2R	75	53D1
dsub80	$(TG)_{24}$	268	70B-C	3	38	65D	2R	23	60C5[b]
dsub55	$(CA)_{16}$	760	70D	3	72	68A	2R	23	55C4[b]
dsub28	$(TG)_9$	463	73C	3	76	81B	2R	77	54A2
dsub48	$(CA)_{19}$	357	74A	3	130	81C	2R	98	58A2
dsub41	$(TA)_4(GT)_8ATG(TA)_4$	452	74C	3	176	81D	2R	120	56E1
dsub13	$(GT)_6TG(GT)_8$	453	74D	3	120	75B	2R	59	60F5
Chromosome J (Muller element D)									
dsub23	$(CT)_{18}$	591	19D	XR	120	38D	3L	111	79C1
dsub16	$(GA)_{32}$	429	21C	XR	182	29C	3L	90	67E3
dsub18	$(GT)_{21}GC(GT)_3$	382	21C	XR	70	30A	3L	42	67E5
dsub59	$(CA)_{33}$	376	23D	XR	66	28A	3L	45	67D5
dsub69	$(TG)_6G(TG)_9$	543	24D	XR	134	23A	3L	91	66A1
dsub74	$(GA)_{13}GC(GA)_3$	662	25B	XR	382	32B	3L	59	76D4
dsub62	$(GA)_{19}$	513	29C-D	XR	183	27B	3L	53	63E5
dsub60	$(CA)_{15}$	384	31B	XR	161	33A	3R	26	92C1
dsub07	$(GA)_{11}$	456	32B	XR	116	32C	3L	80	62C2
dsub27	$(TG)_{15}TG(TG)_4$	583	33A	XR	113	42A	3L	59	78C2
dsub65	$(AG)_2AC(AG)_{14}$	553	34C-D	XR	172	24B	3L	68	69D3
dsub08	$(GA)_2GG(GA)_7GTGA$	647	35C	XR	81	36A	3L	29	73A3
dsub81	$(GT)_5$	531	35C	XR	35	36B	3R/3L[c]	_	_[b]
Chromosome O (Muller element E)									
dsub26	$(GA)_4CA(GA)_{18}AT(GA)_4$	412	75B	2	137	50C	3R	74	96F10
dsub02	$(GA)_{14}GCGA$	512	78A	2	164	45D	X	23	07B2[b]
dsub25	$(CT)_4CG(CT)_{26}TG(CT)_3$	464	78C	2	88	43C	3R	44	92A2
dsub51	$(AG)_2ACAGAT(AG)_{36}$	404	79A	2	66	46D	3L/2L/2R[c]	_	_[b]
dsub14	$(AG)_8$	480	82C	2	198	47C	3R	75	87B4
dsub47	$(CA)_{13}$	370	82D-83A	2	154	47E	3R	132	82E1
dsub38	$(TG)_{13}AA(TG)_3$	577	86E	2	80	52B	3R	82	87E12
dsub34	$(GT)_{14}$	324	88B	2	106	52D	3R	68	92E8
dsub71	$(CA)_9$	412	88C	2	223	53C	3R	130	95D10
dsub01	$(TG)_{14}$	483	90A	2	109	58C	3R	46	93F7
dsub73	$(GA)_{14}GC(GA)_3$	461	90A	2	177	58C	3R	70	93F10
dsub04	$(GT)_{15}$	550	92D	2	131	59C	3R	45	100B5
dsub29	$(GT)_{15}CGA(GT)_3$	512	96A-B	2	198	59D	3R	176	98B3
dsub12	$(CA)_{19}$	484	98B	2	49	56B	3L	30	69D2
mean ± SD		475.83±98.90			130±72			70±46	

The repeat motif of the largest microsatellite and the size of the non-repeat flanking region are given for each *D. subobscura* clone. BLAST identity (Id) is given in base pairs. [a] Chromosome bands for *Drosophila pseudoobscura* were inferred from Flybase; [b] BLAST expected value ≥0.0001; [c] BLAST gave more than one possible location with the same expected value

