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Distribution and evolution of mobile elements in the *virilis* species group of *Drosophila*

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Abstract. The distributions of *Penelope* and *Ulysses*, two transposable elements that can induce hybrid dysgenesis, were studied in several species groups of *Drosophila*. No significant hybridization to *Penelope* and *Ulysses* probes was detected by Southern blot analyses of species outside the *virilis* group. In contrast, both element families have had a long residence in all species of the *virilis* species group, as indicated by their strong presence in the heterochromatic chromocenter. Except for *D. kanekoi*, *D. lummei*, and some strains of *D. virilis*, species of the group carry full-sized, and at least potentially functional, copies of both element families. Consistent with the occurrence of recent transposition, *Penelope* and *Ulysses* elements are located at different chromosomal sites in different geographical strains of the same species. A total of 79 *Penelope* and 47 *Ulysses* euchromatic insertion sites were localized to chromosomal subsections in species of the *virilis* group. Highly significant deviations from independence of the distributions of *Penelope* and *Ulysses* and previously established inversion breakpoints were documented, suggesting that these transposable elements may have played an important role in genomic reorganization and evolution of the *virilis* species group, which is especially rich in karyotypic variation.

Introduction

The genomes of eukaryotic organisms contain many families of transposable elements (TEs), which have the potential to act as spontaneous mutagens. TEs have been shown to induce a wide variety of mutations, chromosomal aberrations and other genetic changes, including insertions, deletions, inversions, translocations, nondisjunction and reduced fertility. When these genetic changes occur in germ line cells, they have the potential to be transmitted to future generations and to serve as raw material for gene and genomic evolution.

An accumulation of data on the variety and genomic distribution of TEs in both laboratory and natural populations suggests that TEs are an important source of new genetic variation. However, the focus of the majority of studies has been on TE-induced variation at the level of coding and regulatory sequences of individual genes, and only very limited attention has been paid to the role of mobile elements in the restructuring and evolution of whole genomes, in natural populations, and their possible role in speciation. Due to the small numbers of studies carried out, reliable estimates of the frequency with which TEs are implicated in the formation of chromosomal structural changes, such as inversions, are not available. The challenge is to determine to what extent TE activity is responsible for genomic structural changes, most of which have occurred at different times in the past.

The activation of some TEs in *Drosophila melanogaster* and other insects leads to a syndrome of aberrant traits collectively known as hybrid dysgenesis (Kidwell et al. 1977). In early studies with this species, three independent systems were shown to be associated with the activation of three different TE families (Bingham et al. 1982; Bucheton et al. 1984; Blackman et al. 1987; Yannopoulos et al. 1987). Additional examples of hybrid dysgenesis have been reported in *Drosophila* and other Diptera. Among these is an unusually interesting system of hybrid dysgenesis described in species of the *virilis* group. Several TE families appear to be involved whose mobility seems to be interrelated (Lozovskaya et al. 1990; Scheinker et al. 1990; Evgen'ev et al. 1992; Petrov et al. 1995). While at least five unrelated TE families, including *Ulysses*, *Penelope*, *Paris*, *Helena* and *Telemac*, are simultaneously mobilized nonreciprocally in the progeny of *D. virilis* dysgenic crosses, the *Penelope* family was shown to play a pivotal role in this hybrid dysgenesis system (Petrov et al. 1995; Evgen'ev et al. 1997; Vieira et al. 1998). This comobilization of multiple families of elements is surprising given the indepen-

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dent mobilization of single elements in previously described hybrid dysgenesis systems (Kidwell 1979; Yannopoulos et al. 1987).

The *virilis* species group of *Drosophila* (Hsu 1952; Patterson and Stone 1952; Spicer 1992) includes 12 species and is divided into two subgroups, or phylads, *virilis* and *montana* (Throckmorton 1982). Classical cytogenetic studies of the *virilis* group established that the *D. virilis* karyotype is the most ancestral based on chromosomal phylogenies (Hsu 1952; Patterson and Stone 1952; Spicer 1992). The *virilis* group is rich in karyotypic variation, which has been described in the classical works of Patterson and Stone (1952). An interesting possibility is that the observed karyotypic variation may be associated with the activity of mobile elements, especially of those that are coordinately mobilized during hybrid dysgenesis, in this species group. In *D. melanogaster*, the mechanism of aberration formation has recently been solved for P-M hybrid dysgenesis. *P* element-induced insertion and recombination can produce chromosomal rearrangements using well-defined mechanisms (Gray et al. 1996; Gloor and Lankenau 1998).

The purpose of this paper is to describe the distribution of mobile elements in species of the *virilis* group, with particular emphasis on *Penelope* and *Ulysses*. These two element families were chosen, not only because they are comobilized in dysgenic crosses, but also because *Penelope* is able to induce *Ulysses* transposition when injected into early embryos of a *Penelope*-free *D. virilis* strain (Evgen'ev et al. 1997). This study was designed to be the first step in testing the hypothesis of an association between TE activity and inversion formation in the *virilis* species group. Evidence is presented that *Penelope* and *Ulysses* have had a long history in the *virilis* species group. Furthermore, the sites of insertion of these TEs show a statistically significant association with one another, as well as with breakpoints of inversions previously established in species of the group.

Materials and methods

Species and strains. The species used in the study are listed in Tables 1 and 2 for the *Drosophila* and *Sophophora* subgenera, respectively. The geographical strains of species of the *virilis* group that were used are listed in Table 1. The numbers used to refer to these strains correspond either to numbers assigned by the Bowling Green Center or by the Stock Center of the Institute of Developmental Biology, Moscow. Flies were maintained on standard medium at 25°C.

DNA isolation and Southern analysis. DNA from adults of different wild-type strains of *D. virilis* and other species of the group was prepared as described by Zelentsova et al. (1986). Aliquots (20 µg) of genomic DNA were used for typical restriction enzyme digestion and Southern blotting following electrophoresis on 1% agarose gels. Gels were denatured, capillary-blotted onto nylon membranes according to the manufacturer's protocol, and fixed by UV cross-linking using a UV Stratalinker 2400 (Stratagene) protocol. Standard high-stringency hybridization and wash conditions were used for Southern blot analysis of species of the *virilis* group. Hybridization was carried out at lower stringency (52°C, 4×SSC) for species other than the *virilis* group. (1×SSC is 0.15 M NaCl, 0.015 M sodium citrate.)

In situ hybridization to polytene chromosomes and cytological analysis. Salivary glands were dissected from male and female third instar larvae in 45% acetic acid and squashed according to procedures developed by Lim (1993). For in situ hybridization studies, larvae were grown at 18°C and live yeast solution was usually added to the culture 2 days before the larvae were analyzed. DNA probes were biotinylated by nick translation using biotin 14-dATP essentially as described (Lim 1993). In some series of experiments, a ^{3}H-labeled probe was used as described (Scheinker et al. 1990). All localizations were made using the cytological map of *D. virilis* chromosomes (Hsu 1952) that is divided into 1371 subsections. Note that the accuracy of localization in our data was to a single subsection, not to a single band. Although each subsection usually includes one prominent band, the total number of subsections is less than the total number of bands. This method is similar to that used by Mukabayire and Besansky (1996) to study the distribution of *T1*, *Q*, *Pegasus* and *mariner* transposable elements in *Anopheles gambiae.* The genome of the latter species was divided into only 170 subdivisions, with 5 bands per subdivision, and hence the total number of bands was 850. Therefore, although these authors considered the unit of localization to be a distinct band, rather than a single subdivision, the degree of resolution was relatively low per unit length of chromosome and was probably no better than that in the present study. This is because the *A. gambiae* genome is almost double the size of that of *D. melanogaster* (260 Mb for *A. gambiae*, versus 140 Mb for *D. melanogaster*). Therefore accuracy to one of the 850 bands of *A. gambiae* is expected to provide less resolution than accuracy to one of the 1371 subsections of *D. virilis.* Breakpoint determinations of most of the inversions of the *virilis* species group were originally made by Hsu (1952) and later confirmed by one of the authors (H.P.).

