Multigene Family of Ribosomal DNA in *Drosophila melanogaster* Reveals Contrasting Patterns of Homogenization for IGS and ITS Spacer Regions: A Possible Mechanism to Resolve This Paradox

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ABSTRACT

The multigene family of rDNA in Drosophila reveals high levels of within-species homogeneity and between-species diversity. This pattern of mutation distribution is known as concerted evolution and is considered to be due to a variety of genomic mechanisms of turnover (*e.g.*, unequal crossing over and gene conversion) that underpin the process of molecular drive. The dynamics of spread of mutant repeats through a gene family, and ultimately through a sexual population, depends on the differences in rates of turnover within and between chromosomes. Our extensive molecular analysis of the intergenic spacer (IGS) and internal transcribed spacer (ITS) spacer regions within repetitive rDNA units, drawn from the same individuals in 10 natural populations of *Drosophila melanogaster* collected along a latitudinal cline on the east coast of Australia, indicates a relatively fast rate of *X-Y* and *X-X* interchromosomal exchanges of IGS length variants in agreement with a multilineage model of homogenization. In contrast, an *X* chromosome-restricted 24-bp deletion in the ITS spacers is indicative of the absence of *X-Y* chromosome exchanges for this region that is part of the same repetitive rDNA units. Hence, a single lineage model of homogenization, coupled to drift and/or selection, seems to be responsible for ITS concerted evolution. A single-stranded exchange mechanism is proposed to resolve this paradox, based on the role of the IGS region in meiotic pairing between *X* and *Y* chromosomes in *D. melanogaster*.

MEMBERS of multigene families typically show a much higher degree of sequence homogeneity than would be expected if they were evolving independently. This phenomenon, known as concerted evolution (Arnheim 1983), suggests the existence of genomic mechanisms of turnover capable of achieving sequence homogeneity among the various repeat units of a multigene family, whether they are located on the same or on different (homologous and nonhomologous) chromosomes (Dover 1982). Several genomic mechanisms of turnover have been proposed as responsible for the observed patterns of concerted evolution. They are involved with the nonreciprocal transfer of information between members of a family, and prominent among them are unequal crossing over and gene conversion (Dover *et al.* 1993). Collectively, the mechanisms of turnover underpin the process of molecular drive, which is the concomitant spread of new variants both through a family (homogenization) and through a sexual species (fixation) with the passing of the generations (Dover 1982). The process of molecular drive gives rise to the observed interspecific pattern of concerted evolution among redundant genetic elements.

The dynamics of spread of variant repeats through a genome (homogenization) and through a population of genomes (fixation) depends on the differences in rates of turnover within and between chromosomes (Dover 1982; for detailed analyses and simulations see Ohta and Dover 1983, 1984). For example, if unequal chromosome exchanges or gene conversion events were restricted to occur only between sister chromatids, then new mutations would be homogenized among the repeats of a single replicating chromosome; *i.e.*, a haplotypic lineage. The observation that all chromosome lineages, nevertheless, carry the same mutant repeats (concerted evolution), suggests that either selection or neutral drift, or both, have promoted the spread of one particular lineage (carrying the homogenized variant repeats) in the population at the expense of all other lineages. In such an extreme scenario, the population genetics of a multigene array can be treated somewhat, but not entirely, as that of a single gene (Dover 1982).

At the other extreme, if turnover between repeats occurs primarily between the relevant chromosomes (homologous or nonhomologous), then the population dynamics of the family as a whole has to be treated as a multilineage phenomenon. Given that measured rates of turnover are several orders of magnitude faster than the generation of variant repeats, yet still several orders slower than the rate at which sex shuffles chromosomes amongst individuals at each generation, turnover between chromosomes ensures a similar degree of homog-

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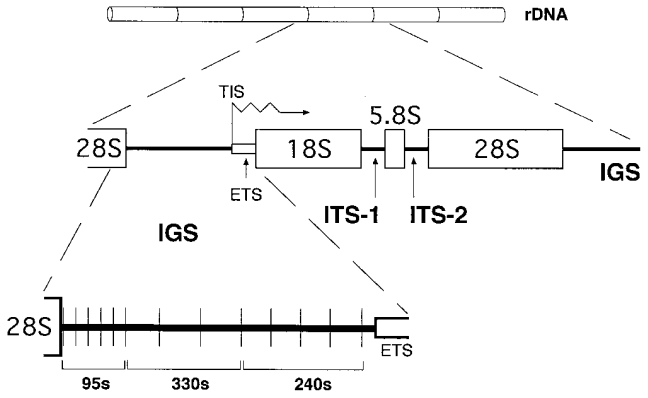

Figure 1.—Structure of ribosomal DNA (rDNA) repeat units in *D. melanogaster*. 28S, 18S, and 5.8S boxes indicate rRNA genes; IGS, intergenic spacer region; ETS, external transcribed spacer; ITS-1 and ITS-2, internal transcribed spacer regions; TIS, transcription initiation site; 95s, 330s, and 240s indicate the organization of subrepeat units (named after their size in bp) present in the IGS region.

enization amongst individuals at any given generation (Dover 1982; Ohta and Dover 1984). Hence, the potential interaction between molecular drive and natural selection, or neutral drift, depends on the extent of such genetic cohesion during a period of homogenization, which, in turn, depends on the differences in rates between intra- and interchromosome exchanges.

In order to understand the evolutionary dynamics of multigene families, it is important to assess the types, locations, rates, and biases of any mechanisms of turnover, and whether or not the family in question can be essentially monitored as a multi- or single-lineage progression.

The ribosomal DNA (rDNA) gene family of *Drosophila melanogaster* offers an excellent system for the study of gene family evolution on different chromosomes because it consists of ~250 tandem repeat units located in the nucleolar organizers of both *X* and *Y* chromosomes (Ritossa 1976). Each unit comprises the coding sequences for the 18S, 5.8S, and 28S rRNAs, as well as spacer sequences between them (see Figure 1).

Most studies on homogenization processes in Drosophila rDNA have analyzed the intergenic spacer IGS (formally called the "nontranscribed spacer") located between 28S and 18S genes (Coen *et al.* 1982a,b; Coen and Dover 1983; Tautz *et al.* 1987; Williams *et al.* 1987; for review see Williams and Robbins 1992 and Dover *et al.* 1993). The IGS is composed of different types and numbers of small, tandemly arrayed subrepeat units giving rise to IGS length polymorphism (Figure 1). The distribution of IGS length variants shared between rDNA arrays of *X* and *Y* chromosomes, and direct observations of unequal exchanges (Coen and Dover 1983; Gillings *et al.* 1987), showed that interchromosomal events were participating in the evolution of this region. Unequal crossing over both at the level of the subrepeat sequences within the IGS and at the level of the complete rDNA units is thought to be the main mechanism leading to the concerted evolution pattern of Drosophila rDNA (Dover *et al.* 1993).

Previous data obtained from the IGS spacer region suggest a multilineage model for *D. melanogaster* rDNA because variants are shared by arrays located on different *X* and *Y* chromosomes, which is suggestive of a simultaneous homogenization process among both types of chromosome (for references see Dover *et al.* 1993). However, in contrast to the situation of the IGS, Schlötterer and Tautz (1994) reported the presence of chromosomal rDNA arrays homogenized for different ITS variants in interbreeding populations of *D. melanogaster*. The ITS contains within it the 5.8S gene and separates the 18S from the 28S gene (see Figure 1). These authors proposed that intrachromosomal exchanges (unequal crossing over or gene conversion between sister chromatids) drive concerted evolution because the chromosome-specific homogenization of ITS mutations suggests an evolutionary process along separate chromosomal lineages. Individual lineages would then be fixed by selection or drift. For a discussion of the various parameters affecting single-lineage *vs.* multilineage homogenization processes, see Schlötterer and Tautz (1994), and Dover (1989, 1994).