water, both at room temperature. Chromosomes were finally stained with 30 μL of a solution containing 1 μg of DAPI (Roche) in 50 μL of Vectashield mounting medium (Vector Lab Inc.). The probes were localized using a vertical fluorescence microscope Leica DMRB from the Serveis Científico-Tècnics of the University of Barcelona equipped with a cooled digital camera CCD Micromax of RTE 782-Y format. Hybridization signals were photographed twice, first using a blue filter for DAPI and second using a red filter for rhodamine. Thus, chromosome bands showed up in blue after approximately 300-ms exposure, and probes showed up in red and were exposed for approximately 10 s. For each microsatellite, three to ten nuclei were photographed and images overlapped using Adobe Photoshop program (Fig. 1a–e). To better assign the position of each microsatellite loci to each cytological band, chromosome images were saved in black and white and, subsequently, colors inverted to better fit the banding pattern of polytene chromosomes maps (Fig. 1f). The probes were localized by comparison to the banding pattern of the standard chromosomal inversions of *D. subobscura* established by Kunze-Mühl and Müller (1958) and modified as in Krimbas (1993). For the O chromosome, the bands were reorganized to match the O_{3+4} arrangement present in the *chcu* strain (Fig. 2).

Localization comparison between *Drosophila* species

Orthologous sequences of our microsatellite loci were searched in the genome of *D. melanogaster* (Adams et al. 2000) and *D. pseudoobscura* (Richards et al. 2005) using the UCSC genome browser (http://genome.ucsc.edu/) and Flybase (http://www.flybase.org) for information of the alignments of the *D. pseudoobscura* sequence scaffolds with their polytene chromosome maps (Schaeffer et al. 2008). Homologous sequences with expected values higher than 0.0001 were not considered for comparison between species since they could be artifactual results due to the small identity encountered. Only searches including microsatellite flanking region were considered reliable.

Data analysis

Microsatellite density was inferred by dividing the number of microsatellites located to each chromosome by the relative length of the chromosome of the Kunze-Mühl and Müller (1958) map as measured in Krimbas and Loukas (1980). A chi-square test was carried out in order to assess whether microsatellite density was the same among chromosomes, as well as between the A chromosome (sexual) and the autosomes. To evaluate the differences on the number of repeats in the clones among the five chromosomes, we used the non-parametric Kruskal–Wallis test. The number of repeats used corresponded to the largest perfect repeat given in Pascual et al. (2000). Differences in repeat length between autosomes and the sex chromosome were assessed by the Mann–Whitney *U* test. To test for differences in the proportion of AC and AG repeats between chromosomes, chi-square tests were carried out. For clones presenting more than one microsatellite loci, only one dinucleotide repeat was considered for the analysis corresponding to that having the larger number of repeats (Pascual et al. 2000). The Kolmogorov–Smirnov test was used to test whether microsatellite were uniformly distributed along each chromosome.

Results

Microsatellites localization and distribution on *D. subobscura* chromosomes

The overlapping of DAPI-stained banding pattern of chromosomes with the FISH signals of rhodamine-labeled probes enabled an accurate assignment of each probe to its specific cytological location (Fig. 1). Multiple nuclei for each locus were scored giving the same precise band location on the Kunze-Mühl and Müller (1958) *D. subobscura* map reorganized to match the O_{3+4} arrangement present in the *chcu* strain (Fig. 2). All 72 microsatellite loci were unambiguously assigned to a chromosome and precisely located in a given band (Table 1).

The relative length of each chromosome was used to compute microsatellite density (Table 2). Since no microsatellite probes were detected in the dot chromosome, we will only refer to the other five chromosomal elements. Although microsatellites appeared on all five chromosomes, their density significantly varied among them ($\chi^2 = 22.57$, $P<0.0002$). There were no differences in density between autosomes ($\chi^2 = 3.22$, $P<0.359$); however, there were clear differences between autosomes and chromosome A, the sexual chromosome, with a significantly higher density in the

