Heredity (2005) 94, 388–395

www.nature.com/hdy

Evolution of the chromosomal location of rDNA genes in two *Drosophila* species subgroups: *ananassae* and *melanogaster*

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The evolution of the chromosomal location of ribosomal RNA gene clusters and the organization of heterochromatin in the *Drosophila melanogaster* group were investigated using fluorescence *in situ* hybridization and DAPI staining to mitotic chromosomes. The investigation of 18 species (11 of which were being examined for the first time) belonging to the *melanogaster* and *ananassae* subgroups suggests that the ancestral configuration consists of one nucleolus organizer (NOR) on each sex chromosome. This pattern, which is conserved throughout the *melanogaster* subgroup, except in *D. simulans* and *D. sechellia*, was observed only in the *ercepeae* complex within the *ananassae* subgroup. Both sex-linked NORs must have been lost in the lineage leading to *D. varians* and in the *ananassae* and *bipectinata* complexes, whereas new sites, characterized by intra-species variation in hybridization signal size, appeared on the fourth chromosome related to heterochromatic rearrangements. Nucleolar material is thought to be required for sex chromosome pairing and disjunction in a variety of organisms including *Drosophila*. Thus, either remnant sequences, possibly intergenic spacer repeats, are still present in the sex chromosomes which have lost their NORs (as observed in *D. simulans* and *D. sechellia*), or an alternative mechanism has evolved.
Heredity (2005) **94,** 388–395. doi:10.1038/sj.hdy.6800612
Published online 23 February 2005

Keywords: *Drosophila*; rDNA; heterochromatin; evolution; fluorescence *in situ* hybridization

Introduction

In all eukaryotes, the genes encoding ribosomal RNA (rDNA) are present in multiple copies, arranged as clusters embedded in heterochromatin and cytologically visible as nucleolus organizers (NORs) when they are transcriptionally active. Typically, each repeat unit within an rDNA locus contains a gene region encoding the 18S, 5.8S and 28S rRNAs, and an intergenic spacer consisting of different subrepeats. Investigation of a wide range of organisms, including fungi, animals and plants, has revealed that the organization, size and degree of repetition of the basic unit are highly variable (reviewed in John and Miklos, 1988). Changes in the chromosomal position and number of NORs have sometimes been reported between closely related species, and even within a given species (Andronico *et al*, 1985; Dubcovsky and Dvorak, 1995; Galian *et al*, 1995; Hirai *et al*, 1996; Shishido *et al*, 2000), showing that these genes can undergo rapid rearrangements due to their heterochromatic location. It follows that studying rDNA genes should help us to understand how genes evolve in the heterochromatin, and also to resolve the phylogeny of closely related species.

Data on the number and chromosomal locations of NORs are very fragmentary in the genus *Drosophila*, which includes about 3500 known species and which is thought to have originated 61–65 million years ago (Powell, 1997). They are only available for 20 species, scattered across three of the 15 subgenera. The two members of the *Idiomyia* subgenus studied, *D. heteroneura* and *D. sylvestris*, exhibit only autosomal NORs (Stuart *et al*, 1981), whereas, in most of the species examined in the *Sophophora* and *Drosophila* subgenera, the NORs appear to be restricted to the sex chromosomes. In the subgenus *Drosophila* and in the *repleta* group, both *D. repleta* and *D. hydei*, as well as two of their closest relatives, possess one NOR on the X chromosome and two on the Y chromosome (Hennig *et al*, 1975, 1982). One of these species also possesses rDNA sequence clusters on several autosomes, but these appear to be transcriptionally inactive. Again in the *repleta* group, molecular and genetic data indicate that an NOR is present on each sex chromosome of *D. mercatorum* and *D. buzzatii* (Templeton *et al*, 1985; Knibb *et al*, 1989). The arrangement is different in the sibling species *D. arizonensis* and *D. mulleri*, which possess only an X-linked NOR together with a cluster of not very active rDNA genes on the dot chromosome (Bicudo, 1981). In *D. tumiditarsus*, a single

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Received 22 May 2003; accepted 30 July 2004; published online 23 February 2005

NOR is found on the dot chromosome (Sinibaldi and Cummings, 1981). In the *Sophophora* subgenus, eight species belonging to the *melanogaster* subgroup have been examined. All of these have an NOR on each sex chromosome, with the exception of *D. simulans* and *D. sechellia*, which were found to possess only an X-linked NOR (Lohe and Roberts, 2000). However, the Y chromosome of *D. simulans* has been shown to carry a large block of 240-bp nontranscribed spacer repeats (Lohe and Roberts, 1990). The only other species studied in the *Sophophora* subgenus, *D. ananassae* (*ananassae* subgroup), carries NORs on its Y and fourth chromosomes (Kaufmann, 1937; Kikkawa, 1938).