To evaluate the two different models, we have analyzed for the first time both IGS and ITS regions in the same individual flies drawn from natural populations of *D. melanogaster*, using PCR. This technique allows us to analyze both regions from the same individuals, because only small amounts of DNA are required. The high levels of variation detected in both IGS length and copy number between males and females indicate relatively fast rates of interchromosomal exchanges. On the other hand, the absence in *Y* chromosomes and some *X* chromosomes of an ITS mutation, which is apparently almost fully homogenized in other *X* chromosome arrays of the same inbreeding populations, indicates an haplotypic evolution of *D. melanogaster* rDNA units.

Here, we suggest a mechanism to explain the contrasting evolutionary progressions that are occurring in the two spacer regions of the same rDNA units, taking into account the new discoveries on the role of the IGS region in achiasmatic meiotic pairing between *X* and *Y* chromosomes in *D. melanogaster*.

MATERIAL AND METHODS

Fly stocks: Natural populations of *D. melanogaster* collected along a 4000-km latitudinal cline down the east coast of Australia at the beginning of 1993 were a gift of Professor L. Partridge (University College London, UK). Immediately after collection, the flies were subdivided into isofemale lines and maintained at 18° on oatmeal and treacle medium. They were routinely transferred to fresh bottles at 3-wk intervals. A total of 48 isofemale lines from 10 populations located ~400 km apart (see Figure 2) have been analyzed. DNA was extracted

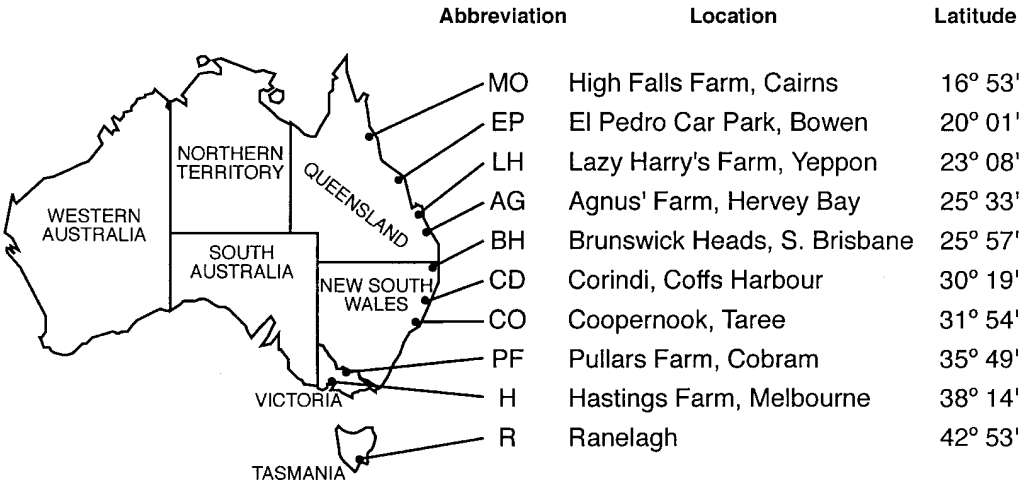

Figure 2.—Geographical location of the 10 populations from eastern Australia which were analyzed for ITS and IGS variability.

from five individual males and five individual females for each of the isofemale lines studied.

DNA extraction and PCR amplification: Genomic DNA was extracted from single flies following the method of Ashburner (1989), with the exception that flies were homogenized directly in the lysis buffer.

Complete IGS were amplified using a forward primer located in the 3′ end of the 28S rDNA gene, IGS-F (AATGGATG TGATGCCAATGTA), and a reverse primer located within the external transcribed spacer (ETS) region, ETS-R (GAGC CAAGTCCCGTGTTC). ~0.5 ng of DNA was added to 50 μl of amplification mix (73 mm Tris-HCl pH 9.0; 20 mm $(NH_4)_2SO_4$; 0.1% Tween-20; 1.4 mm $MgCl_2$), which contained 10 pmol of each primer and one unit of *Taq* polymerase (Advanced Biotechnologies, Surrey, UK). The thermocycling profile consisted of a previous incubation at 94° for 3 min, plus 30 cycles with 30 sec at 94° and 10 min at 60° that was performed in a GenAmp PCR System 9600 (Perkin Elmer, Norwalk, CT). The amplified products were separated in 21-cm-long 1% agarose gels, visualized by ethidium bromide staining and photographed in Tmax 100 4 × 5 film (Eastman Kodak Co., Rochester, NY). A mixture of *Hin*dIII-digested λ DNA and *Hae*III-digested θX174 DNA fragments were used as molecular markers.

A primer located in the 3′ end of 18S rDNA gene, ITS-F (ACAAGGTTTCCGTAGGTGA), and a primer located in the central region of the ITS-1 (ITS which lay between 18S and 5.8S genes), ITS-R (ATGCCCATAACTAAGATGA), were used to detect the polymorphic 24-bp deletion described in ITS-1 of *D. melanogaster* by Schlötterer and Tautz (1994). Amplification was achieved using the same conditions as above but the thermocycling program consisted of 30 cycles with 30 sec at 94°, 30 sec at 60°, and 30 sec at 72°, respectively, and the reaction volume was 10 μl. The reaction products were electrophoresed in 2.5% Nusieve agarose (FMC, Rockland, ME), using *Pst*I-digested λ DNA as a molecular marker. All primers were synthesized at the Protein and Nucleic Acids Chemistry Laboratory facility at the University of Leicester.

ITS-PCR products were cloned using the pGEM-T vector system (Promega, Madison, WI) following manufacturer's instructions and sequenced using the method of Sanger *et al.* (1977).

IGS length variants identification and comparison: Phenotypic IGS profiles were obtained for individual males and females after identification of IGS length variants by direct comparison of PCR products present in the same gels, and by comparison of densitometric graphics obtained from scanned gel film negatives using NIH Image software (National Institutes of Health, USA). Males from line CD4 were used as internal markers in all the gels, and all the individual male and female IGS profiles were analyzed using at least two gels where different samples (~20) were loaded to facilitate identification of IGS variants. Number, position, and relative intensity of PCR products were reproducible for each sample.

A code of one letter and two numbers was assigned to each variant according to their relative gel migration between molecular markers. Series A corresponds to variants longer than 4361 bp; series B includes variants from 4361 to 2322 bp; series C from 2322 to 2027 bp; series D from 2027 to 1353 bp; series E from 1353 to 1078 bp; and series F corresponds to variants shorter than 1078 bp. Numeration inside the series was assigned by relative migration of the variants between the limits of a series in a scale from 0 to 99, where the smaller figures indicate the largest variants (see Figure 3).

Similarities between phenotypic profiles were estimated using the Jaccard (1901) coefficient, and the resulting pairwise similarities were expressed as a distance matrix. Present bands were encoded as 11111, 11110 or 11100 according to high, medium or low intensity, and absent bands were encoded as 00000. Relative intensity was established by densitometry analysis using NIH Image software. Band-sharing coefficients between IGS profiles were calculated using the Kulczynski (1927) expression:

$$x = 0.5\,[(Nab/Na) + (Nab/Nb)],$$

where *Na* and *Nb* are the number of present bands in profile *a* and *b*, respectively, and *Nab* is the number of bands shared by both profiles (Jeffreys *et al.* 1985).