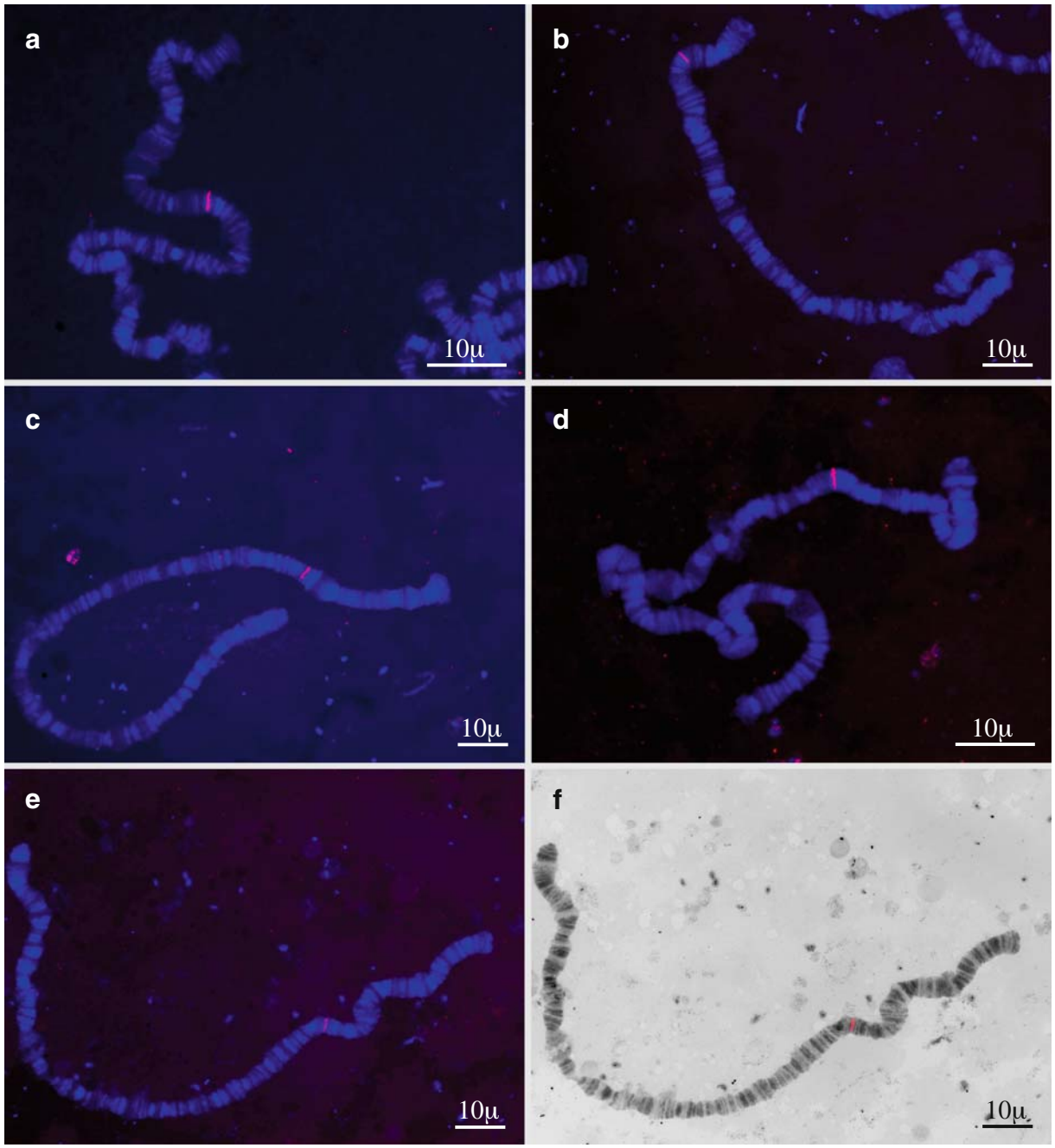

Fig. 1 Localization of microsatellite probes by fluorescence in situ hybridization to *D. subobscura* polytene chromosomes. **a** dsub54 probe hybridized to chromosome A (Muller element A). **b** dsub15 probe hybridized to chromosome U (Muller element B). **c** dsub79 probe hybridized to chromosome E (Muller element C). **d** dsub22 probe hybridized to chromosome J (Muller element D). **e** dsub12 probe hybridized to chromosome O (Muller element E). **f** The same figure as **e** treated to facilitate probe localization

latter (χ^2 = 8.92, P<0.003). Chromosome A, though the smallest one, presented 36% of the analyzed microsatellites. For all autosomes, the localized microsatellites fitted a uniform distribution, as assessed by the Kolmogorov–Smirnov test (Table 2). On the contrary, microsatellites located in chromosome A significantly deviated from that distribution (Kolmogorov–Smirnov, D=0.297, P=0.016). No differences in the repeat