Probes used for in situ hybridization and Southern blot analysis. The *Penelope* probe is 8 kb in length and represents the largest copy of this element isolated so far (Evgen'ev et al. 1997). It has an organization suggestive of a tandem array of two copies of *Penelope*, one of them containing the complete open reading frame of the element (Evgen'ev et al. 1997). This copy, along with flanking sequences of about 1 kb, was cloned into a Puc18 SacI site. Sometimes we isolated the 2.6 kb XhoI-XhoI fragment of this clone representing a full-sized copy of the element and used it in our in situ and Southern blot studies. In most cases, we used the whole clone, which does not contain other repetitive sequences. Therefore a good internal control for intensity of hybridization and efficiency of blotting is provided by a unique flanking sequence present in the clone.

One *Ulysses* probe contains 2.5 kb of the internal part of the element bounded by the EcoRI and BamHI restriction sites and cloned into Puc18. A second *Ulysses* probe contains the NdeI-ClaI fragment of *Ulysses*, representing a complete long terminal repeat (LTR) isolated from agarose after restriction digestion and electrophoresis (Evgen'ev et al. 1992). In contrast to the *micropia* retrotransposon, in which members of the same family frequently differ in the structure of their LTRs (Lankenau et al. 1989), all species of the *virilis* phylad (with the exception of *D. lumme* and *D. kanekoi*) have the same LTR structure, based on Southern blot analysis (see Fig. 1b). In addition, we used the 2.9 kb HindIII-ApaI internal fragment of the *D. virilis gypsy* element (M.B. Evgen'ev, unpublished results) and a probe obtained from Dr. H. Lozovskaya containing the 3' part of the *Helena* retrotransposon (Petrov et al. 1995).

The *Paris* probe represents an internal 1 kb fragment of this element obtained by us by the polymerase chain reaction (PCR) from genomic DNA of the *D. virilis* Batumi strain, using the following primers: 5' GTTCACATCTTGCCTAA and 3' CCATTACAGCCCGTAGAC, which delimit the internal part of *Paris* (Petrov et al. 1995). The 100 µl PCR mixture contained 1×PCR buffer (10 mM TRIS-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin), 0.2 mM of each of the dNTPs, 0.1 M of each primer, 300 ng of *D. virilis* (Batumi strain) genomic DNA and

2.5 U AmpliTaq DNA polymerase (Perkin Elmer Cetus). After an initial 2 min at 94°C, the following sequence was repeated 35 times: denaturation 2 min 94°C, annealing 1 min 60°C, extension 2 min 72°C. The final extension was 10 min at 72°C. Ten microliters of each PCR product was analyzed on 1% agarose gels. Subsequently the resulting fragment of the *Paris* element was cloned using a TA-cloning kit. Clones of interest were sequenced with Sequenase (Amersham). Genomic libraries from *D. texana* and *D. montana* strains were prepared by partial Sau3A digestion, with subsequent ligation into the BamHI site of Lambda Dash (Stratagene) as previously described (Evgen'ev et al. 1997). *Penelope*-containing clones were isolated from these libraries using the 2.6 kb XhoI-XhoI fragment of *Penelope* from *D. virilis* (see above).

Results

Penelope *and* Ulysses *are clearly identified only in the* virilis *species group*

The results summarized in Tables 1 and 2 indicate that sequences clearly identifiable to the *Penelope* and *Ulysses* families of elements appear to be restricted to the *virilis* species group. However, several species belonging to the *repleta*, *willistoni* and *saltans* species groups showed weak hybridization to the *Penelope* or *Ulysses* probes. These cases may reflect the presence of members of element families that are highly diverged from the *Penelope* and *Ulysses* sequences isolated from *D. virilis*. Alternatively, they may reflect nonspecific binding, because the signals were weak, despite hybridization with heterologous species DNA under conditions of low stringency. In all these exceptional cases, the signal was only observed after long exposure. The intensity of the hybridization signals was very faint. Therefore, until the corresponding sequences are determined, it will be impossible to know whether these species really contain any sequences related to *Penelope* or *Ulysses*.

Penelope *and* Ulysses *have recently been active in most members of the* virilis *species group*

Figure 1A shows the distribution of *Penelope* in species of the *virilis* group as determined by Southern blot analysis. DNA was digested with XhoI and hybridized with a labeled *Penelope*-containing probe (Materials and methods). XhoI sites are located at both ends of the complete *Penelope* element (Evgen'ev et al. 1997). Thus, the thickness of the 2.6 kb band, indicated by an arrow in Fig. 1A, provides an estimate of the number of potentially full-sized *Penelope* elements in each species. Most species of the group exhibit a comparatively high copy number and *Penelope* distribution and structure exhibit significant polymorphism. However, two species,

Table 1. Distribution of *Penelope* and *Ulysses* in species of the *repleta*, and *virilis* species groups of the *Drosophila* subgenus

Species group	Species	*Penelope*[a]	*Ulysses*[a]	Species group	Species	Strains	*Penelope*[a]	*Ulysses*[a]
repleta	*repleta*	+	–[b]	*virilis*	*virilis*		+++	+++
	neorepleta	+	–		*lummei*	200, 201, 234, 1101, ST, 211	+	+
	mercatorum	–	–		*texana*	418, 419, 420, 423	+++	+++
	paranaensis	–	–		*americana*	405, 0951.0, 0951.9	+++	+++
	peninsularis	–	–		*novamexicana*	400, 1031.42	+++	+++
	meridiana	+	–		*montana*	1021.0, 1021.22, 1021.19, 1021.13, 1022.18, 502		
						D. m. ovivororum	+++	+++
	buzzatti	+	–		*kanekoi*	1051.0	+	++
	mojavensis	–	–		*flavomontana*	0981.2, 565	+++	+++
	arzonae	+	–		*borealis*	0961.3, 0961.0, 520	++	++
	mulleri	–	–		*lacicola*	540, 0991, 13, 0991.0	+++	+++
	hydei	–	–		*littoralis*	1013, 1001.3, 1001.0, RX, 4-11	+++	+++
	neohydei	–	–		*ezoana*	570, 0971.0	+++	+++
	euneohydei	–	–					

[a] The number of + symbols indicates the strength of hybridization to the probe
[b] – indicates no detectable hybridization

Table 2. Distribution of *Penelope* and *Ulysses* in species of the *willistoni, saltans* and *melanogaster* species groups of the *Sophophora* subgenus

Species group	Species	*Penelope*[a]	*Ulysses*	Species	Species group	*Penelope*[a]	*Ulysses*[a]
willistoni	*willistoni*	–[b]	–	*melanogaster*	*simulans*	–	–
	pavlovskiana	–	–		*yakuba*	–	–
	tropicalis	–	–		*nikananu*	–	–
	equinoxalis	+	–		*lutescens*	–	–
	paulistorum	+	–		*mimetica*	–	+
	fumipennis	–	–		*biauraria*	–	–
	succinea	–	–		*ficusphila*	–	–
saltans	*saltans*	–	–		*ananassae*	+	–
	emarginata	–	–		*malerkotliana*	–	–
	australosaltans	–	–		*quadraria*	–	–
					rufa	–	–
					tscasi	–	–

[a] The number of + symbols indicates the strength of hybridization to the probe
[b] – indicates no detectable hybridization

D. lummei and *D. kanekoi*, appear to be free of complete elements and carry very few, if any, *Penelope*-related sequences.