RESULTS

The chromosomal distribution of IGS variability: A total number of 59 IGS length variants with sizes between 4.3 and 0.9 kb have been identified in the 10 Australian populations. Amplification of larger variants does not appear possible using standard PCR protocols. Although small variants can be amplified preferentially by PCR, there was no correlation between the length of each variant and its intensity. The reproducibility of band number, position, and relative intensity confirms that PCR-IGS band patterns do not depend as much upon the length of the variants as upon the relative

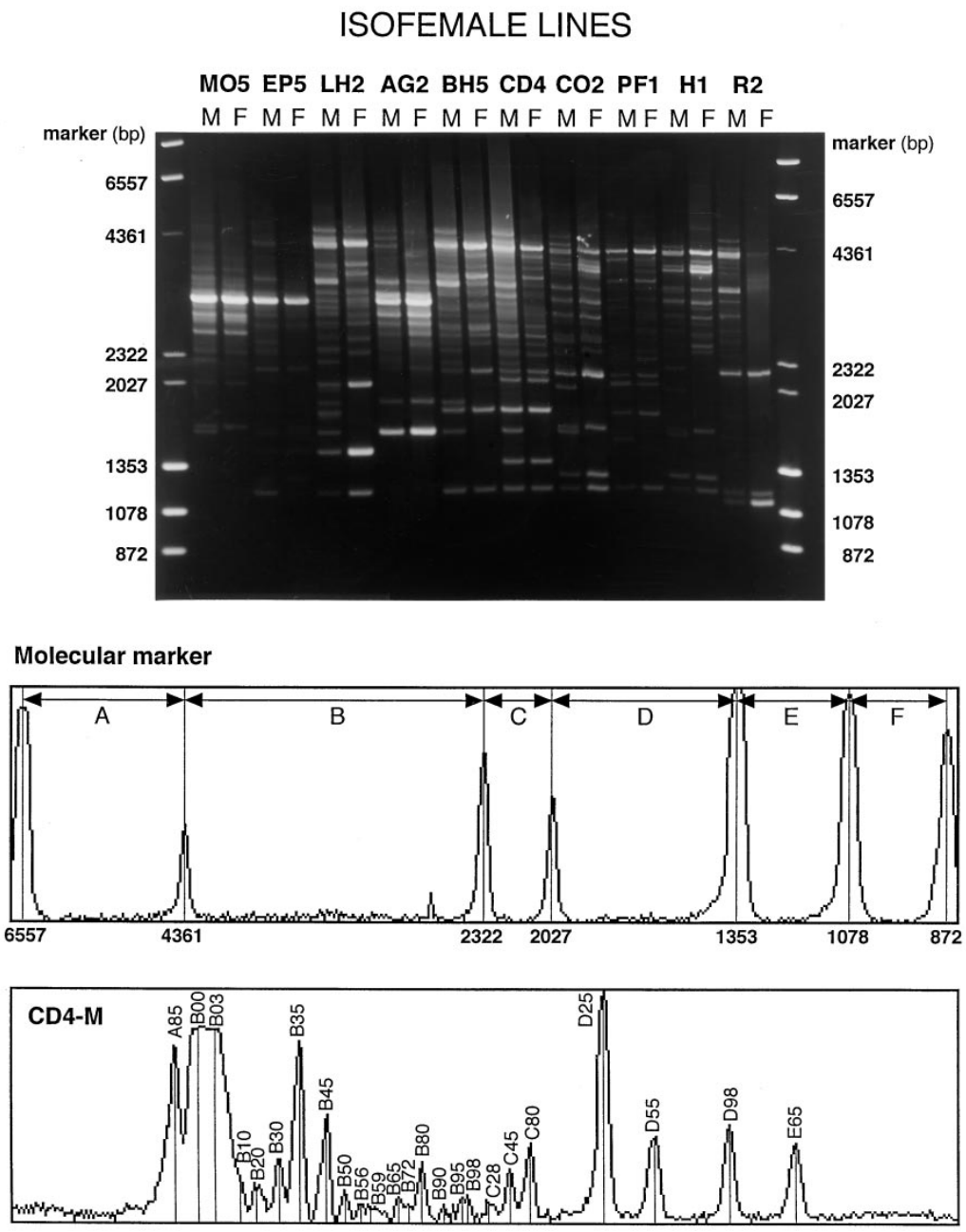

Figure 3.—Amplification of IGS regions using IGS-F and ETS-R primers and genomic DNA extracted from individual males (M) and females (F) of selected isofemale lines from each of the populations analyzed. Comparison and identification of IGS length variants was facilitated by the use of densitometry plots as shown in the bottom part of the figure: males from line CD4 were used as internal markers, and relative gel migration between known molecular markers was used to assign codes to the IGS length variants (see materials and methods).

number of original copies of each variant, as indicated in previous studies (Ruiz Linares *et al.* 1994; Bowen and Dover 1995; Polanco and Pérez de la Vega 1997).

Variability in both presence-absence and copy number of length variants has been found not only between isofemale lines (see Figure 3), but also within them. Variability within an isofemale line can be expected if the founder female was heterozygous for rDNA loci and/or the mating male carried an rDNA array in its *X* chromosome that was different to the two *X* arrays carried by the female. However, 21 isofemale lines (44%) showed no within-line variability between both male and female IGS profiles. This result suggests that either the mating male carried the same *X* chromosome rDNA array as the homozygous founder female or, more probably, that one of the *X* chromosomes present in the isofemale line has been fixed by drift during the ~50 generations of time between fly collection and DNA extraction. Such isofemale lines with, apparently, only one *X* and one *Y* chromosome were found in all the populations.

The analysis of distribution of IGS length variants between populations revealed that 20 variants (33.9%) were present in all populations, and another 10 variants were missing in no more than two populations, giving a total of 30 (50.8%) highly shared variants along the Australian east coast populations.

TABLE 1

Frequencies in male profiles of IGS length variants which were present as male-specific in at least one population

IGS length variant	Population									
	MO	EP	LH	AG	BH	CD	CO	PF	H	R
A85	*71.4*	*33.3*	42.9	*50.0*	*100.0*	*88.9*	*88.9*	16.7	*60.0*	*100.0*
B00	*85.7*	50.0	57.1	*62.5*	*100.0*	77.8	77.8	16.7	*80.0*	*100.0*
B04	*71.4*	*50.0*	57.1	75.0	*40.0*	55.6	100.0	16.7	*80.0*	100.0
B15	—	*33.3*	57.1	—	80.0	—	—	33.3	80.0	66.7
B25	28.6	*16.7*	42.9	37.5	20.0	22.2	77.8	16.7	80.0	16.7
B48	—	—	14.3	—	—	—	—	—	20.0	*50.0*
B50	100.0	100.0	57.1	87.5	80.0	100.0	100.0	33.3	*20.0*	—
B82	*28.6*	50.0	28.6	87.5	*80.0*	44.4	*77.8*	33.3	80.0	*100.0*
B89	—	*16.7*	57.1	37.5	60.0	—	44.4	33.3	*20.0*	—
B95	85.7	66.7	57.1	*62.5*	100.0	66.7	88.9	33.3	*100.0*	*100.0*
C00	*14.3*	33.3	28.6	12.5	—	*22.2*	—	—	—	—
C28	100.0	66.7	42.9	100.0	100.0	55.6	100.0	50.0	*80.0*	*100.0*
C30	28.6	66.7	28.6	12.5	20.0	*11.1*	33.3	50.0	20.0	100.0
C65	—	—	—	12.5	20.0	*11.1*	—	—	—	—
C80	14.3	50.0	57.1	—	*60.0*	33.3	*22.2*	66.7	20.0	—
D00	42.9	—	28.6	—	—	—	*11.1*	*16.7*	*80.0*	—
D04	14.3	16.7	*14.3*	37.5	—	—	—	*33.3*	—	—
D05	—	—	—	—	—	—	*22.2*	50.0	—	66.7
D15	28.6	16.7	14.3	50.0	60.0	33.3	33.3	—	—	*66.7*
D44	14.3	—	*14.3*	12.5	—	—	—	—	—	—
D45	—	*16.7*	*14.3*	—	—	—	—	—	—	—
D47	—	—	*14.3*	12.5	—	—	—	—	—	—
D55	85.7	83.3	*42.9*	100.0	100.0	88.9	100.0	16.7	*60.0*	*100.0*
D60	—	—	—	—	—	—	11.1	*16.7*	—	—
D70	—	—	*14.3*	—	—	—	—	—	—	—
D95	—	—	—	*25.0*	—	—	—	—	*60.0*	16.7

Frequencies in italics indicate male-specificity in the above population. Only two IGS length variants (in italics) remain as male-specific when all the male and female profiles from all the populations are compared. —, absence of the IGS variant in the above population.