To get a more precise picture of how the rDNA clusters have evolved at the species group and subgroup levels in *Drosophila*, we used fluorescence *in situ* hybridization (FISH) on mitotic chromosomes, to add to the data on the *melanogaster* and *ananassae* subgroups. In the *melanogaster* subgroup, the phylogenetic relationships are clear, except for the *simulans–sechellia–mauritiana* triad. In the *ananassae* subgroup, morphological, molecular, karyotypic and behavioural data strongly support a division into three complexes *ananassae*, *bipectinata* and *ercepeae* (Bock and Wheeler, 1972; Lemeunier *et al*, 1986, 1997; Schawaroch, 2002; Da Lage, personal communication). However, the phylogenetic relationships of the complexes have not been resolved, nor is the positioning of *D. varians*, a species that does not seem to fit into any of these complexes. To shed some light on these issues, we examined *D. varians* and 11 other species chosen from the three complexes.

Materials and methods

Drosophila strains and chromosome spreads

The species studied in the *melanogaster* and *ananassae* subgroups are listed in Table 1. Mitotic chromosomes were obtained from the brain of last-instar larvae using conventional procedures: colchicine and hypotonic treatments, fixation in methanol:acetic acid (3:1) and spreading with 60% acetic acid. Slides were stored in the freezer until use. Between three and six individuals were analysed for each species in the *ananassae* and *melanogaster* subgroups, except for *D. simulans*, for which 11 individuals were studied.

Fluorescence *in situ* hybridization

The rDNA probe used in the study was Py12 isolated from *D. melanogaster* and cloned in the PMB9 plasmid (R Terracol, Institut J Monod, Paris). It contained a complete DNA array unit, that is, the 18S, 5.8S and 28S genes, and the intergenic nontranscribed spacers. A probe containing the 240-bp nontranscribed spacer repeats from *D. simulans* cloned in the A3235PS plasmid (Lohe and Roberts, 1990) was also used. Both probes were labelled with digoxygenin-11-dUTP (Roche Diagnostics) by nick translation.

The slides were pretreated in 2× SSC at 37°C and dehydrated. The probe solution was prepared in 2× SSC, 35% formamide and 10% dextran sulphate. It was then deposited on the slides (10 µl per slide) and covered with a glass coverslip. Chromosome and probe DNA were simultaneously denatured for 5 min at 72°C on a hot plate. Hybridization was performed at 37°C overnight in a humidified chamber. After removing the glass coverslip, the slides were washed for 15 min at room temperature in a 50% formamide/2× SSC solution, and then washed in 2× SSC and 1× PBD (Q-biogene). Fluorescence detection consisted of incubating the slides for 5 min with rhodamine anti-digoxygenin (60 µl per slide) (Q-biogene) followed by washes in 1× PBD, counterstaining and mounting in DAPI/Antifade (Q-biogene).

Slides were observed under a Zeiss Axioplan 2 epifluorescence microscope. Chromosome images were

Table 1 Species and strains used in this study

Species	*Origin*	*Reference of the stock*
melanogaster subgroup		
D. melanogaster		Canton-S, PGE laboratory
D. mauritiana	Chaland Is. (Mauritius)	PGE laboratory, No. 163-1
D. sechellia	Seychelles	PGE laboratory, No. 228
D. simulans	Tunisia	PGE laboratory, No. SR
D. teissieri	Zimbabwe	PGE laboratory, No. 128-2
D. santomea	São Tomé Is.	PGE laboratory
D. yakuba	Cameroon	PGE laboratory, No. 115
ananassae subgroup		
(1) *ananassae* complex		
D. ananassae	Ivory Coast	PGE laboratory, No. 242-1
D. atripex	Thailand	PGE laboratory
D. pallidosa	Samoa	Tucson Stock centre, No. 14024-0433-1
D. phaeopleura	Fiji	Tucson Stock centre, No. 14024-0434-0
(2) *bipectinata* complex		
D. bipectinata	New Caledonia	PGE laboratory
D. malerkotliana	Ivory Coast	PGE laboratory, No. 243-1
D. parabipectinata	Mauritius	PGE laboratory
D. pseudoananassae	Thailand	Tucson Stock centre, No. 14024-0421-0
(3) *ercepeae* complex		
D. ercepeae	La Réunion	PGE laboratory, No. 164-14
D. merina	Madagascar	PGE laboratory, No. 290-1
D. vallismaia	Seychelles	PGE laboratory, No. 206-11
(4) *D. varians*	Philippines	PGE laboratory

generated using a highly sensitive CCD camera (Princeton Instruments, Evry, France) and the Metaview 4.1.7 image-analysing system (Universal Imaging Corporation).