When the same species were probed with the 2.5 kb EcoRI and BamHI internal fragment of *Ulysses*, *D. lummei* again seemed to have very few hybridizing sequences. However, *D. kanekoi* clearly exhibited a significant level of hybridization (data not shown). When species of the group were checked for the presence of intact *Ulysses* LTRs, using the second probe, only a weak signal was seen at the expected position in *D. lummei* DNA and no hybridization at all was detected at the expected position in *D. kanekoi* (Fig. 1B). Essentially the same picture was seen when the reverse transcriptase-encoding region of *Ulysses* was used as a probe (data not shown). When *gypsy* and *Helena* probes were used for comparison in Southern blot analyses of the same species, significant hybridization is evident in all species studied, including *D. kanekoi* and *D. lummei* (Fig. 1C, D). The *Paris* element also hybridized with all species of the *virilis* group in a relatively uniform way (data not shown).

Taken altogether, the results of this series of experiments suggest that, in contrast to other species of the group, *D. lummei* and *D. kanekoi* lack full-sized functional copies of both the *Penelope* and *Ulysses* elements. Additional evidence in favor of recent transposition of *Penelope* and *Ulysses* in the other species of the group comes from in situ hybridization analyses that revealed significant interstrain polymorphism in chromosomal distribution of these elements (described in detail below).

Penelope *and* Ulysses *are predominantly found in the chromocenter in most* virilis *group species*

To investigate further the distribution patterns of *Penelope* in *D. lummei*, we carried out Southern blot analysis of individual *D. lummei* strains of widely different geographical origin in comparison with a reference *P*-like strain of *D. virilis* (Fig. 2A). Note that the bands of *Penelope*-hybridizing DNA in the *D. lummei* lanes are usually very faint and are only seen after a long exposure time. This pattern is in contrast to the darker bands that are evident at approximately the same positions at the top of the gel, in all lanes of Fig. 2A. The latter are due to hybridization with unique flanking sequences that are present in the probe (Materials and methods). The results indicate that most *D. lummei* strains have an almost identical pattern of hybridization, suggesting that *Penelope* elements are mostly located in the centric heterochromatin in this species. It is necessary to emphasize, however, that not all *D. lummei* strains are identical in this respect. Thus the Moscow strain (lane 4) exhibits some polymorphism with a weak signal corresponding to the position of the 2.6 kb band, indicating the presence of a complete, potentially active *Penelope* copy.

All other *virilis* group species analyzed for the *Penelope* element were characterized by a rather high level of polymorphism when strains of different geographical origin were examined. Figure 2B provides an example of the results of Southern blot analysis when different geographical strains of *D. montana* were investigated. All strains contain the prominent 2.6 kb band, indicating the presence of a full-sized *Penelope* copy. Significant polymorphism in the patterns of hybridization bands is probably due to different positions being occupied by *Penelope* elements in the chromosomes of the strains studied. Similar results were seen when the same *D. montana* strains were examined for the presence of *Ulysses* (Fig. 2C). A similar high level of polymorphism was indicated by hybridization patterns for *Penelope* and *Ulysses* elements in different geographical strains of *D. littoralis*, another representative of the *montana* phylad (data not shown). Therefore our Southern hybridization experiments have confirmed that, while multiple complete and incomplete copies of *Penelope* and *Ulysses* are present in most species of the *virilis* group, complete copies of both of these elements are absent in *D. lummei* and *D.*