Paucity of Y-specific IGS variants: The comparison between male and female profiles at the level of populations revealed that 26 (44%) of the IGS length variants were male-specific in at least one population (Table 1), and the percentage of male-specific variants in the populations ranged from 7.7 to 29.4% (Table 2). However, 24 of these 26 variants were also present in female profiles from other populations, leaving only two variants (D45 and D70 in Table 1) as *Y* chromosome-specific across all the populations.

Average band-sharing coefficients at the populational level were always higher for male than female profiles (except for population PF), as also were the average values obtained between all the populations (Table 3). The same *X* chromosomes responsible for female variability are present in the males, indicating that there is a higher similarity between *Y*-linked rDNA arrays than between *X*-linked rDNA arrays in nearly all the populations when the IGS regions were compared.

Geographical distribution of IGS profiles: The 142 different IGS profiles identified in males and females from the 48 isofemale lines were compared using the Jaccard coefficient and unweighted pair-group method (UPGMA) for clustering (Figure 4I). Two subgroups, A and B in Figure 4I, were found in which all the profiles, except five female and two male profiles (subgroup C in Figure 4I), were included. All the profiles from the three southern populations (R, H, and PF) were located in subgroup A, while the majority of profiles from the two northern populations (MO and EP) were included in subgroup B. Profiles from central Australian populations (LH, AG, BH, CD, and CO) were distributed in both subgroups. A similar North-South grouping can be seen when only female profiles are compared (Figure 4III), but no such grouping could be formed when only male profiles were compared (Figure 4II).

This difference between male and female profile distributions could be due to the different sample size for *X* and *Y* chromosomes. Accordingly, the comparative analysis was repeated using only male and female profiles obtained from the 21 lines, where only one *X* and one *Y* chromosome was responsible for the single female and male profiles present in these isofemale lines. Again, analysis of male and female profiles together, as

TABLE 2

Percentage of male-specific IGS length variants per population

Population	Number of male-specific variants	Total variants in male profiles	Percentage
MO	5	35	14.3
EP	6	38	15.8
LH	6	47	12.8
AG	4	40	10.0
BH	5	40	12.5
CD	3	38	7.9
CO	5	38	13.2
PF	3	39	7.7
H	10	34	29.4
R	8	32	25.0

TABLE 3

Average band-sharing coefficients for male and female profiles

	Band sharing	
Population	Males	Females
MO	0.704	0.579
EP	0.530	0.497
LH	0.458	0.456
AG	0.584	0.487
BH	0.651	0.569
CD	0.586	0.519
CO	0.662	0.607
PF	0.517	0.625
H	0.700	0.681
R	0.821	0.682
[a]Australian east coast	0.621	0.570

[a]Average for the 10 populations analyzed.

well as female profiles alone, produced phenograms in which the North and South subgroups could be observed, but these two subgroups could not be obtained when male profiles were compared alone (data not shown).

Is the distinction between group A and B distributions due to the presence of specific length variants unique to North or South populations, or is it due to specific profiles representing different combinations of length variants in each rDNA array? In the latter possibility, the individual length variants are extensively shared across the populations but the profiles (*i.e.*, the combination of length variants in a single chromosomal array) are unique and population-specific.

The comparison between female IGS profiles included in subgroups A (South) and B (North) from Figure 4III, revealed the presence of 41 shared IGS variants; 10 specific variants in the South subgroup, and four specific variants in the North subgroup (Table 4). From the 41 shared variants, only nine showed significant differences in frequency between South and North subgroups. The frequencies of the 14 subgroup-exclusive variants were lower than 20% in their corresponding subgroup, with the exception of one variant (B15), which was present in more than half of the profiles (54.8%) included in the South subgroup.

The low frequencies of the specific variants and the similar frequencies of the majority of IGS variants between the North and South profiles indicate that the geographical differences are due to different combinations from the same pool of shared length variants. This suggests frequent swopping of length variants between rDNA arrays with relatively little intra-array homogenization of specific length variants at the population level.

ITS variability: PCR amplification of a region of ITS-1 using primers ITS-F and ITS-R revealed the presence, in the same set of individuals drawn from the same Australian populations, of a polymorphic 24-bp deletion described previously by Schlötterer and Tautz (1994). Two different PCR products, with an ~20-bp difference, estimated by gel migration, were obtained (see Figure 5). Cloning and sequencing of the products from one of the isofemale lines confirmed the large product as the expected 481-bp fragment according to the published sequence from Oregon-R strain. The smaller PCR product was 457-bp long due to a 24-bp deletion in Oregon-R sequence, and was located in the same position indicated by Schlötterer and Tautz (1994).

The chromosomal distribution of these two ITS variants is indicated in Table 5 as deduced by the analysis of phenotypes obtained from five males and five females from each of the isofemale lines, as well as the analysis of F_1 males from crosses between Oregon-R females and males from the appropriate Australian lines.

The 24-bp deletion was not found in 17 (35%) isofemale lines (Figure 5A), including all the five lines from the Tasmanian population R, the four lines from the southeast Australian population H, four out of five lines from the neighboring population PF, two lines from CD, and one line each from BH and the northeast population MO. The shorter PCR product, corresponding to the 24-bp deletion, was found in all the other 31 isofemale lines analyzed.

In 13 isofemale lines, all the female flies showed only the 24-bp deletion PCR product, while all the corresponding males showed both the 481- and 457-bp PCR products (Figure 5B). Males from these lines were crossed with Oregon-R females, and the F_1 males were analyzed in order to locate the chromosomal origin of the 24-bp deletion. All the F_1 males of these crosses showed only the 481-bp product (Figure 5E). Therefore, in these 13 isofemale lines, the ITS regions carrying the 24-bp deletion are only present in the *X*-linked rDNA arrays. Such arrays are apparently almost fully homogenized for