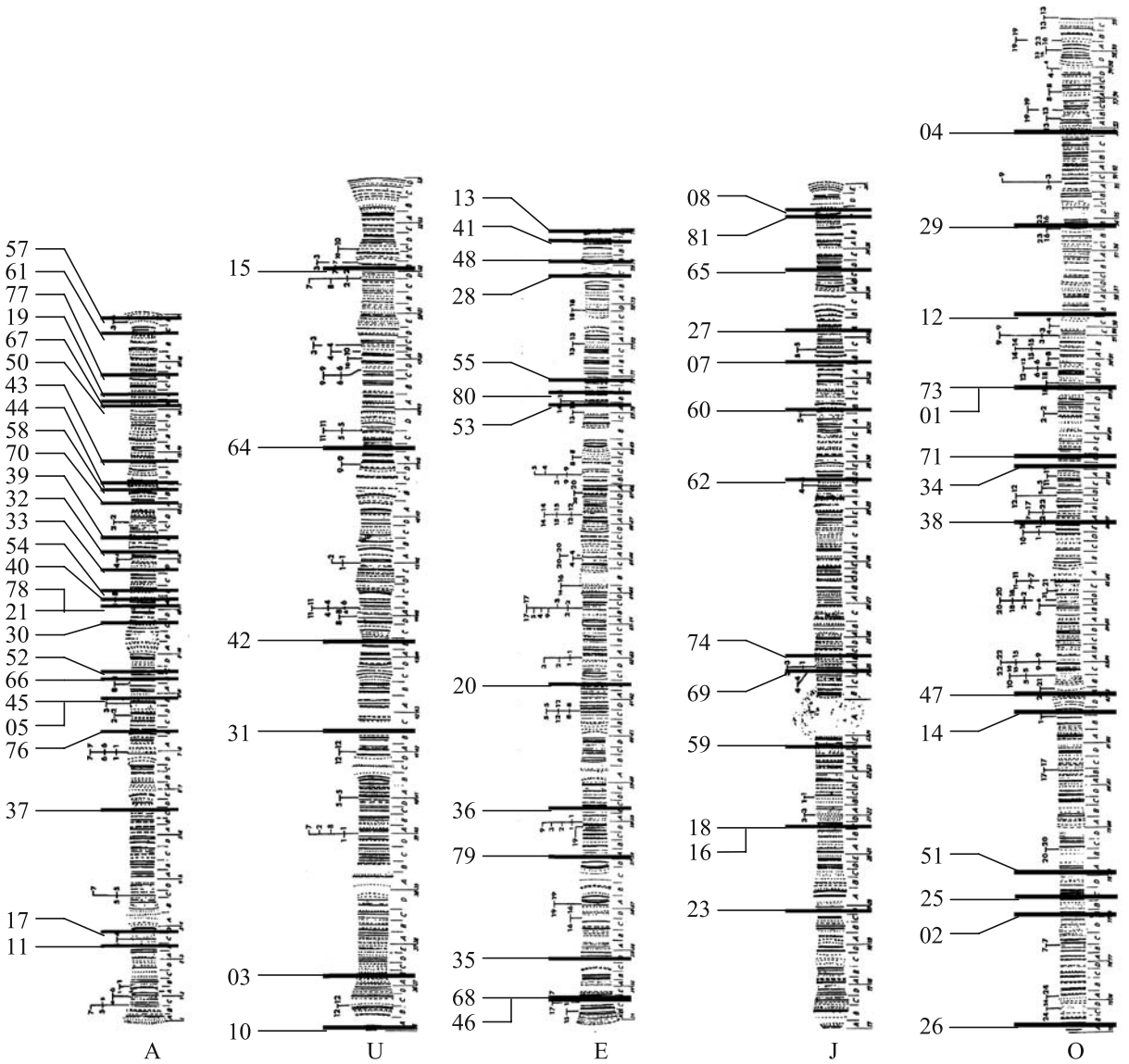

Fig. 2 Cytological location of 72 microsatellite markers of *D. subobscura* using Kunze-Mühl chromosomal maps. All chromosomes represent the standard inversion with the exception of the O chromosome rearranged to match the O_{3+4} arrangement present in the *chcu* strain. *Numbers* represent each microsatellite loci

Table 2 Percentage, relative density, and Kolmogorov–Smirnov *D* test for uniformity distribution of microsatellite *loci* in each *D. subobscura* chromosome

Chromosome	Relative length	No. of microsatellites	Microsatellite density	*D*	*P* value
A	0.17	26	0.081	0.297	0.016
E	0.19	13	0.036	0.318	0.114
J	0.20	13	0.031	0.168	0.768
O	0.24	14	0.030	0.144	0.896
U	0.20	6	0.015	0.264	0.712

length of the clone were observed among the five chromosomes as computed by the Kruskal–Wallis test (H=1.05, P=0.902) or between autosomes and the sexual chromosome as given by the Mann–Whitney test (U=544.5, P=0.629).