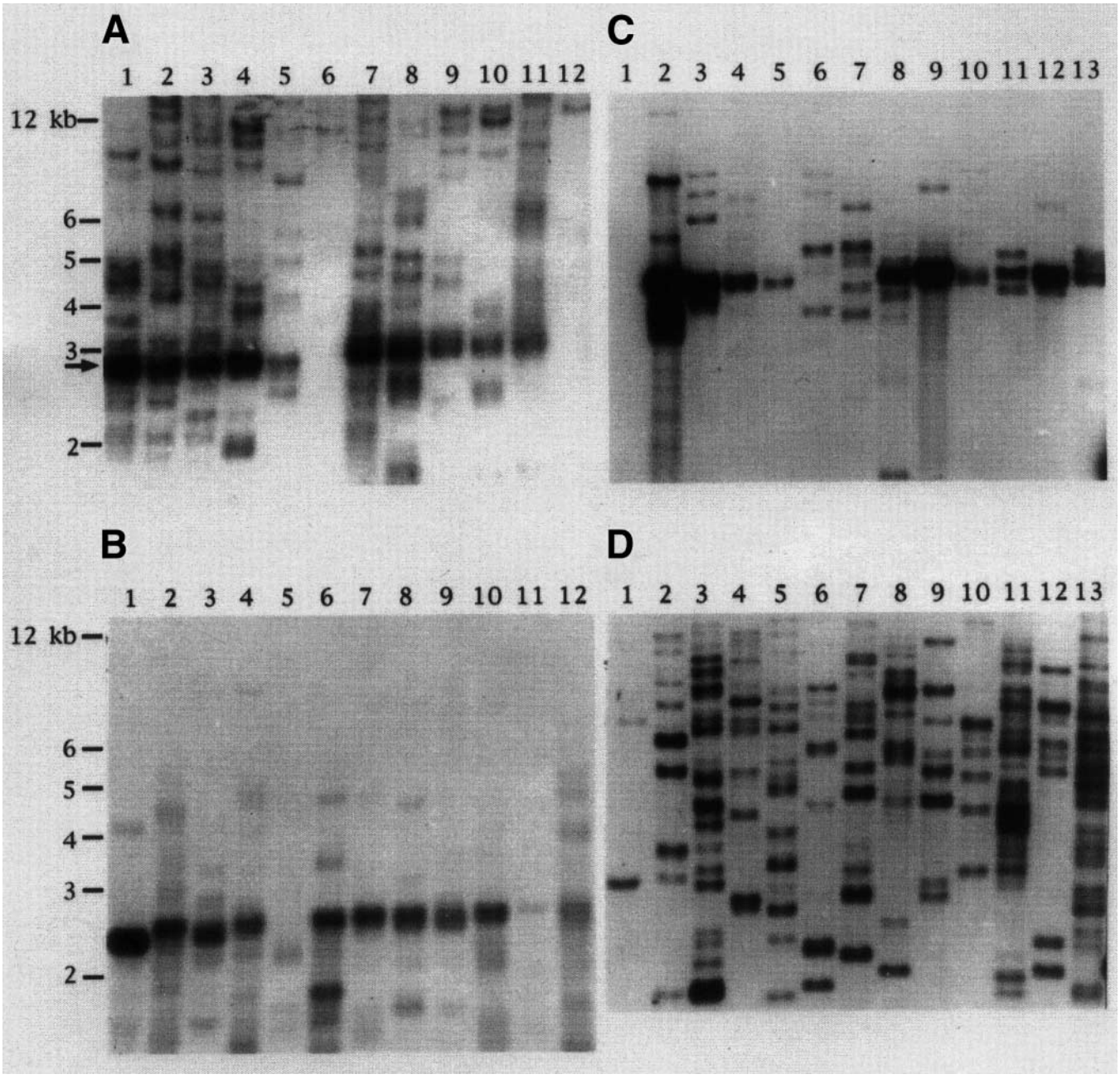

Fig. 1A–D. Southern blot analysis of genomic DNA from species of the *virilis* group of *Drosophila* probed with different *D. virilis* transposable elements. **A** A blot of XhoI-digested genomic DNA hybridized with a *Penelope* probe. *Lanes:* (*1*) *D. virilis* strain 160; (*2*) *D. montana*; (*3*) *D. flavomontana*; (*4*) *D. americana*; (*5*) *D. borealis*; (*6*) *D. kanekoi*; (*7*) *D. ezoana*; (*8*) *D. lacicola*; (*9*) *D. novomexicana*; (*10*) *D. texana*; (*11*) *D. littoralis*; (*12*) *D. lummei*. The position of the 2.6 kb band is indicated by an *arrow*. **B** Analysis of NdeI+ClaI-digested DNA probed with the NdeI-ClaI fragment of *Ulysses*. The 2.2 kb band corresponds to the long terminal repeats. *Lanes:* (*1*) *D. virilis* strain 160; (*2*) *D. flavomontana*; (*3*) *D. americana*; (*4*) *D. borealis*; (*5*) *D. kanekoi*; (*6*) *D. ezoana*; (*7*) *D. lacicola*; (*8*) *D. novomexicana*; (*9*) *D. texana*; (*10*) *D. montana*; (*11*) *D. lummei*; (*12*) *D. littoralis*. **C** Analysis of *Hin*dIII-digested genomic DNA probed with the *gypsy* clone. *Lanes:* (*1*) *D. melanogaster* Oregon R (control); (*2*) *D. virilis* strain 160; (*3*) *D. flavomontana*; (*4*) *D. americana*; (*5*) *D. borealis*; (*6*) *D. kanekoi*; (*7*) *D. ezoana*; (*8*) *D. lacicola*; (*9*) *D. novomexicana*; (*10*) *D. texana*; (*11*) *D. montana*; (*12*) *D. lummei*; (*13*) *D. littoralis*. **D** The same blot as in **C** was stripped of label by boiling and rehybridized with a probe containing a region of the *Helena* element (Materials and methods)

kanekoi, and the numbers of incomplete copies are relatively small in comparison with those for other species of the group.

Further evidence for the predominant localization of *Penelope*-related sequences to the chromocenter in most *virilis* group species is provided by in situ hybridization experiments. The heavy labeling of the *Penelope* probe to the chromocenter that was observed in *D. littoralis* and *D. montana* (Fig. 3B, C) is typical for all species of the *virilis* group, except for *D. virilis* (Fig. 3E). The label is usually concentrated predominantly in the heterochromatic chromocenter, but a few euchromatic sites are

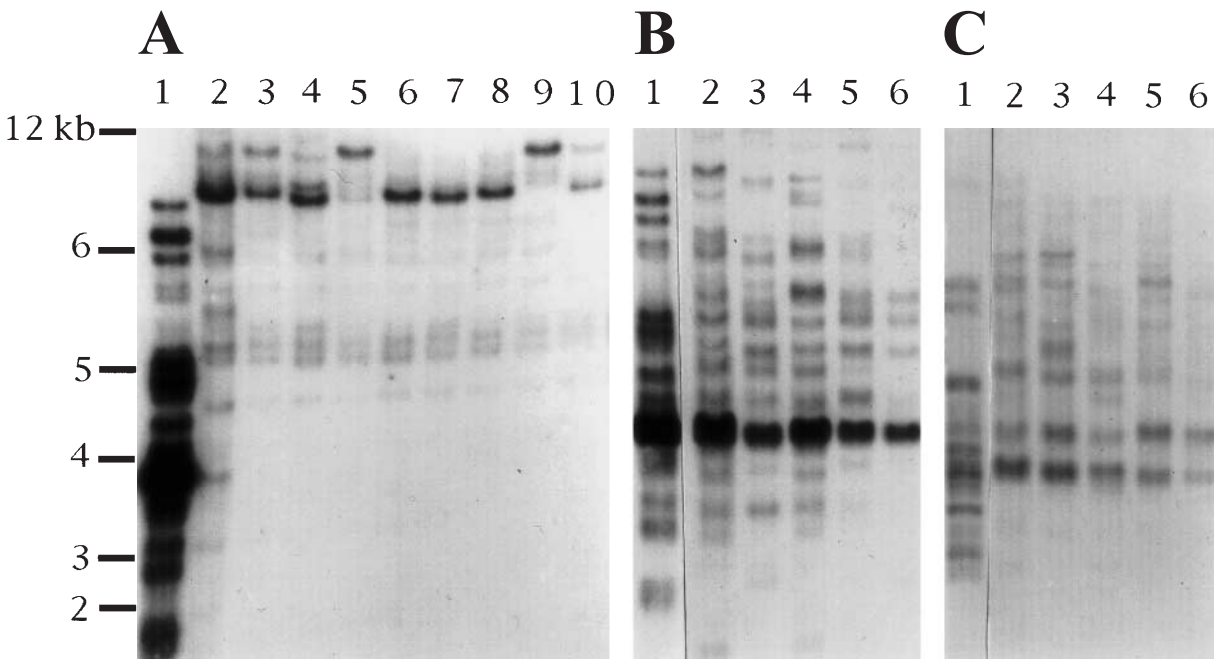

Fig. 2A–C. Southern blots of different geographical strains of *D. lummei* and *D. montana* probed with *Penelope* and *Ulysses*. **A** XhoI-digested genomic DNA isolated from different strains of *D. lummei* and probed with a *Penelope*-containing clone. *Lanes:* (*1*) *D. virilis* strain 160; (*2*) *D. lummei* strain 200, collected in Moscow region; (*3*) *D. lummei* strain 201, collected in Moscow region; (*4*) *D. lummei* strain 203, collected in Moscow region; (*5*) *D. lummei* strain 223, collected close to Krasnodar town, South Russia; (*6*) *D. lummei* strain 1102, from Finland; (*7*) *D. lummei* strain 1100, from Finland; (*8*) *D. lummei* strain 1109, from Sweden; (*9*) *D. lummei* strain of unknown origin carrying an *sv* mutation; (*10*) *D. lummei* strain of unknown origin carrying a *w* mutation. High molecular weight bands evident in all lanes are due to hybridization with flanking genomic sequences present in the *Penelope*-containing clone used for hybridization (Materials and methods). **B** XhoI-digested DNA isolated from different geographical *D. montana* strains blotted and hybridized with a *Penelope* probe. The position of the 2.6 kb band indicative of the presence of full-sized *Penelope* copies is indicated by the *arrow*. *Lanes:* (*1*) *D. virilis* strain 160; (*2*) *D. montana* strain 1021.9, from Colorado; (*3*) *D. montana* strain 1021.19, from Oregon; (*4*) *D. montana* strain 1021.14, from Utah; (*5*) *D. montana* strain 1021.22, from Alaska; (*6*) *D. montana-ovivororum*. **C** The same blot as in **B** was stripped of DNA by boiling and rehybridized with the EcoRI-BamHI internal fragment of the *Ulysses* element

also seen. Similar experiments using a *Ulysses*-containing probe generally showed the same pattern for *D. littoralis*, *D. texana* and *D. lummei* (Fig. 3A, D and F, respectively). The *D. texana* chromosomes were hybridized with a ^{3}H-labeled *Ulysses* probe (see Fig. 3D and Materials and methods). In contrast, in *D. virilis*, the number of *Ulysses* insertion sites in euchromatin has generally been observed to be 15–25, several times higher than for other species of the group (Scheinker et al. 1990; Evgen'ev et al. 1997).