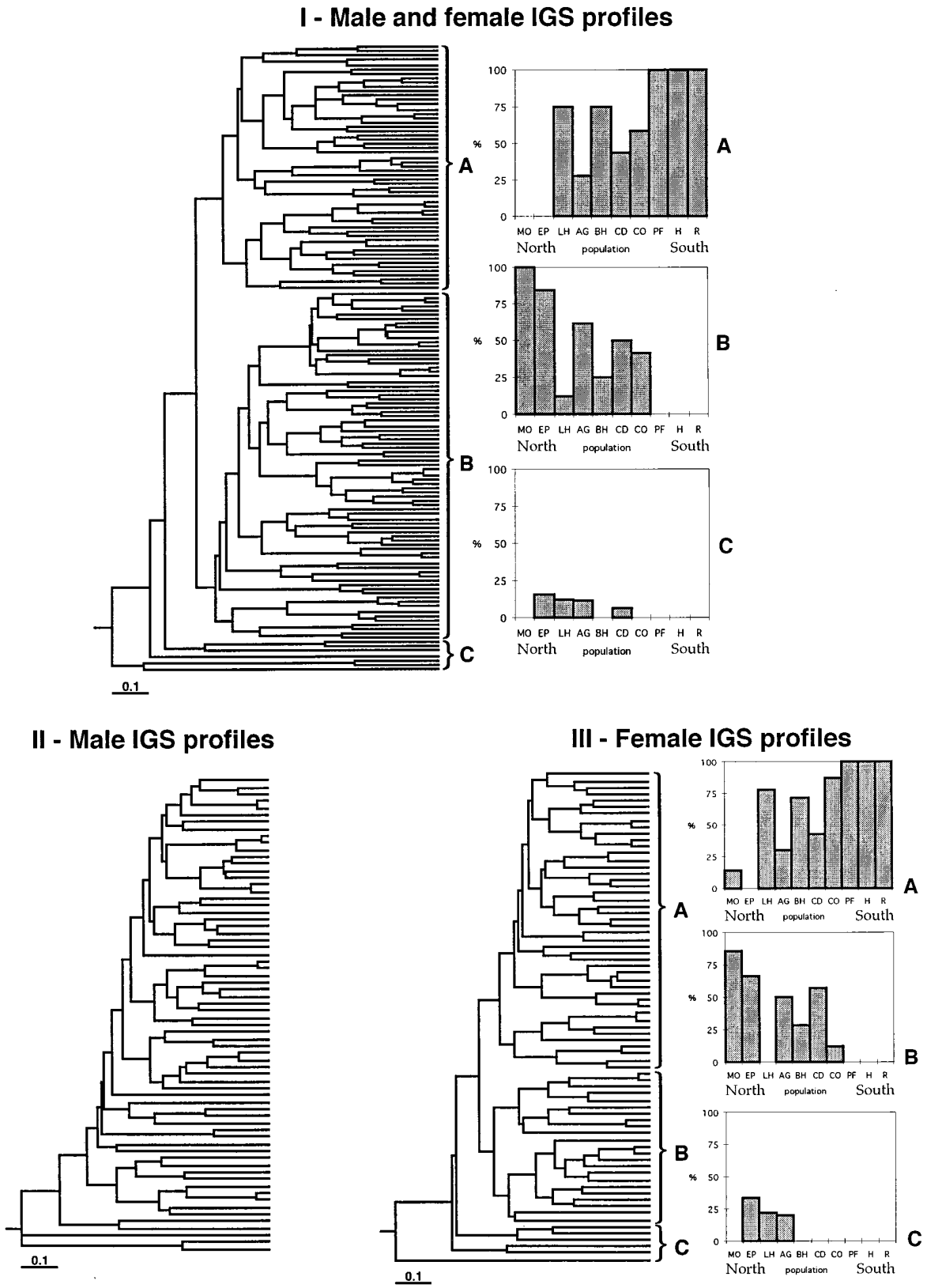

Figure 4.—Phenograms obtained using the UPGMA method and Jaccard similarity for the total 142 different IGS profiles identified in males and females (I), for the 68 male profiles (II), and for the 74 profiles corresponding to females (III). Histograms indicate the population distribution of profiles when subgroups A, B, and C are present.

TABLE 4

Frequencies of IGS length variants in female profiles included in A (South) and B (North) subgroups from Figure 4III

IGS Variant	Subgroup A	Subgroup B	IGS variant	Subgroup A	Subgroup B	IGS variant	Subgroup A	Subgroup B
A85	14.3	0s	B62	61.9	34.6	D05	9.5	0s
B00	16.7	7.7	B65	73.8	88.5	D10	0	15.4n
B03	88.1	30.8*	B66	61.9	57.7	D15	11.9	46.2*
B04	19.0	7.7	B72	45.2	7.7*	D25	28.6	23.1
B06	4.8	0s	B80	64.3	57.7	D44	0	3.8n
B10	78.6	15.4*	B82	9.5	26.9	D50	31.0	50.0
B15	54.8	0s	B85	9.5	0s	D55	7.1	65.4*
B18	64.3	3.8*	B89	23.8	34.6	D60	2.4	7.7
B20	42.9	11.5*	B90	38.1	57.7	D75	11.9	38.5
B25	38.1	15.4	B95	14.3	26.9	D95	2.4	0s
B30	45.2	38.5	B98	45.2	42.3	D98	14.3	0s
B35	92.9	88.5	C00	2.4	11.5	E15	4.8	0s
B40	21.4	38.5	C28	21.4	30.8	E35	81.0	61.5
B45	85.7	57.7	C30	38.1	26.9	E50	4.8	11.5
B48	11.9	0s	C45	31.0	26.9	E65	92.9	19.2*
B50	52.4	100.0*	C65	4.8	3.8	E85	14.3	0s
B54	21.4	23.1	C80	14.3	11.5	F95	0	3.9n
B56	66.7	73.1	D00	7.1	15.4			
B59	69.0	73.1	D04	0	19.2n			

*, significant difference between subgroup frequencies using the Sokal and Rohlf (1969) percentage similarity test; s, A (South) subgroup exclusive variant; n, B (North) subgroup exclusive variant.

this variant, while it is not present in the *Y* chromosome arrays of the same populations. These *X* chromosomes, nearly fully homogenized for the 24-bp deletion, were found in isofemale lines of northern and central populations: four lines from MO, three lines from EP, one line from LH, two lines from AG, and three lines from CD.

The phenotype composed of both PCR products (481 and 457 bp; Figure 6C) was found in all males and females analyzed from nine isofemale lines from populations CO (five lines), BH (three lines), and EP (one line). Analysis of F_1 males from crosses between Oregon-R females and males from these nine lines revealed that the 24-bp deletion was not present in *Y* chromosome rDNA arrays (Figure 5E), and that both ITS variants were located together in the *X* chromosome arrays of these lines.

The polymorphic phenotypes found in males and females from four lines (three from population LH and one from PF) indicated the presence of two types of *X*-linked arrays: one of them carrying only the 481-bp variant and the other carrying both 481- and 457-bp variants. Another three lines (two from AG and one from LH) showed a similar situation of arrays carrying both variants, as well as arrays carrying only the 457-bp variant (Table 5). Additionally, in two lines from populations EP (line 3) and AG (line 2; Figure 5D), *X*-linked arrays were found apparently fully homogenized for each of the ITS variants.

In summary (see Table 5), the 24-bp deletion was found in *X*-linked rDNA arrays from eight populations and was missing in the two southern populations H and R; and *X*-linked arrays have been identified carrying different homogenized ITS variants in five of those populations, as well as *X*-linked arrays composed of mixtures of the deleted and nondeleted forms.

IGS and ITS profile comparisons: As the same fly samples were used for ITS and IGS analysis, it has been possible to directly compare the distribution variability for both spacer regions for the first time. IGS female profiles corresponding to single-banded ITS phenotypes were grouped according to the presence of the 24-bp deletion variant of 457 bp (21 profiles) or to the presence of the 481-bp product (27 profiles) in the ITS region. A comparison of IGS length variants between these two groups revealed the presence of six and nine variants specific for each group, respectively, and 37 shared variants (Table 6). The group-specific IGS variants were present in only 1–6 IGS profiles, except one variant (B18), which was present in 16 (59.3%) profiles associated with the nondeleted phenotype of the ITS region. All other frequent IGS variants (present in $>$50% of the profiles) were found both in profiles corresponding to *X* chromosome rDNA arrays homogenized for the deleted variant and in profiles corresponding to *X* chromosome arrays homogenized for the nondeleted variant.

Comparisons of IGS length variants between male and female IGS profiles (23 and 21 profiles, respectively) from the 13 isofemale lines, in which *X* and *Y* chromosome rDNA arrays are apparently fully homoge-

nized for different ITS variants, revealed the presence of only eight male-specific IGS variants. Two of them were present in 60% of the male profiles but the other six were present in ≤20% of the male IGS profiles.

In total, these results indicate that the distinction which exists between *X* and *Y* chromosomes with respect to the ITS regions does not exist with respect to the IGS regions of the same set of rDNA arrays.