The frequency of microsatellites with AC repeats was significantly higher than the frequency of microsatellites with AG repeats (χ^2 = 5.62, P=0.017). Nevertheless, not all chromosomes presented the same proportion, the frequency of AC repeats being higher than AG repeats in chromosomes A, E, and U (85%, 77%, and 67%, respectively) and similar in chromosomes J and O (57% and 46%, respectively). However, significant pairwise differences, regarding the proportion of AC repeats, were only obtained when comparing the A and the J chromosome, being those showing the more extreme AC proportions (χ^2 = 6.33, P=0.012).

Microsatellite homologies in related *Drosophila* genome projects

The orthologous sequences of the *D. subobscura* 72 microsatellite loci including their flanking regions were searched in the genomes of *D. pseudoobscura* and *D. melanogaster* using the UCSC and Flybase genome browsers. The aligned segments were generally larger when compared to the *D. pseudoobscura* genome (mean = 130±72 bp) than with *D. melanogaster* (mean = 70±46 bp; see Table 1). In *D. melanogaster*, only the flanking regions were detected. On the contrary, in *D. pseudoobscura*, not only the orthologous flanking regions were larger but also the same microsatellite repeat was detected in 88.9% of the loci. Overall, 50% of the loci could be located within introns in *D. melanogaster* and 68% of the loci in *D. pseudoobscura*, the difference being due to predicted genes. All *D. subobscura* microsatellite loci were located in the same chromosomal element in *D. pseudoobscura* (Table 1 and Fig. 3). However, when searching our sequences in the *D. melanogaster* genome, nine loci were located in a different element. Nevertheless, these results were generally associated with a high expected value between the aligned sequences having thus a low probability of being true homologous sequences. Only two loci, dsub60 and dsub12, presenting low expected value and thus with a higher probability of being orthologous were localized in a different chromosomal element in *D. melanogaster*; nevertheless, the homologous detected sequence was small and the number of the aligned base pairs smaller than 30 bp (Table 1).

Discussion

Microsatellite localization and distribution

The results of the present study reveal the adequacy of FISH for localizing individual microsatellite loci on polytene chromosomes. All hybridization signals were unique, indicating high specificity and reproducibility of the technique used and at the same time proving that these microsatellite loci are suitable markers for accurate identification of individual chromosomes or specific regions within them.

In a preliminary study, a few probes had been labeled with biotin as described in Segarra and Aguadé (1992), and while single copy genes were perfectly localized, no signals were observed for microsatellite loci probes with the same length (Pascual, personal communication). Fluorescent in situ hybridization using digoxigenin-labeled DNA probes is more suitable since it comprises no danger and can be similarly efficient to radioactivity in localizing small DNA fragments or less accessible regions. Hybridizations are hard to detect in DNA regions densely packed and under-replicated as observed in intercalary heterochromatin (Belyaeva et al. 2008). Thus, high DNA packaging or under-replication of microsatellite-rich regions seems a plausible explanation for the unsuccessful hybridization with biotin.

The lack of hybridized microsatellites on the dot chromosome of *D. subobscura* is in agreement with the lower frequency of dinucleotide repeats found in that chromosomal element in relation to the other chromosomes in other *Drosophila* species (Lowenhaupt et al. 1989). However, differences among species have been reported since the dot chromosome of *Drosophila virilis* has approximately 20-fold higher level of AC repeats than that of *D. melanogaster* (Slawson et al. 2006). Immunofluorescent staining of the dot chromosome using HP1 antibody showed a banded pattern in *D. pseudoobscura* and *D. melanogaster*, indicating that this element is mostly constituted by heterochromatin, while negative hybridization in *D. virilis* supported the inference of being euchromatic (Riddle and Elgin 2006). Thus, it is more plausible that *D. subobscura*