Another independent line of evidence favoring a predominantly heterochromatic localization of *Penelope* in species of the *virilis* group comes from in situ hybridization studies using different clones of *D. texana* and *D. montana* isolated from corresponding genomic libraries. Five *D. texana* clones, and 11 *D. montana* clones were hybridized with polytene chromosomes of a *D. virilis* M-like strain lacking *Penelope* copies. All hybridization sites observed in the chromosomes were therefore due to the presence of the flanking sequences contained in these *D. montana* and *D. texana* genomic clones. The analysis revealed that only one clone from each of the two species hybridized with just a single chromosomal site in *D. virilis* chromosomes (data not shown). The remaining clones hybridized with multiple sites in *D. virilis* chromosomes, presumably due to the presence of repetitive DNA associated with the majority of elements located in heterochromatin in these species.

Under-representation of *Penelope* sequences in *D. texana* polytene chromosomes is consistent with their prominent heterochromatic location. Previously we have demonstrated by in situ hybridization analysis that the majority of *Penelope* elements in *D. virilis* are located in the euchromatic arms of chromosomes (Evgen'ev et al. 1997). In contrast, we have presented data that demonstrate that, in all other species of the group, *Penelope* elements have a predominantly heterochromatic location (see Fig. 3). Moreover, we have shown that, in contrast to *D. virilis*, most *Penelope* elements in *D. texana* and *D. montana* are embedded in repetitive DNA; almost all *Penelope*-containing clones (including flanking DNA) isolated from these species gave multiple hybridizations with *D. virilis* polytene chromosomes from an M-like strain (see above). Keeping in mind the well-known cases of under-replication of satellite DNA during polytenization in salivary gland cells (Gall and Atherton 1974), and the recently described under-representation of several heterochromatin-associated TEs in polytene chromosome-containing cells (reviewed by Arkhipova et al. 1995), we compared the representation of *Penelope*

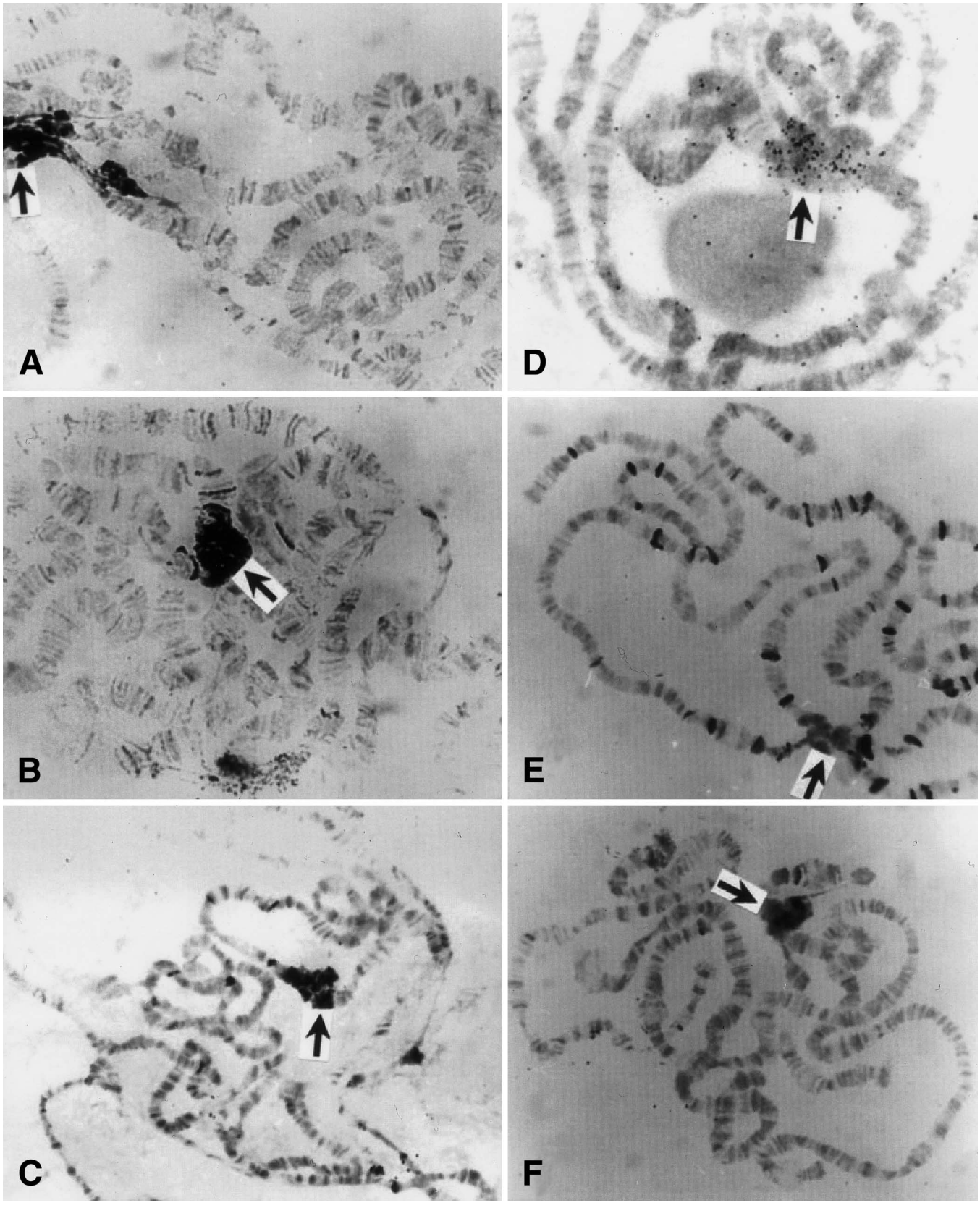

Fig. 3A–F. In situ hybridization of *Penelope* and *Ulysses* DNA probes to polytene chromosomes of selected species of the *virilis* group. **A** *Ulysses* with *D. littoralis* chromosomes. **B** *Penelope* with *D. littoralis* chromosomes. **C** *Penelope* with *D. montana* chromosomes. **D** *Ulysses* with *D. texana* chromosomes. **E** *Penelope* with *D. virilis* chromosomes. **F** *Ulysses* with *D. lummei* chromosomes. In each case, the heavily labeled chromocenter is indicated by an *arrow*