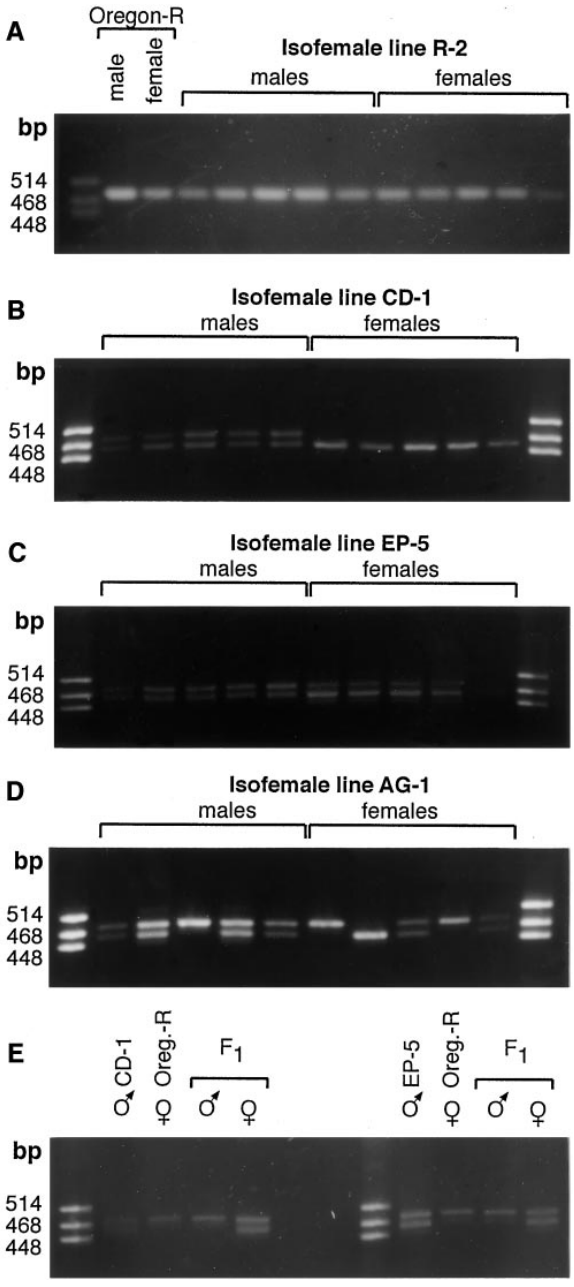

Figure 5.—Agarose gel electrophoresis of PCR products amplified using ITS-F and ITS-R primers. (A) Example of isofemale line where only the 481-bp variant (nondeletion variant as in Oregon-R) was found. (B) Example of isofemale line where females only showed the 457-bp variant (24-bp deletion variant), while males showed both 481- and 457-bp variants. (C) Example of isofemale line where both males and females showed both ITS variants. (D) Example of isofemale line where females showed homogenization of *X* chromosomes for different ITS variants. (E) Parental and F_1 males and females from crosses between CD-1 males (B) and Oregon-R females.

DISCUSSION

How is within-species homogeneity between member genes of multigene families achieved? The roles of a variety of genomic mechanisms of turnover in spreading an initially rare variant gene through a gene family are well documented (Dover 1982; Dover *et al.* 1993). Gene families do not exist, however, on one chromosome in one individual. The dynamics of homogenization is a problem in population genetics; *i.e.*, an explanation is required to explain how all individual members of a family acquire high levels of sequence similarity. One solution to the problem is to assume that a mutant member gene spreads only throughout its chromosome of origin and that selection and/or drift fix that particular chromosome in the population (Kimura and Ohta 1979). This is known as the "double-diffusion" process when neutral drift is evoked at the population level and stochastic gain-and-loss (*e.g.*, by unequal crossing over or unbiased gene conversion) is evoked at the genomic level. Another solution is to invoke nonreciprocal exchanges between homologous (or, where necessary, nonhomologous) chromosomes such that variant genes can spread between chromosomes, which, through the process of sex, subsequently separate and enter new individuals at the next generation in all of which interchromosome exchanges may proceed. This latter process of concomitant spread through a genome (homogenization) and through a population (fixation) is called "molecular drive" (Dover 1982).

Double-diffusion is essentially a single-lineage process; molecular drive is essentially a multilineage process. Given the variety of turnover mechanisms in eukaryotic genomes, each with different rates, bias, units, and locations of operation (depending on the gene family and on the species) often operating one on top of another (Dover 1993), it is not surprising to observe that each redundant set of sequences follows its own evolutionary trajectory.

A popular focus for attempts to understand the forces at play in multigene families is the rDNA of many genera, in particular in Drosophila. Early data showed that unequal crossing over occurs between *X* and *Y* rDNA arrays in *D. melanogaster* (Coen and Dover 1983), and that the existence of species-specific mutations in the IGS, which were shared by all *X* and *Y* chromosomes, also supported the operation of *X*/*Y* exchanges (Coen *et al.* 1982a,b). However, more recently, Schlötterer and Tautz (1994) provided convincing evidence that, unlike the IGS, the ITS region evolves along haplotypic lineages, suggesting that selection or drift must be responsible for the eventual fixation of a newly homogenized array at the population level.

In order to examine this seeming paradox between

TABLE 5

Chromosomal distribution of 481- and 457-bp ITS-1 variants

	Y chromosome phenotype			*X* chromosome phenotype		
	Only 481 bp	481 bp and 457 bp	Only 457 bp	Only 481 bp	481 bp and 457 bp	Only 457 bp
Population MO						
MO-1	+	−	−	−	−	+
MO-2	+	−	−	−	−	+
MO-3	+	−	−	−	−	+
MO-4	+	−	−	−	−	+
MO-5	+	−	−	+	−	−
Population EP						
EP-1	+	−	−	−	+	−
EP-2	+	−	−	−	−	+
EP-3	+	−	−	+		+
EP-4	+	−	−	−	−	+
EP-5				−	−	+
Population LH						
LH-1	+	−	−	−	+	+
LH-2	+	−	−	+	+	−
LH-3	+	−	−	+	+	−
LH-4	+	−	−	+	+	−
LH-5	+	−	−	−	−	+
Population AG						
AG-1	+	−	−	−	−	+
AG-2	+	−	−	+	−	+
AG-3	+	−	−	−	+	+
AG-4	+	−	−	−	−	+
AG-5	+	−	−	−	+	+
Population BH						
BH-1	+	−	−	+	−	−
BH-2	+	−	−	−	+	−
BH-3	+	−	−	−	+	−
BH-4	+	−	−	−	+	−
Population CD						
CD-1	+	−	−	−	−	+
CD-2	+	−	−	−	−	+
CD-3	+	−	−	+	−	−
CD-4	+	−	−	−	−	+
CD-5	+	−	−	+	−	−
Population CO						
CO-1	+	−	−	−	+	−
CO-2	+	−	−	−	+	−
CO-3	+	−	−	−	+	−
CO-4	+	−	−	−	+	−
CO-5	+	−	−	−	+	−
Population PF						
PF-1	+	−	−	+	−	−
PF-2	+	−	−	+	−	−
PF-3	+	−	−	+	−	−
PF-4	+	−	−	+	+	−
PF-5	+	−	−	+	−	−
Population H						
H-1	+	−	−	+	−	−
H-2	+	−	−	+	−	−
H-3	+	−	−	+	−	−
H-4	+	−	−	+	−	−
Population R						
R-1	+	−	−	+	−	−
R-2	+	−	−	+	−	−
R-3	+	−	−	+	−	−
R-4	+	−	−	+	−	−
R-5	+	−	−	+	−	−

Chromosomal distribution was deducted by analysis of five males and five females in each isofemale line (see Figure 6), and analysis of F_1 males from crosses using Oregon-R females. +, indicates presence of the above phenotype; −, absence of the above phenotype in the corresponding isofemale line.