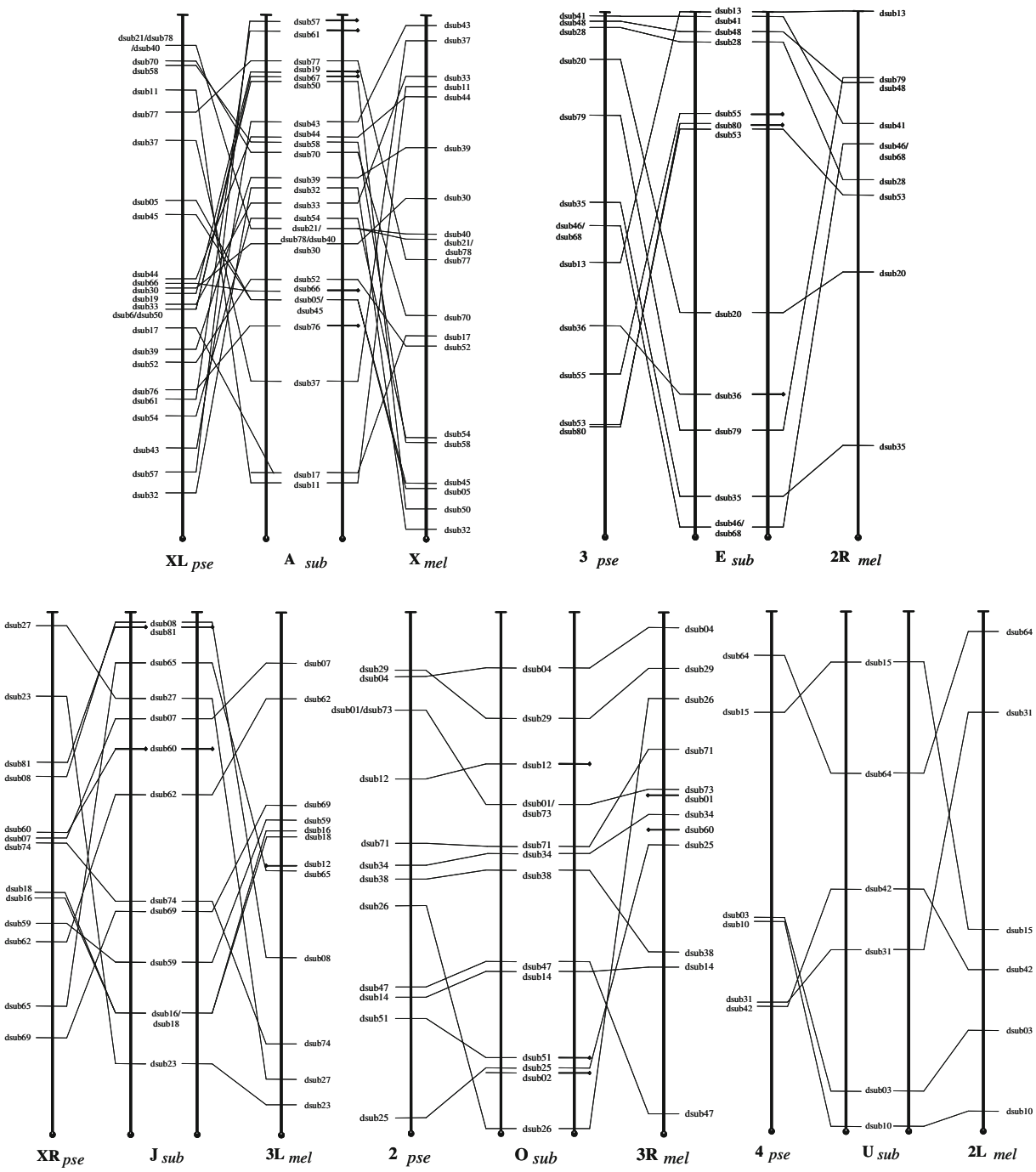

Fig. 3 Microsatellites location comparison across the homologous chromosomes of *D. subobscura*, *D. melanogaster*, and *D. pseudoobscura*. For the last two species, microsatellite position was determined by BLAST in the UCSC and Flybase servers. Each chromosome is oriented so that its centromere (*full dot*) is pointing down. The length of each chromosome is the same to facilitate across-species comparison and does not reflect size differences between chromosomes

is mostly heterochromatic and depleted of dinucleotide repeats as in *D. melanogaster* and *D. pseudoobscura*, the latter being from the same group.

In *D. subobscura*, microsatellite density is significantly higher in the sexual A chromosome when compared to the autosomes, and no bias in the location of the loci was expected from the random isolation of microsatellite loci from a genomic, non-enriched microsatellite DNA library (Pascual et al. 2000). This difference had already been observed in other *Drosophila* species (Pardue et al. 1987; Lowenhaupt et al. 1989; Bachtrog et al. 1999) with 1.5–3 times as many repeats per million base pairs of sequence in the sex chromosome than for the autosomes (Katti et al. 2001).