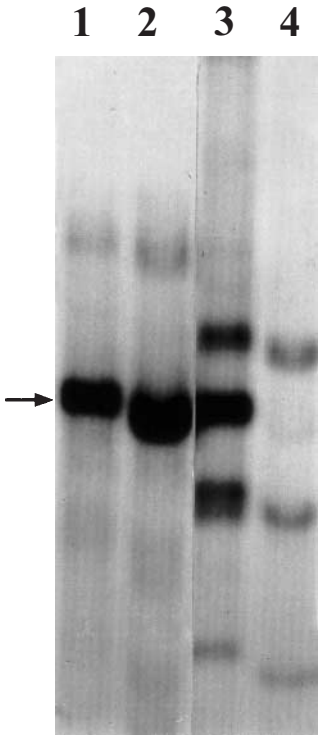

Fig. 4. Comparison of *Penelope* DNA content in adult flies with that obtained from salivary glands. DNA from *D. virilis* strain 160 and *D. texana* was isolated essentially as described in Materials and methods, digested with XhoI and blotted. The blot was hybridized with a *Penelope* probe. *Lanes:* (*1*) adult flies of *D. virilis* strain 160; (*2*) salivary glands of *D. virilis* strain 160; (*3*) adult flies of *D. texana*; (*4*) salivary glands of *D. texana*. The position of the 2.6 kb band indicative of the presence of full-sized *Penelope* copies is shown by an *arrow*. The slight difference observed in the molecular weight of corresponding bands between adult flies and salivary gland DNA is due to the fact that DNA extracted from whole flies is more prone to degradation during the extraction procedure because of the presence of more nucleases (Franco et al. 1993)

in two species, i.e., *D. virilis* and *D. texana*, that have strikingly different distributions of this element. Figure 4 illustrates the results of such a comparison. Evidently, in the case of *D. virilis* strain 160, in which *Penelope* elements are found mostly in euchromatic sites, there is no observable difference between *Penelope* content in the DNA isolated from whole adult flies and salivary gland DNA (lanes 1 and 2). On the other hand, when we compared equal amounts of DNA isolated either from adult flies, or from larval salivary glands (lanes 3 and 4) in *D. texana*, a species in which *Penelope* copies are located predominantly in the heterochromatic chromocenter, a clear difference between the two DNA samples is demonstrated. Severe under-representation of *Penelope* sequences in *D. texana* salivary gland DNA is evident (lane 4).

The Penelope *and* Ulysses *locations are correlated with each other and with inversion breakpoints*

Cytological analysis of different geographical strains of *virilis* group species was performed. Usually five to six larvae were examined for each strain. In situ hybridization to polytene chromosomes revealed a total of 79 euchromatic hybridization sites for *Penelope* and 47 sites for *Ulysses* for all species and strains studied in the *virilis* species group, with the exception of *D. virilis* itself. Figure 5A and B shows the chromosomal distributions of these sites for *Penelope* and *Ulysses*, respectively. Initial visual inspection of Fig. 5A and B suggests that the distributions of both *Penelope* and *Ulysses* insertion sites are far from random. The highest numbers of *Penelope* insertion sites are found in chromosomes *2* and *4* (26 and 20 sites, respectively), with an internal region of chromosome *2* (bands L through U) having the highest density. Chromosomes *X, 3, 5* and *6* (the microchromosome) have 10, 12, 11 and 0 *Penelope* sites, respectively. The highest numbers of *Ulysses* insertion sites are found in chromosomes *2* and *3* (13 and 15 sites, respectively). Chromosomes *X, 3, 5* and *6* have 7, 3, 6 and 3 *Ulysses* sites, respectively. In addition some sites are occupied by more than one species, as would be expected if insertions into these sites pre-date speciation events.

It was of interest to compare the chromosomal localization of the euchromatic insertion sites identified in Fig. 5A and B among different geographical strains of the same species and among different species. The data summarized in Table 3 clearly show significant interstrain polymorphisms for both *Penelope* and *Ulysses*. The vast majority of *Penelope* and *Ulysses* sites were occupied in only one geographical strain of a given species; we failed to detect any fixed hybridization sites common to all geographical strains of a given species. In contrast, three fixed sites for the *gypsy* element are present in all species of the group (M. B. Evgen'ev, unpublished results). Surprisingly, common *Penelope* and *Ulysses* sites are more likely to be shared by species in the same phylad than by strains in the same species. This situation is particularly evident for *Ulysses* for which nine species have insertion sites shared among species of the same phylad. In contrast, only one of the nine has a site shared with other strains of the same species. Although eight species have at least one *Penelope* site shared with another strain in the same species, in most cases the number shared with other species is greater, i.e., they show the same tendency as the *Ulysses* data.

The high frequency of association of *Penelope* and *Ulysses* hybridization sites with inversion breakpoints previously documented for species of the group is surprising. In Fig. 5A and B, both the numbers and locations of *Penelope* or *Ulysses* insertions are shown above and the locations of inversion breakpoints below the chromosomal map (Table 4). In all cases reported, the measure of coincidence is defined as occurrence of a site within the same chromosomal subsection, according to the *D. virilis* map of Hsu (1952). It is noteworthy that sites occupied by *Penelope* (or *Ulysses*) in one species (e.g., *D. littoralis*) frequently coincide with inversion breakpoints characteristic for another species of the group. For example, the site Yf in the *X* chromosome carries *Penelope* in *D. ezoana*, *Ulysses* in *D. littoralis* and coincides with the cosmopolitan inversion "a", which is typical for *texana-americana-novamexicana*;