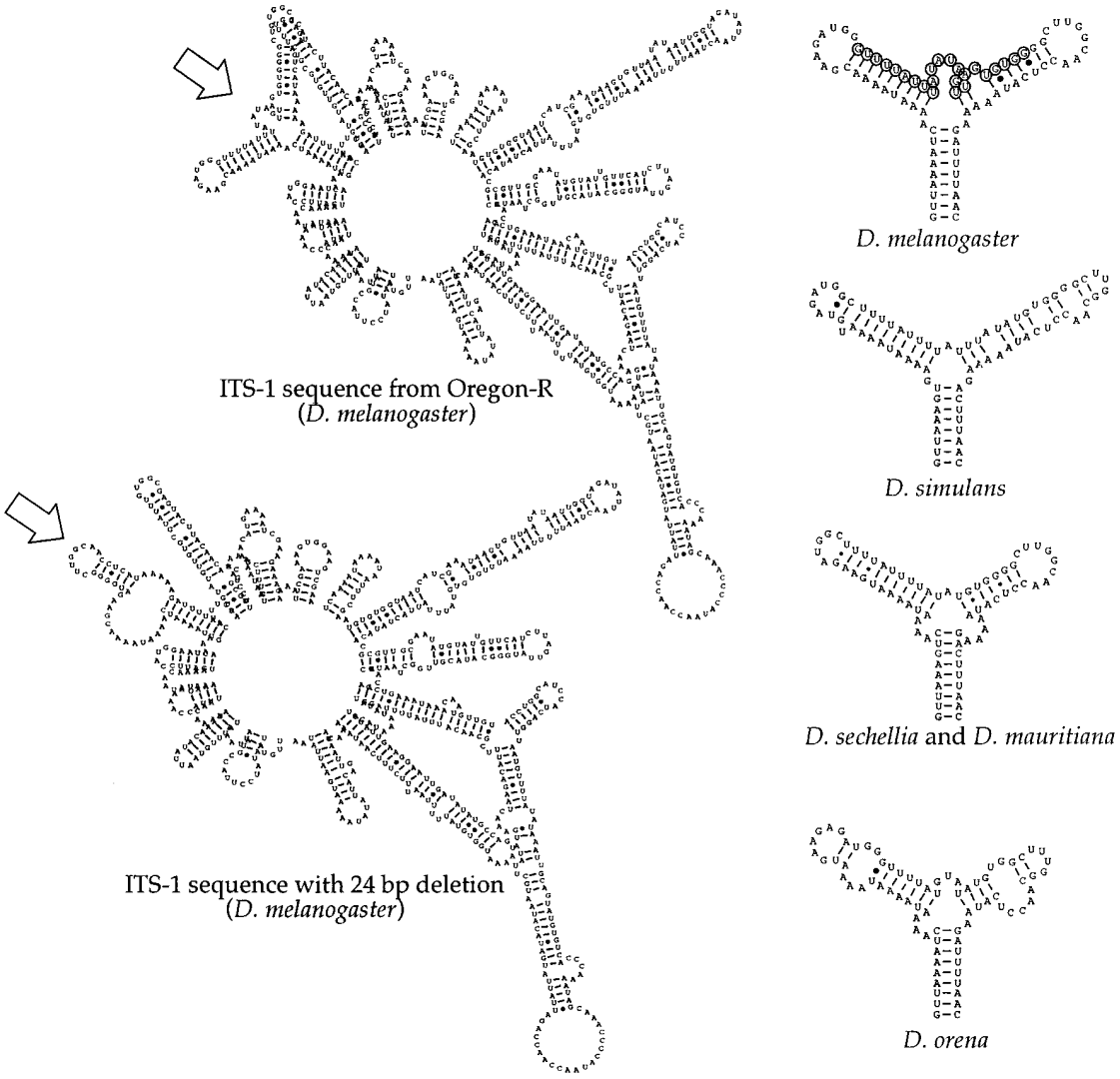

Figure 6.—Computer-simulated foldings at 25° in the ITS-1 region using MFOLD software (Zuker 1989). Upper left: folding of complete ITS-1 region using the published sequence of *D. melanogaster* for Oregon-R strain (Tautz *et al.* 1988). Bottom left: folding of the ITS-1 region of *D. melanogaster* carrying the 24-bases deletion described by Schlötterer and Tautz (1994). Arrows point to the differences between both foldings: the *Y*-shaped structure modified by the deletion is enlarged on the upper right side; the bases involved in the deletion are circled. Similar *Y*-shaped structures for the same region in four Drosophila species [according to Schlötterer *et al.* (1994) alignment of ITS-1 sequences] are represented below.

the IGS and ITS, we have analyzed patterns of mutant distribution in both regions from within the same individuals, which were drawn systematically from a long latitudinal transect of populations of *D. melanogaster* along the east coast of Australia. Notwithstanding the recent Australian colonization by *D. melanogaster*, our results indicate that selection pressures might be affecting the distribution of IGS variants in these populations. North-South grouping of IGS profiles is found only when female, but not male, profiles are compared, indicating that selection might be operating only on *X*-linked rRNA genes as suggested before (Cluster *et al.* 1987; Williams *et al.* 1987; Clark *et al.* 1990).

The presence of the ITS 24-bp deletion only in *X*-linked rDNA arrays, and the presence of *X* chromosomes carrying arrays which are either fully or partially homogenized for different ITS variants in interbreeding populations, suggest that intrachromosomal exchanges are driving the homogenization process, leading to the concerted evolution pattern of rDNA, as suggested by Schlötterer and Tautz (1994). The presence of *X*-linked arrays with mixtures of deleted and nondeleted ITS variants also suggests that interchromosomal exchanges take place but that these are restricted to *X-X* exchanges. A rapid homogenization within chromosomal lineages provides an explanation for how selection may operate in the rDNA family, as the whole chromosome rather than the individual repeat units may come under selective pressure (Dover 1982).

The different frequencies along the Australian east coast of *X* chromosomes homogenized for different ITS variants (see Table 5) could be the result of such selec-

TABLE 6

Frequencies of IGS length variants in female IGS profiles which were associated to two different ITS phenotypes

	ITS phenotype			ITS phenotype			ITS phenotype		
IGS variant	Non-deletion[a]	24 bp deletion[b]	IGS variant	Non-deletion[a]	24 bp deletion[b]	IGS variant	Non-deletion[a]	24 bp deletion[b]	
A85	18.5	0 Nd	B62	66.7	52.4	D04	3.7	14.3	
B00	22.2	0 Nd	B65	85.2	71.4	D05	11.1	0 Nd	
B03	85.2	4.8	B66	70.4	38.1	D10	0.0	9.5 24d	
B04	14.8	9.5	B72	40.7	14.3	D15	0.0	42.9 24d	
B10	96.3	9.5	B80	51.9	66.7	D25	40.7	4.8	
B15	66.7	4.8	B82	7.4	28.6	D50	11.1	47.6	
B18	59.3	0 Nd	B85	14.8	0 Nd	D55	7.4	38.1	
B20	33.3	9.5	B89	14.8	33.3	D60	0.0	9.5 24d	
B25	37.0	4.8	B90	33.3	42.9	D75	11.1	28.6	
B30	59.3	19.0	B95	11.1	23.8	D95	3.7	0 Nd	
B35	88.9	85.7	B98	40.7	42.9	D98	14.8	0 Nd	
B40	29.6	47.6	C00	0	9.5 24d	E35	66.7	42.9	
B45	96.3	47.6	C28	14.8	33.3	E50	3.7	9.5	
B48	14.8	0 Nd	C30	40.7	19.0	E65	92.6	9.5	
B50	22.2	95.2	C45	22.2	33.3	E85	18.5	0 Nd	
B54	33.3	23.8	C65	0	9.5 24d	F25	0.0	4.8 24d	
B56	66.7	66.7	C80	22.2	9.5				
B59	74.1	52.4	D00	3.7	23.8				

Nd, IGS variant associated exclusively to nondeletion ITS variant; 24d, IGS variant associated exclusively to 24-bp deletion ITS variant.

[a] Only the 481-bp variant (as in Oregon-R sequence) is present.