Results

The *melanogaster* subgroup

Mitotic chromosomes hybridized with the Py12 probe and counterstained with DAPI are shown in Figure 1. The heterochromatic structure of the sex chromosomes is presented in Figure 2(a).

The *melanogaster* complex: The FISH results confirm the paracentromeric position of the ribosomal RNA genes on the X chromosome of *D. sechellia* and *D. simulans* (Figure 1a and b) that had previously been described using a unique 28S gene probe (Lohe and Roberts, 2000). The Y chromosomes of these two species have different heterochromatic banding patterns, with two large heavily positive DAPI blocks in *D. simulans* versus four blocks in *D. sechellia*, a finding that is consistent with the quinacrine banding (Lemeunier and Ashburner, 1984) (Figure 2a). The pattern of hybridization on the Y chromosome is similar in the two species. Repeated observations of highly stretched prometaphase chromosomes show that there are two hybridization sites on the long arm with much lower intensity than the X signal. The location of the signals differs between species: one is close to the tip and the other in a less distal position in *D. simulans*, one is in the middle of the long arm and the other near the centromere in *D. sechellia*. The results obtained with the 240-bp repeat probe differ between the species with regard to the signal intensity. On the X chromosome, the signal is strong in *D. sechellia* and weak in *D. simulans*, whereas the reverse is observed for the Y hybridization sites (boxes in Figure 1a and b).

In *D. mauritiana*, the heterochromatic structure of the Y chromosome differs greatly from that observed in *D. sechellia* and *D. simulans* (Figure 2a). The Py12 probe produces a strong signal, close to the centromere, on both sex chromosomes (data not shown), which is consistent with the known location of NORs in this species (Lohe and Roberts, 2000).

The *D. santomea*, *D. yakuba* and *D. teissieri* triad: The heterochromatic structure of the X chromosome, determined after DAPI staining, is very similar in *D. santomea* and *D. yakuba* (Figure 2a). A fluorescent heterochromatic block on the long arm of the X chromosome previously described by Lemeunier *et al* (1978) in *D. yakuba* is also present in *D. santomea*, but appears to be fainter, and it does not exist at all in *D. teissieri*. In all three species, the NOR on the X chromosome, determined using the Py12 probe, is in a similar position: on the long arm, and close to the centromere (Figure 1c and d).

The heterochromatic structure of the Y chromosome is different in *D. santomea* and *D. yakuba*. In the latter species, the short arm of the Y chromosome shows two bright blocks, one close to the centromere, the other at the tip of the short arm, and the long arm presents a short bright block close to the centromere. In *D. santomea*, the short arm of the Y chromosome shows two bright blocks, which are identical in size and not adjacent to the centromere, and the long arm also has two bright blocks,

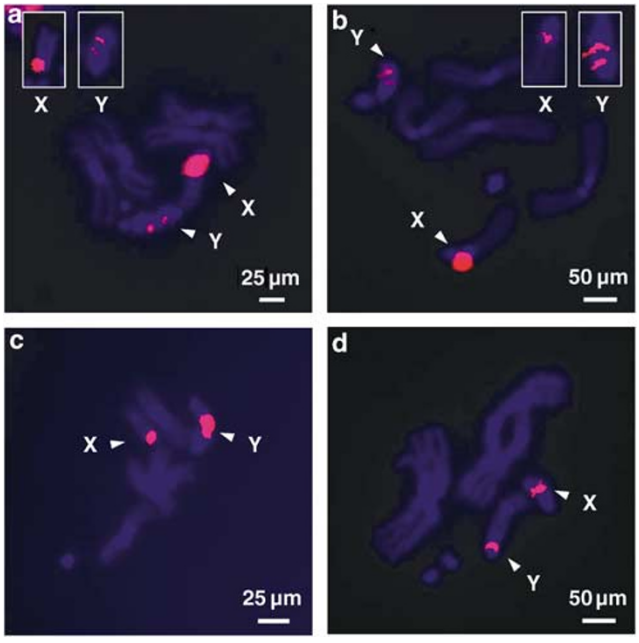

Figure 1 *melanogaster* subgroup: mitotic chromosomes are counterstained with DAPI and hybridized with the Py12 probe and the 240-bp probe (in boxes). (**a**) *D. sechellia*, (**b**) *D. simulans*, (**c**) *D. santomea* and (**d**) *D. yakuba*.