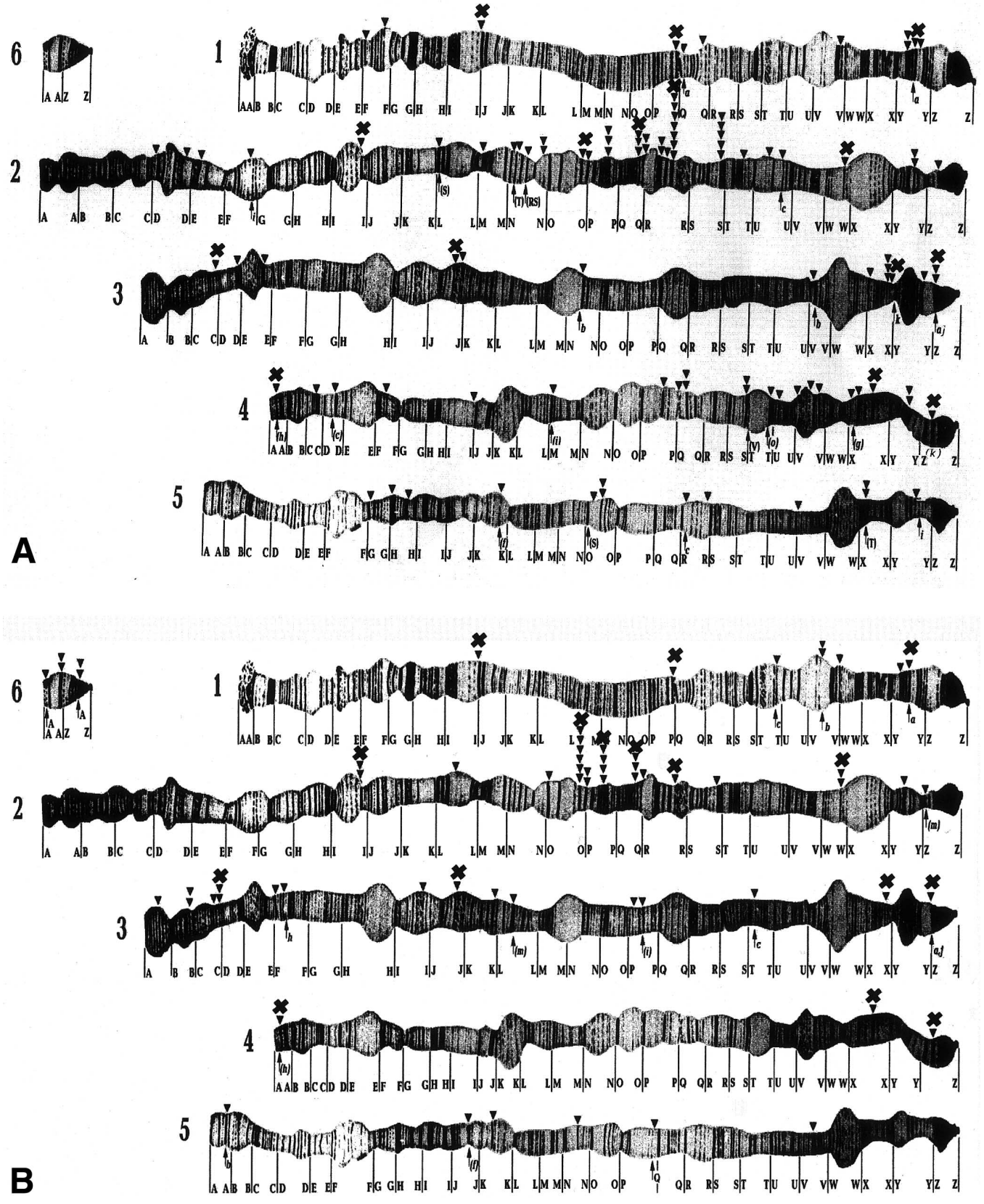

Fig. 5. The pooled distributions of *Penelope* (**A**) and *Ulysses* (**B**) in chromosomes of *virilis* group species. The positions of the elements are shown by *filled triangles* on a map of *D. virilis* chromosomes (Hsu 1952). The *number of these triangles* above a given subsection of a chromosome represents the number of *virilis* group species in which hybridization was detected at this site in at least one geographical strain. The 16 positions at which *Ulysses* insertions coincide with those of *Penelope* are indicated by *X* above the chromosomes (see also Table 4). The positions at which *Ulysses* and *Penelope* positions coincide with inversions breakpoints known for species of the group are indicated by *arrows* below the chromosomes. The *letters in parentheses close to the arrows* indicate endemic inversions that are restricted to only some geographical strains. The *letters without parentheses* indicate cosmopolitan inversions that are characteristic for a certain species or group of species

Table 3. The occurrence of *Penelope* and *Ulysses* hybridization sites in species of the *virilis* group

Phylad	Species	No. strains examined per species	Total no. of hybridization sites for all strains of a species	
			Penelope	*Ulysses*
virilis	*D. americana*	3	11	6 [3][a]
	D. texana	4	8 (1)[b]	–[c]
	D. novamexicana	2	4	8 [5]
	D. lummei	6	9 (3)	20 [8]
montana	*D. montana*	7	17 (5) [8]	2 [1]
	D. borealis	3	6 (2) [6]	2 [2]
	D. lacicola	3	4 (1) [3]	6 (1) [3]
	D. flavomontana	1	16 (6) [3]	2
	D. littoralis	5	18 (2) [6]	13 [3]
	D. kanekoi	1	1 [1]	1 [1]
	D. ezoana	2	15 (1) [3]	2 [1]

[a] Square brackets indicate the number of sites shared with different species in the same phylad
[b] Round brackets indicate the number of sites shared with other members of the same species
[c] – indicates hybridization to the chromocenter only

Table 4. Common chromosomal sites of *Penelope* and *Ulysses* hybridization and coincidence with inversion breakpoints (*BP*) in species and strains of the *virilis* species group

Site	*Penelope* hybridization	*Ulysses* hybridization	Inversion BPs
X Ij	*D. ezoana* 570, *	*D. lacicola* 0991.13	
X Ph	*D. ezoana* 570, *	*D. americana* 0951.9, *	
X Yf	*D. ezoana* 570, *	*D. littoralis* 4-11, *	a
2 Ii	*D. flavomontana* 0981.2	*D. lummei* 201, *D. littoralis* 4-11, *	
2 Og	*D. ezoana* 570, *	*D. lummei* ST, *D. montana* 1021.19, *D. borealis* 0961.3, *D. littoralis* 4-11, *D. kanekoi* 1051.0, *D. ezoana* (all), *	
2 Pi	*D. montana* 502, *D. flavomontana* 0981.2, *D. littoralis* 1013,*	*D. lummei* 200, 201 and 234, *D. lacicola* 0991.0 *D. lummei* ST and 234, *D. americana* 0951.0, *	
2 Qi	*D. lummei* 201, *D. littoralis* 1013 and 4–11, *		
2 Rh	*D. montana ovivorum*, *D. montana* 502, 1029.19, 1021.0, *D. lacicola* 540, *D. borealis* 0961.3, *D. flavomontana* 0981.2, *	*D. lummei* 234	
2 Wf	*D. lummei* 200, 201, 234, ST, *	*D. novamexicana* 1031.42, *D. lacicola* 0991.13, *	
3 Cj	*D. littoralis* 4-11	*D. borealis* 0961.3, *D. lacicola* 0991.0, *	
3 Ji	*D. americana* 405	*D. lummei* 1101S, *	
3 Xi	*D. littoralis* 4-11, *	*D. lummei* 1101S, *	
3 Za	*D. littoralis* 1001.3, *D. ezoana* 570	*D. littoralis* 4-11	a, j
4Ad	*D. texana* 423, *	*D. americana* 0951.9, *	h
4 Xk	*D. littoralis* 4-11, *	*D. littoralis* 4-11	
4 Zc	*D. americana* 0951.0, *	*D. littoralis* 4-11, *	(k)

* Represents sites also present in one or more of 16 *D. virilis* strains examined (M.B. Evgen'ev et al., unpublished results).

also site Ad in chromosome *4* carries *Penelope* in *D. texana*, *Ulysses* in *D. americana* and coincides with the cosmopolitan inversion "h" found in both *D. montana* and *D. lacicola* (see Table 4). The total numbers of insertions that appear to coincide with known inversion breakpoints in the same phylad (Hsu 1952) are eight for *Penelope* and nine for *Ulysses* within the *virilis* phylad and 13 for *Penelope* and six for *Ulysses* within the *montana* phylad. The total numbers of insertions that appear to coincide with the locations of known inversion breakpoints in different phylads are seven for *Penelope* and five for *Ulysses*.

Table 4 provides another surprising result. Cytological analysis indicates that 16 chromosomal subsections are shared by at least one insertion of both *Penelope* and *Ulysses* in one or more strains of species of the *virilis* group. Furthermore, a large majority of these 16 sites are also shared with insertion sites of both elements in at least one strain of *D. virilis* (Zelentsova et al., unpublished results). In addition, 4 of the 16 insertion sites that are shared by *Penelope* and *Ulysses* are also shared by an inversion breakpoint (Table 4).

The data on association of chromosomal locations of *Penelope* and *Ulysses* insertion sites with one another

Table 5. Association of mobile elements *Penelope* (*Pen*) and *Ulysses* (*Uly*) with inversion breakpoints in genomes of the *virilis* group

Comparison type	C_{obs}	C_{exp}	Significance
Pen vs breakpoint	22	8.30	$P<<0.001$
Uly vs breakpoint	15	4.94	$P<<0.001$
Pen vs Uly	16	2.71	$P<<0.001$

The number of subsections on the chromosomal map of *D. virilis* is 1371. The total number of inversion breakpoints found in species of the *virilis* group is 144 (Hsu 1952; Poluektova et al. 1991; Zhdanov et al. 1994). The total numbers of *Penelope* and *Ulysses* insertion sites found are 79 and 47, respectively. C_{obs} and C_{exp} are, respectively, the observed and expected numbers of mobile element insertion sites that coincide with inversion breakpoints

and with breakpoints of inversions previously identified for species of the group (Hsu 1952) were subjected to statistical analysis using the method described (see Appendix). The results of this analysis are presented in Table 5. For all three types of comparison, statistically significant differences were found between the observed and expected numbers of coincident sites, assuming that insertions occur independently of one another.