[b] Only the 457-bp variant (originated by a 24-bp deletion) is present.

tive forces. However, it is not clear how selection can act on the ITS-1 spacer sequence that is transcribed and removed in the rRNA maturation process. One possibility is that the ITS variants analyzed are linked to different mutations in the coding regions. This is unlikely given the high conservation of rRNA genes. Another possibility is that mutations in this region can modify the folding pattern of the 45S precursor molecule and affect the maturation process of rRNAs. Computer-simulated folding using MFOLD software (GCG Package, Madison, Wisconsin) (Zuker 1989) showed that the 24-bp fragment involved in the above deletion is located in a *Y*-shaped structure which can also be similarly folded for *D. simulans*, *D. mauritiana*, *D. sechellia*, and *D. orena* ITS-1 sequences. Deletion of these 24 bp turn the *Y*-shaped structure into a stem-loop (see Figure 6). If the maturation process is affected by this change, selection pressures may be responsible for the distribution of the two ITS variants. Kirby *et al.* (1995) present evidence that secondary structures of pre-mRNA of the *Adh* locus in *D. melanogaster* are maintained by selection.

Fluctuations in copy number of individual rDNA length variants within an entire rDNA array are probably achieved by unequal crossing over at the level of the entire rDNA unit (Dover *et al.* 1993), but our data show that partial or complete homogenization within the ITS region is not coupled to homogenization within the IGS region, although both are located in the same rDNA repeat unit. Furthermore, IGS length variants are not distributed to specific chromosomal lineages; they are instead shared by *X* and *Y* chromosomes.

We do not believe that extensive sharing, revealed by our analysis, is due to the frequent coincidental production of the same length variants in all *X* and *Y* chromosomes. The only known incidences of coincidental production of mutant length variants are to be found among microsatellite arrays. Analyses of the frequency distribution of microsatellite alleles often reveal that the process of production of allelic length variants follows a step-wise mutation model rather than an infinite allele model. In other words, there are equal frequencies of length variants, plus or minus one, two, or three repeats, *etc.*, from the most frequent length allele.

In the case of IGS length variants, there is no simple numerical relationship between the size and abundance of the numerous length variants shared by *X* and *Y* chromosomes. If we were to rely on mutation alone as being responsible for the observed distribution patterns, then we would need to propose a bizarre process of biased mutation, which happens to produce arbitrary and unrelated lengths of IGS, frequently and coincidentally, on all examined chromosomes. In the past we have observed transition stages in the homogenization of mutant repeats of two large satellite DNA families, spread

over several chromosomes, in species of Drosophila (Strachan *et al.* 1985). Such transition stages cannot be easily explained by either the double diffusion process or by coincidental mutation. In a similar vein, and following on from further arguments below, we explain the IGS distribution patterns as a consequence of frequent *X-X* and *X-Y* unequal recombination. Work in progress on the detailed analysis of mutations in given same length alleles should shed light on this issue.

Furthermore, we need to answer the critical question: Why are specific length variants in specific rDNA units not also homogenized during the homogenization of specific ITS variants in the same units? How is it possible that such different evolutionary trajectories are followed by two regions of the same unit? We suggest that the answer lies in the presence of a recombinatorial "hotspot" involved with frequent *X-X* and *X-Y* sharing of IGS length variants but not in *X-Y* sharing of ITS variants.

McKee *et al.* (1992) present evidence that the 240-bp repeats within the IGS function as *X-Y* chromosome pairing sites in male meiosis, and that *X-Y* pairing is stimulated only when the 240-bp subrepeats from the IGS region are present, with a positive correlation between copy number of 240-bp subrepeats and ability to stimulate pairing. Further data (for review see McKee 1996) confirmed that a specific sequence, or property, of the 240-bp repeats is more important for pairing than the overall length of rDNA homology. This sequence is probably the RNA polymerase I active promoter contained in each 240-bp subrepeat (Coen and Dover 1982; Murtif and Rae 1985).

McKee *et al.* (1992) present a model for *X-Y* achiasmatic chromosome pairing based on the presence of active promoters and high-affinity cleavage sites for topoisomerase I (Christiansen *et al.* 1987) in the 240-bp subrepeats. This model concerns the formation of a stable heteroduplex known as a "hemicatenane," in which single strands from different chromatids are interwound without free-end transfer due to transient nicks introduced by topoisomerase I. Resolution of the "hemicatenane" is suggested to occur by reversal of the formation process, again using topoisomerase I. Topoisomerase I sites are present not only in every 240-bp subrepeat in the IGS region but also in every 330-bp subrepeat. It has been demonstrated that topoisomerase I can promote heterologous transfer of DNA strands, because the transient single-stranded breaks introduced by the enzyme could serve both as the donor and as the acceptor (Halligan *et al.* 1982). Thus, it is possible that exchanges of single-strand fragments between nonsister chromatids of *X* and *Y* chromosomes could occur during the resolution process of hemicatenane structures if more than one topoisomerase I site is involved. The minimum of 5–6 240-bp subrepeats required for a detectable stimulation of pairing (McKee 1996) may indicate the steric requirement for two active topoisomerase I sites. Such exchanged fragments would include several 240- and/or 330-bp repeats, depending on which topoisomerase I sites are used.

The above hypothesis implies the presence of breakage points in the 330- and 240-bp subrepeats, and that the 95-bp subrepeat region is not involved in the single-strand exchange between chromatids. Williams *et al.* (1987) demonstrate that differences between *X*-linked and *Y*-linked IGS variants accumulate only in the 95-bp subrepeat region. Ruiz Linares *et al.* (1994) analyzed in detail the structure of four spacer length variants obtained from an Oregon-R strain. All of them showed five copies of the 95-bp subrepeat, and a breakage hotspot was detected in the first 330-bp subrepeat. Other breakage points were located in several positions within the 240-bp subrepeat array.

Ruiz Linares *et al.* (1994) describe an unexpected bipartite structure for the 240-bp subrepeat array: the repeats in the left half of the array consisted of one sequence variant, while a different variant occupied the right half. This distribution is not expected from models of randomly positioned unequal crossing over, and was the first indication that independent homogenization processes could be affecting different regions of the rDNA units. If the 330- and 240-bp subrepeat arrays are transferred together by a process such as that proposed here, the bipartite structure could have arisen by single-strand exchange between two IGS arrays carrying different numbers of 240-bp repeats, which had been homogenized for different mutations in the 240-bp repeats.

The model of meiotic exchange of single-stranded fragments can explain the shared distribution of IGS mutations between *X* and *Y* chromosomes, while leaving the other regions of the rDNA unit to evolve mostly along haplotypic *X* chromosome lineages but with the occasional *X-X* exchanges. The model also allows for the presence of chromosome-specific IGS variants in some populations as the whole rDNA array does not take part in the *X-Y* chromosome pairing. Park and Yamamoto (1995) demonstrate that the pairing site is undoubtedly in the central portion of the long tandem arrays of rDNA, although its exact location was not possible to determine. IGS variants located outside this central portion would not be involved in the meiotic exchange and could remain isolated to specific chromosomes until subsequent internal reorganization brings them closer to the exchange region.

It has been suggested that *X* and *Y* chromosome rDNA arrays evolve independently as selection pressures affect only *X* chromosome arrays in which the rDNA genes are preferentially expressed (Cluster *et al.* 1987; Williams *et al.* 1987; Clark *et al.* 1990). However, *Y* chromosome arrays can be expressed when rDNA is removed from *X* chromosomes, so their expression capabilities are maintained without selection pressures. The meiotic exchange of fragments containing 240-bp repeats could provide functional promoter regions to the *Y* chromosome arrays from the *X* chromosomes.

In summary, our data show that there is no single course of evolution for the rDNA: different regions follow different trajectories depending on the locations of reciprocal and nonreciprocal exchanges along the rDNA unit.

Further detailed analysis of the internal organization of a large number of individual IGS length variants isolated from *X* and *Y* chromosome-linked rDNA arrays should provide useful information about the internal distribution of IGS variability among the 240-bp subrepeat arrays and its chromosomal distribution. Such studies, as well as an analysis of the postmeiotic rate of generation of new IGS length variants, are in progress and could provide direct evidence for the meiotic exchange model proposed here.

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