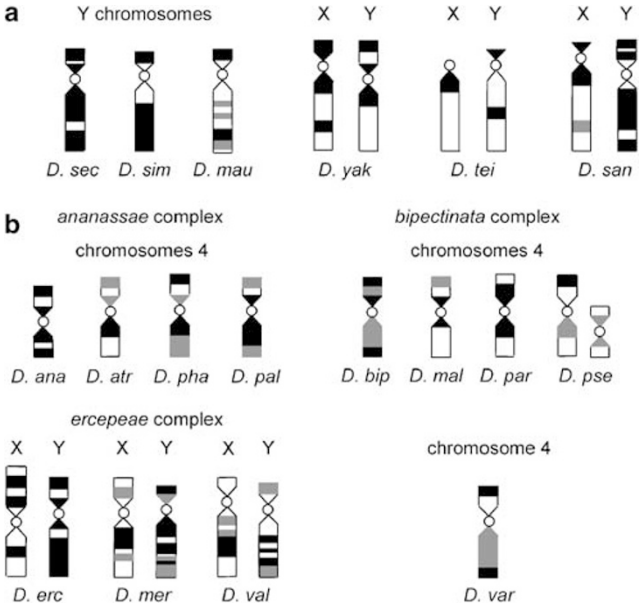

Figure 2 Schematic representation of the heterochromatic structure of the chromosomes bearing an NOR within the *melanogaster* (**a**) and the *ananassae* (**b**) subgroups. DAPI staining: faint (white box), medium (grey box), intense (black box). (*D. sec: D. sechellia, D. sim: D. simulans, D. mau: D. mauritiana, D. yak: D. yakuba, D. tei: D. teissieri, D. san: D. santomea, D. ana: D. ananassae, D. atr: D. atripex, D. pha: D. phaeopleura, D. pal: D. pallidosa, D. bip: D. bipectinata, D. mal: D. malerkotliana, D. par: D. parabipectinata, D. pse: D. pseudoananassae, D. erc: D. ercepeae, D. mer: D. merima, D. val: D. vallismaia, D. var: D. varians.*)

one of them is very large and not adjacent to the centromere, and the other is smaller and terminal. In *D. teissieri*, the observation of stretched chromosomes revealed the existence of a short arm on the Y chromosome, which had never been described before. The long arm carries a bright block in the middle. Hybridization with the Py12 probe showed that the Y-linked NOR lies on the long arm in the three species, close to the centromere in *D. santomea* and *D. yakuba* (Figure 1c and d), and lies within the bright block in *D. teissieri* (data not shown).

The ananassae *subgroup*

Mitotic chromosomes hybridized with the Py12 probe and counterstained with DAPI are presented in Figures 3 and 4. The heterochromatic structure of the sex chromosomes is shown in Figure 2b.

The *ananassae* complex: The four species studied are devoid of NORs on the X chromosome, and *D. ananassae* is the only one to display a NOR on the Y chromosome (Figure 3a–c). All have an NOR on the metacentric (or submetacentric) chromosome 4. In *D. ananassae* and *D. phaeopleura*, the hybridization site is terminal, whereas in *D. atripex* and *D. pallidosa* the site appears to be closer to the centromere. The centromere is always between two heterochromatic blocks, and the NOR is located in a DAPI bright-terminal block. In *D. ananassae*, there is an additional block at the tip of one arm of chromosome 4, opposite to the arm bearing the NOR (Figure 2b).

In *D. atripex* and *D. phaeopleura*, we observed differences in the size of the rhodamine signals between the homologues of chromosome 4 (Figure 3b and c). The differences are strongly marked in *D. phaeopleura*, for which the largest rhodamine signal is associated with additional heterochromatic material, as can be observed after DAPI staining (Figure 3c).

The *bipectinata* complex: The heterochromatic structure of the chromosome 4 and the sex chromosomes is highly variable within the complex (Figure 2b). *D. pseudoananassae* carries an additional and smaller pair of chromosomes 4. There is only one NOR, located on chromosome 4 (the larger one in *D. pseudoananassae*), in a telomeric or subtelomeric position (Figure 4c). Differences in the size of the hybridization signals between the homologues are observed in some individuals of *D. malerkotliana* and *D. parabipectinata* (not shown).