Moreover, *D. subobscura* microsatellites located in the sex chromosome showed a non-uniform distribution with more microsatellites being detected near the telomere (distal end), contrary to a uniform one in autosomes. These differences could be an artifact of the number of loci hybridized. Nonetheless non-random distribution of AC repeats have been observed in other studies with the highest concentration in the distal two thirds of the X chromosome of *D. melanogaster* (Pardue et al. 1987; Cuadrado and Jouve 2007b), confirming our results and suggesting some functional significance of these repetitive sequences. Abundance of repetitive DNA might be related to the higher order structures and the packaging level and expression of DNA, although there is still a great gap in the literature concerning these issues.

A general significantly higher proportion of AC than AG was found in the present work along the genome of *D. subobscura*, being the largest proportion detected in the sex chromosome as reported in other *Drosophila* species (Pardue et al. 1987; Bachtrog et al. 1999; Cuadrado and Jouve 2007b). Why these two dinucleotide repetitions (AC/TG and AG/TC), with the same proportion of each nucleotide in the double-stranded DNA and similar slippage synthesis potential (Schlötterer and Tautz 1992), show different frequency and genomic distribution has been attributed to an old genomic component conferring some type of evolutionary advantage (Cuadrado and Jouve 2007b). However, this hypothesis was not confirmed by our observations since no repeats were found in the homologous *D. subobscura* sequences observed in *D. melanogaster* (see below) in spite of the similar pattern of dinucleotide distribution in the X chromosome of both species. Consequently, independent distribution of the same elements points to convergence and suggests a functional role of microsatellite loci in the chromosomal architecture.

Faster evolutionary rate in the sex chromosome has been hypothesized to be caused by higher mutation rate, higher recombination rate, dosage compensation mechanisms, or purifying selection leading to higher accumulation of mutations in the sex chromosome. For the mutation rate hypothesis, no differences in the average levels of nucleotide diversity or different rates of amino acid evolution were observed between the sex chromosomes and autosomes in *Drosophila*, although silent and intron sites show slightly more divergence in the X chromosome (Bauer and Aquadro 1997; Thornton et al. 2006). Nonetheless, fourfold differences in evolutionary rate among chromosomal elements were reported in some *Drosophila* species, with the sex chromosome exhibiting the highest rate of rearrangement (González et al. 2002). Regarding the recombination rate hypothesis, no significant effect was observed that could explain the different microsatellite density on the sex chromosome (Bachtrog et al. 1999). In addition, no consistent effect was encountered between recombination rate and autosome evolution, although a higher average recombination rate for chromosome X was observed (González et al. 2002).

Support for the dosage compensation mechanism causing faster X evolution, which in *Drosophila* consists of an increased expression of sex-linked genes in males, comes from autosomal arms that have fused to sex chromosomes, such as in *D. pseudoobscura* and *Drosophila miranda* (Pardue et al. 1987). These chromosomes, having newly acquired dosage compensation, were also found to have an increased density of microsatellites in comparison to the homologous autosomal elements in the other *Drosophila* species. It was indicated that the X chromosome evolves differently from autosomes as a result of its hemizygosity in males, X chromosome inactivation during spermatogenesis, and sexual antagonism, supporting that selection can also be driving gene content evolution of sex chromosomes (Gurbich and Bachtrog 2008).

Purifying selection was proposed to present greater efficiency on the X chromosome than on the autosomes across the *Drosophila* phylogeny since codon usage bias is consistently greater for X-linked genes (Singh et al. 2008). Moreover, for the three *Drosophila* species

compared in the present work, it was indicated that in ancestral populations, purifying selection could explain the higher microsatellite polymorphism for X-linked loci (Kauer et al. 2002; Reiland et al. 2002; Pascual et al. 2007). As pointed out by Kauer et al. (2002), recessive deleterious mutations are purged more efficiently from the populations when located on the X chromosome. As they are removed more rapidly, there is less chance for recombination to link different neutral alleles with the deleterious mutations. Thus, the background selection model gives a good fit to the observed results since it predicts more neutral variation on the X chromosome than on the autosomes (Aquadro et al. 1994) and could thus explain the higher polymorphism and density of microsatellites located in the sex chromosome without needing to invoke that the effective population size of the X is higher than the expected three fourths of the autosomal effective population size (Vicoso et al. 2008).