The *ercepeae* complex: In the *ercepeae* complex, the NORs are located on the sex chromosomes (Figure 4a and b). In each species, the X chromosome always displays three heterochromatic blocks, the position and intensity of which differ between species (Figure 2b). The X chromosome of *D. ercepeae* is almost metacentric and shows three blocks with the same intensity of DAPI staining. The NOR is located in the middle of the arm of the X chromosome, which has a single DAPI-positive block. In *D. vallismaia* and *D. merina*, the NOR is situated on a weaker DAPI band, close to the centromere, but on different arms (the long arm of the X chromosome in *D. vallismaia*, and the short arm in *D. merina*).

The Y chromosomes of *D. vallismaia* and *D. merina* are both submetacentric and their banding patterns display some similarities. They show two hybridization signals of the Py12 probe, differing in size, one at each tip of the chromosome (Figure 4a). The Y chromosome of *D. ercepeae* appears slightly different in its heterochromatic banding pattern. It is characterized by a unique

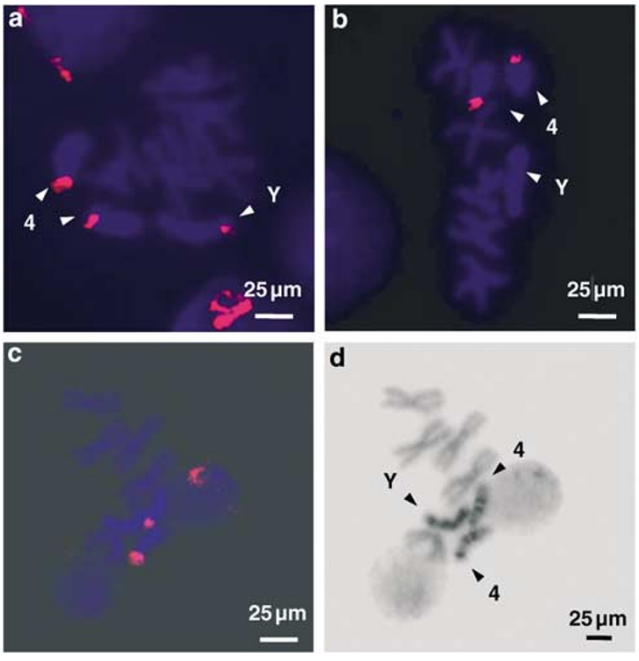

Figure 3 *ananassae* subgroup (*ananassae* complex): mitotic chromosomes are counterstained with DAPI and hybridized with the Py12 probe. (**a**) *D. ananassae*, (**b**) *D. atripex*, (**c**) *D. phaeopleura*, (**d**) *D. phaeopleura*, DAPI staining with inverted contrast.

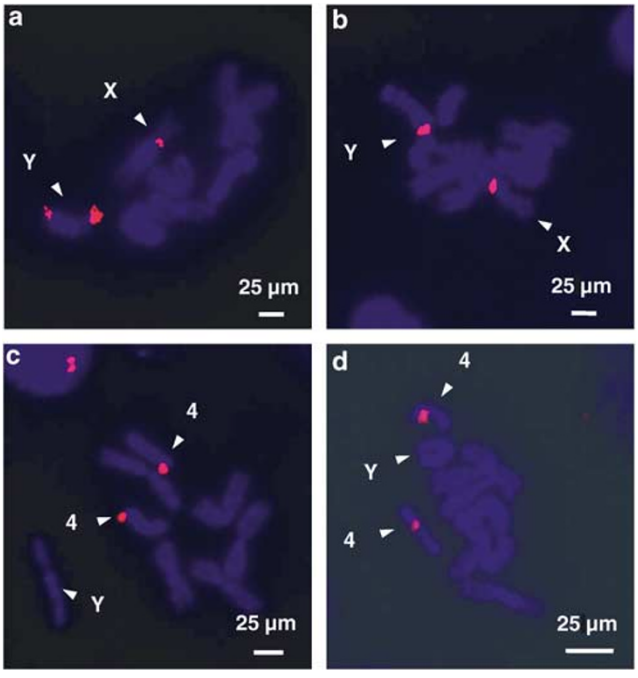

Figure 4 *ananassae* subgroup: mitotic chromosomes are counterstained with DAPI and hybridized with the Py12 probe. (**a**) *D. vallismaia*, (**b**) *D. ercepeae* (*ercepeae* complex), (**c**) *D. malerkotliana* (*bipectinata* complex), (**d**) *D. varians*.

hybridization site, in a distal position on the long arm (Figure 4b).

D. varians: *D. varians* possesses sex chromosomes and a chromosome 4, which have heterochromatic banding patterns very different from those of the species belonging to the three complexes (Figure 2b). *D. varians* has a unique NOR, situated on the shorter arm of the submetacentric chromosome 4, close to the centromere (Figure 4d). In some individuals, we observed differences in the sizes of the signals between the homologues.

Discussion

rDNA evolution and *Drosophila* phylogeny

In the light of the phylogenetic relationships previously established in the *melanogaster* group, we can see how the data reported here help to clarify the placement of the species studied. This leads us to propose the diagram presented in Figure 5, which shows the most parsimonious scenario for NOR evolution, as detailed below.

In the *melanogaster* subgroup, the relationships among the sibling species *D. simulans*, *D. sechellia* and *D. mauritiana* are still obscure. The allozymic data (Cariou, 1987) and introgression experiments (Palopoli *et al*, 1996) tend to group *D. simulans* and *D. sechellia* together. The assigning of *D. sechellia* and *D. mauritiana* to the same group is supported by DNA–DNA hybridization and gene sequence data (Caccone *et al*, 1996). Finally, phylogenic reconstruction using microsatellites provides evidence that *D. sechellia* arose first, followed by a split between *D. simulans* and *D. mauritiana* (Harr *et al*, 1998). The data on NORs (Lohe and Roberts, 2000) and the present findings strengthen the hypothesis that *D. sechellia* is the closest relative of *D. simulans*. Not only do both these species have no NOR on the Y chromosome, they also reveal two weak hybridization sites on this chromosome with the Py12 probe. The same pattern was observed in two other stocks of *D. simulans* from different geographic locations (unpublished data). The hybridization with the 240-bp repeat probe in *D. simulans*

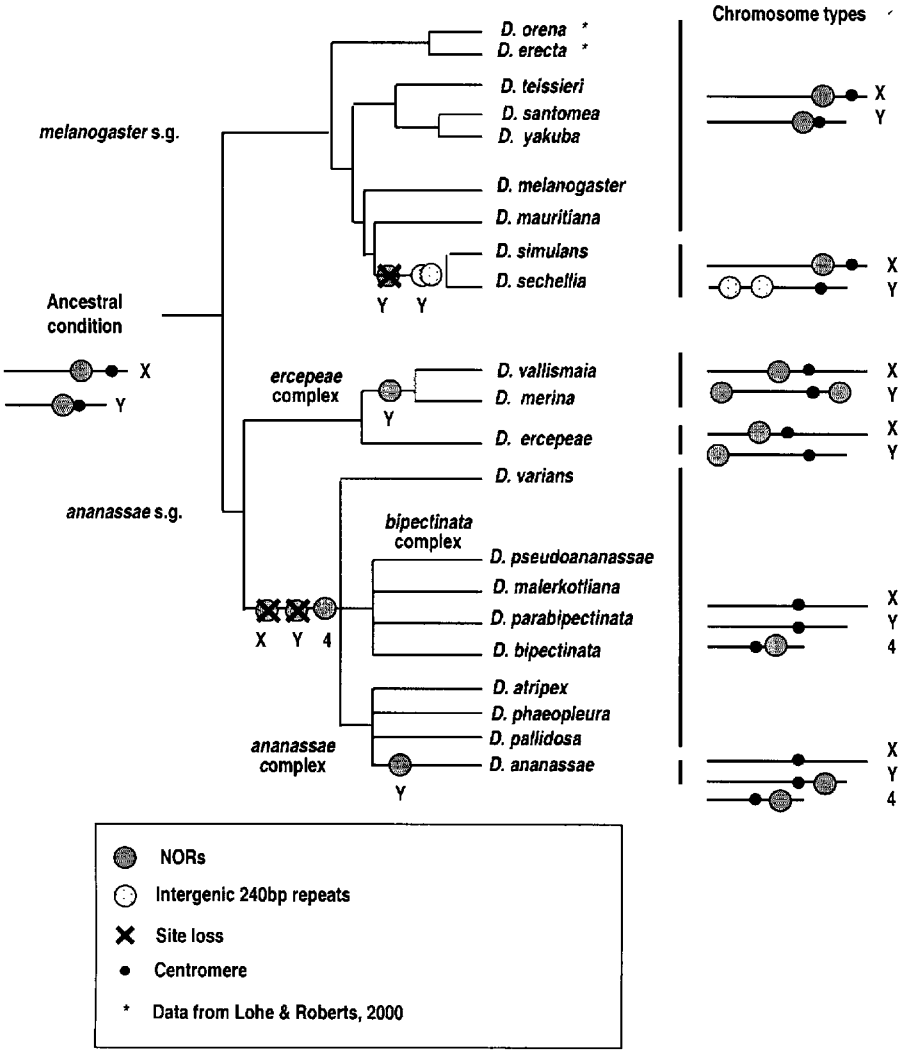

Figure 5 Parsimonious scenario for NORs' evolution (see text). When the relationships between species are not well supported by data of the literature, these are represented as unresolved branching. Chromosome types represent the different NOR configurations observed across species.