Homologies between *Drosophila* species

The comparisons of the locations of the 72 *D. subobscura* dinucleotide microsatellite loci in the genomes of *D. pseudoobscura* and *D. melanogaster* showed high conservation of chromosomal elements, as previously observed with gene markers (Segarra and Aguadé 1992; Segarra et al. 1996; Papaceit et al. 2006). However, great internal shuffling by paracentric inversions had to occur inside each chromosomal element during *Drosophila* species divergence to explain for the great lack of conservation in markers position within each chromosomal element (González et al. 2002; Papaceit et al. 2006; *Drosophila* 12 Genomes Consortium 2007; Bhutkar et al. 2008). With the high level of physical rearrangement encountered in the present study (Fig. 3), we observed numerous syntenic breaks, although it would be necessary to localize a higher number of markers on each chromosomal element to detect the evolutionary breakpoints disrupting syntenic segments between species. Though highly reorganized, the wide conservation of homologous chromosomal content between species corroborates the existence of low inter-arm translocations during the evolution of the *Drosophila* genus. Only two of the 72 microsatellite loci gave indications of translocation between chromosomal arms when comparing *D. subobscura* and *D. melanogaster*. In *D. subobscura*, these two loci (dsub12 and dsub60) are found in chromosomes O and J, respectively (Table 1 and Fig. 2), and their orthologous elements are presently fused in *D. melanogaster*, producing the metacentric chromosome 3. Pericentric inversions in the *D. melanogaster* lineage would explain the exchange in their chromosomal position, as was invoked to explain an exchange of two markers between the fused 2R and 2L arms of *D. melanogaster* with respect to their location in *D. subobscura* (Papaceit et al. 2006). However, we cannot assure the occurrence of a pericentric inversion in chromosome 3 of *D. melanogaster* based on our data since in spite of the small expected value, the number of aligned base pairs was rather small, and thus, the locations could be artifactual.

The divergence time differences between *D. subobscura* and the other two species are in agreement with the amount of genetic homologies found between them since greater homologies were detected when comparing *D. subobscura* with *D. pseudoobscura* (Ramos-Onsins et al. 1998). Consequently, there is a higher probability for the *D. subobscura* microsatellite itself to be found in the *D. pseudoobscura* genome. Given that 88.9% of the *D. pseudoobscura* orthologous sequences also contained the microsatellite repeat, this would represent a divergence time of approximately 8 Mya if we apply the regression equation and evolutionary rate derived from fish (Carreras-Carbonell et al. 2005, 2008), in accordance to the divergence time estimate given by Ramos-Onsins et al. (1998). Using the same rationale, if we consider that the melanogaster group diverged from the obscura group about 30 Mya as proposed by Throckmorton (1975), no detection of polymorphism would be expected and thus the absence of the repeats. Nevertheless, the microsatellite repeat itself can be functional since the density in microsatellite loci in the distal part of the sex chromosome both in *D. subobscura* and *D. melanogaster* (Cuadrado and Jouve 2007b) seems to be convergent and not dependent on their common ancestry. Furthermore, it has been suggested that microsatellite can even serve as regulatory elements of transcription (Kashi and Soller 1999) and have been reported from expressed sequence tags (EST-SSRs; Ellis and Burke 2007) and even related to scaffold attachment regions influencing their attaching potential (Lenartowski and Goc 2002). Intronic SSRs can affect gene transcription, mRNA splicing, export to cytoplasm, or induce heterochromatin-mediated-like gene silencing (Li et al. 2004). Approximately half of

the *D. subobscura* microsatellites were localized in intronic regions, as inferred from the *D. melanogaster* annotated genome, and thus, its expansion in repeat number could be submitted to functional constraints.

In summary, with this work, we were able to assign by fluorescent in situ hybridization precise and specific locations to 72 microsatellites in *D. subobscura*. Greater microsatellite density and non-uniform distribution was observed in the sex chromosome when compared to the autosomes, which could be explained by the greater effect of purifying selection on the sex chromosome, although further assays should be carried out to clarify this issue. Great conservation of the chromosomal element content between *Drosophila* species was obtained using these non-codifying markers. Nevertheless, great shuffling within each element is necessary to explain the chromosomal location of these microsatellites in the assembled chromosomes of both *D. pseudoobscura* and *D. melanogaster*. Finally, the localization of these highly polymorphic markers will allow deeper analysis at the population level, inferring genomic recombination rates, tracking the genetic basis of chromosomal inversion adaptation, or disentangling the role of selection and drift in adaptive laboratory evolution among other future studies.

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