shows that both sites result from an amplification of this sequence (shown by the very strong signal). In *D. sechellia*, the signal on the Y chromosome with this probe remains very weak, whereas the signal on the X chromosome is still strong. This suggests that there has been an amplification of intergenic sequences on the X chromosome, but not on the Y chromosome. Lohe and Roberts (2000), using a 28S probe, failed to detect any hybridization signal on the Y chromosome of *D. sechellia*, which suggested a complete or nearly complete loss of the coding sequences. Whether sequences other than the 240-bp repeats are present on this chromosome remains an open question. The most parsimonious scenario is that the events involving the loss of the Y-linked NOR and the acquisition of a second site possessing rDNA-related sequences on the Y chromosome occurred only once. This would mean that *D. sechellia* and *D. simulans* originated from a common ancestor after the split that led to *D. mauritiana*. The difference in the hybridization signal size between the two species would therefore result from heterochromatin rearrangements after the speciation event.

In the *ananassae* subgroup, only the species belonging to the *ercepeae* complex were found to carry NORs on both sex chromosomes, which is the general pattern in the *melanogaster* subgroup. We propose that this pattern is ancestral in the *melanogaster* group, and that the *ercepeae* complex was the first to evolve within the subgroup. Although the evolutionary scheme we propose needs to be confirmed by further studies, we can already see that it is consistent with recent data on gene sequence divergence (Schawaroch, 2002; Da Lage, personal communication). The loss of the Y- and X-linked NORs, and the acquisition of an NOR on the fourth chromosome, would seem to have occurred in the lineage leading to the *ananassae* and *bipectinata* complexes, before their split. Owing to the major heterochromatic rearrangements that have occurred, we cannot, however, exclude the possibility that an NOR may have been acquired by chromosome 4 several times independently. According to our scenario, the presence of a Y-linked NOR in *D. ananassae* results from a secondary acquisition. The absence of a Y-linked NOR in *D. pallidosa*, which is thought to be its closest relative (Singh, 2000), supports this hypothesis. This would imply that the NOR on the fourth chromosome did not necessarily arise as a result of transposition from the X chromosome, as proposed by Hinton and Downs (1975), but may have originated from the Y chromosome.

Within the *ercepeae* complex, the Y chromosomes of *D. vallismaia* and *D. merina* appear to be structurally very similar to each other and also both bear two rDNA hybridization sites, whereas the Y chromosome of *D. ercepeae* showed a single site. *D. vallismaia* and *D. merina* appear to be the closest relatives in this triad, as judged from their ability to produce fertile hybrids, from the structure of the autosomes and X chromosome, and from morphological and molecular data (Lemeunier *et al*, 1997; Da Lage, personal communication). If they are, then the shared pattern observed in *D. vallismaia* and *D. merina* would require at least two events: either two independent acquisitions of a Y-linked NOR or the acquisition of a Y-linked NOR in the lineage leading to the three species, followed by the secondary loss of a Y-linked NOR in the lineage leading to *D. ercepeae.* This latter hypothesis, which requires *D. vallismaia* and *D. merina* to be the closest relatives, is more parsimonious because it requires only one acquisition of NOR.

The phylogenetic position of *D. varians* is still unclear. Its inclusion in the *ananassae* subgroup was initially challenged, on the basis of some characteristics of its periphallic organs (Bock and Wheeler, 1972). Recent molecular data support this assignation, but do not enlighten us about the relationships of this species with the three complexes (Schawaroch, 2002). The structure of its mitotic chromosomes, which have been examined here, does not provide any further information about its possible position within the subgroup. However, the presence of an NOR on its fourth chromosome tends to bring *D. varians* closer to the *bipectinata* and *ananassae* complexes.

Functional constraints on the chromosomal location of NORs?

Although sequences unrelated to rDNA might also be involved (Park and Yamamoto, 1995), cytological and molecular studies in *D. melanogaster* have produced evidence that the NORs, and more precisely the 240-bp repeats of the intergenic spacer, function as X–Y pairing sites during meiosis (McKee and Karpen, 1990; McKee *et al*, 1992). In this context, Lohe and Roberts (2000) argued that the persistence of NORs on both sex chromosomes within the *melanogaster* subgroup results from this functional constraint. The cytological observations of Ault and Rieder (1994) strongly suggest that the 240-bp repeats on the Y chromosome of *D. simulans* do play this role, thus compensating for the lack of a Y-linked NOR in this species. Our study shows that the situation on the Y chromosome of *D. sechellia* may be similar to that in *D. simulans*. The situation in the *melanogaster* subgroup is consistent with a role for NORs in pairing, whereas their apparent absence from the sex chromosomes in species of the *bipectinata* and *ananassae* complexes (except *D. ananassae*, which carries a Y-linked NOR) conflicts with this hypothesis. The sequences of the rDNA genes have been proven to be highly conserved during evolution and, moreover, we obtained strong hybridization signals on the fourth chromosome of these species, with the Py12 plasmid cloned in *D. melanogaster*. Thus, the lack of signal on the sex chromosomes can be definitely ascribed to a lack of rDNA genes. We also tested one species of each complex with the 240-bp repeat probe from *D. simulans* (data not shown). No signal was detected, even at the NOR location on the fourth chromosome, which strongly suggests a marked divergence of this sequence at the species group level. This means that we cannot exclude the possibility that intergenic spacer repeats are present on the sex chromosomes of the *ananassae* subgroup species, and function as pairing sites. Observations of multivalents composed of the X, Y and fourth chromosomes during male meiosis in *D. ananassae* (Hinton and Downs, 1975; Matsuda *et al*, 1983) are consistent with this hypothesis. Although the X chromosome is devoid of NORs in this species, it has been seen to pair with other chromosomes at sites that may correspond to their NORs (figures in Tobari *et al*, 1993). Further studies will require the use of spacer probes cloned from the *ananassae* subgroup.

Heterochromatin and rDNA evolution

The changes in the number and location of NORs among *Drosophila* species studied at the group scale have proved to be quite revealing about the rearrangements affecting heterochromatin. This is particularly clear for the X-linked NORs. These have a similar paracentromeric position in all but one of the nine species of the *melanogaster* subgroup, a situation that can be linked to the limited degree of heterochromatic changes on this chromosome. *D. orena* is an exception, in that it has acquired a considerable amount of heterochromatic material; a change that was accompanied by a change in NOR location (Lemeunier *et al*, 1978). In contrast, within the *ananassae* subgroup, the X chromosome is characterized by appreciable heterochromatic rearrangements. Only the *ercepeae* complex has retained an X-linked NOR, the position of which differs considerably between species. The positions of the Y-linked NORs, which have sometimes been lost and sometimes duplicated, are highly variable in both subgroups. This is consistent with the major organizational changes undergone by the Y chromosome, which is entirely heterochromatic. Even at the species-complex scale, the banding pattern of this chromosome is never maintained.

In *D. melanogaster*, as in many other species, the fourth chromosome appears as a small dot chromosome that is considered atypical compared to the other autosomes, mainly because of its heterochromatic structure. In the *ananassae* subgroup, as shown here, and in some other species, such as *D. kikkawai* (Baimai and Chumchong, 1980) and *D. birchii* (Baimai, 1969), the fourth chromosome is large and metacentric, and an additional pair of heterochromatic chromosomes 4 is sometimes observed. In several species of the *ananassae* subgroup, we observed size differences in NOR signals between the homologous chromosomes 4. The case of *D. phaeopleura* is particularly remarkable, with a translocation of a part of the tip, bearing an NOR, from one chromosome to its homologue, and the presence in some individuals of a supernumerary chromosome 4. The heteromorphism of homologous NORs has been well documented both in animals (Delany *et al*, 1991; Abuin *et al*, 1996; Hirai *et al*, 1998; Mandrioli *et al*, 1999) and plants (Miller *et al*, 1980; Moscone *et al*, 1995; Zoldos *et al*, 1999). This may be related to differences in the ribosomal gene copy number at homologous sites, which has been attributed to unequal crossing over (Fitch *et al*, 1990; Dover *et al*, 1993; Delany and Krupkin, 1999; Mandrioli *et al*, 1999). In *D. melanogaster*, several lines of evidence suggest that the chromosome 4 may have derived from an ancestral X chromosome (Hochman, 1976; Larsson *et al*, 2001). So far, few studies have investigated the nature of the rearrangements that have affected the fourth chromosome. Further investigation of this issue should help to elucidate the role of heterochromatin in the process of chromosome evolution.

Acknowledgements

We would like to thank Dr Lohe for kindly providing a 240-bp probe, and D Ogereau for the preparation of the probes.

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