Discussion

Although at least five transposable elements are known to be involved in *D. virilis* hybrid dysgenesis (Vieira et al. 1998), this study has mainly focused on the distributions of the *Penelope* and *Ulysses* families of elements. Functional members of these two families appear to be restricted to the *virilis* species group, but a broader distribution of homologous elements that are highly diverged from the *D. virilis* elements cannot be excluded. Both *Penelope* and *Ulysses* appear to have had a long history in the *virilis* species group and their properties in present-day natural populations vary from one species to another. However, with the exception of *D. lummei* and *D. kanekoi*, some fraction of *Penelope* and *Ulysses* elements appear to be at least potentially functional in all species of the group. Also, active *Penelope* elements appear to have only recently invaded *D. virilis*, but the evidence for this is presented in detail elsewhere (Evgen'ev et al. 1997; M.B. Evgen'ev, in preparation).

The data presented here suggest that both *Penelope* and *Ulysses* have had a very long presence in the *virilis* species group. The most compelling evidence for this is the strong hybridization of *Penelope* and *Ulysses* probes to the chromocenter as exemplified in Fig. 3. Even *D. virilis*, a species that shows relatively weak hybridization of *Penelope* to the chromocenter (see Fig. 3E), has been shown to carry highly divergent *Penelope*-homologous sequences in an M-like strain (M. B. Evgen'ev, unpublished results). A general working hypothesis to explain the distribution of *Penelope* and *Ulysses* in species of the *virilis* group posits the presence of both *Penelope* and *Ulysses* in the ancestor of the whole *virilis* species group. Accordingly, the descendants of these ancient diverged forms are present in the chromocenters of all *virilis* group species, including *D. virilis*, *D. lummei* and *D. kanekoi*. It can be postulated further that either active forms of *Penelope* and *Ulysses* persisted in other species of the group, or that there were one or more instances of horizontal transfer of active elements into the other lineages at different times in the past. Horizontal transfer provides the most likely explanation for different subfamilies of the element being present in the same species. Observations of highly diverged subfamilies of the same element family within a single species have frequently been made for the *mariner* and *P* families of transposable elements (Robertson and Lampe 1995; Clark and Kidwell 1997). Detailed speculation about the historical relationship or dependence of the two element families must await the identification of the molecular mechanisms involved in co-mobilization.

The distributions of *Penelope* and *Ulysses* insertion locations, shown in Table 3, are unexpected and difficult to explain. It is not surprising that a number of *Penelope* and *Ulysses* insertion sites are shared among strains belonging to more than one species. However, it is surprising that these common sites are more frequently shared with other species in the same phylad than with other strains in the same species. This situation is particularly evident for *Ulysses* (see Table 3), for which nine species have insertion sites shared among species of the same phylad; however, only one of the nine has a *Ulysses* site shared with other strains of the same species.

Two explanations for the Table 3 data are possible if insertion of an element pre-dated, or coincided with, speciation. The first possibility is that the unexpected distribution of shared insertion sites reflects ancestral polymorphisms that pre-date speciation. However, in the case of insertion sites that were fixed at the time of speciation, the majority of strains of a species might be expected still to retain them, unless a loss had subsequently occurred (e.g., by ectopic recombination). In this case, insertions present at the same site in different descendent species are expected to be identical by descent. Furthermore, to explain the *Ulysses* data in Table 3, the rate of loss would have had to have been extremely high, and the species that share a site would be expected to be more closely related to one another than to a species with which the site was not shared. However, examination of the data did not reveal such a correlation.

A second possible explanation of the data in Table 3 is that *Penelope* and *Ulysses* insertions that are shared among different species are not necessarily identical by descent. Such insertions would represent transpositions occurring since strain divergence, with sites that are common among different species arising independently as a result of some type of site specificity (discussed below). This might be true for at least some of the shared insertion sites, but it seems to be unlikely that all the shared *Ulysses* insertions reported in Table 3 resulted from relatively recent independent transpositions. In any case, the two hypotheses are not mutually exclusive and the data reported in Table 3, particularly for *Penelope*, seem likely to represent a mixture of old and young insertion sites.

The high frequency of coincidence of *Penelope* and *Ulysses* insertion sites and of both with inversion breakpoints is one of the most surprising and least easily explained aspects of the results of this study. The existence of mechanisms for site specificity of integration, for both unrelated families of repeats, may be one possible explanation for the high frequency of coincidence between *Ulysses* and *Penelope* insertion sites. However, hot spots for chromosomal breakage may be a more likely explanation, because of the additional coincidence of some of these breakpoints with those of species-specific inversions. An alternative, not mutually exclusive, hypothesis is that the sites of mobile element insertions are directly related to the formation of inversion breakpoints.

A possible scenario to explain the coincidence of *Penelope* and *Ulysses* insertions in genomes of species belonging to the *virilis* group is as follows. We postulate that chromosomes of all *virilis* group species have a number of sites that are particularly sensitive to chromosomal breakage associated with a product of *Penelope* activity, such as an integrase. Sensitive sites are postulated to be hot spots for insertions of *Penelope, Ulysses* and possibly other mobile elements that are activated in *Penelope*-induced hybrid dysgenesis. The reality of the postulated specificity is reinforced by our original genetic studies (Lozovskaya et al. 1990), in which we clearly demonstrated that a highly specific spectrum of mutations is observed in the progeny of dysgenic crosses. Furthermore, confirmation is provided by our recent injection experiments in which we introduced *Penelope* into an M-like strain and were able to induce a spectrum of mutations very similar to that observed in the progeny of dysgenic crosses (Evgen'ev et al. 1997). Typically, when we used a *Penelope* clone having a deleted integrase region in our injection experiments, we failed to observe any mutants in the progeny of injected individuals.

The hypothesis that co-mobilization of other *D. virilis* TEs is related to the presence of a functional *Penelope* integrase can also be extended to the production of inversions. Following the invasion of a species, such as *D. virilis,* lacking *Penelope* elements, transposition of *Ulysses*, *Helena*, *Paris* and probably other quiescent transposons into staggered nicks made by activated *Penelope* integrase may represent the first step in a possible 'cytological revolution'. Later, ectopic pairing and recombination between copies of elements belonging to the same family of repeats may lead to formation of inversions and various other rearrangements. According to this scenario, other elements, besides *Penelope*, are expected to be associated with rearrangement breakpoints that arose during hybrid dysgenesis. This prediction was realized by an inversion having *Ulysses* sequences at the breakpoints in the progeny of *D. virilis* dysgenic crosses (Scheinker et al. 1990). The presence of *Ulysses* at both breakpoints of inversion 3h in *D. littoralis* (M.B. Evgen'ev, unpublished results) provides additional independent evidence for this idea.

We have previously described three AT-rich target site duplications resulting from three independent *Penelope* transpositions for three different copies of the element (Evgen'ev et al. 1997). However, loose consensus sequences derived from these three duplications indicate that base content alone is not the overriding factor in selection of *Penelope* target sites. It is more likely that the preferred sites for *Penelope* integration are influenced by local chromatin structure, as for many retroviruses in higher eukaryotes (Sandmeyer et al. 1990).

It is widely accepted that the induction of new inversions may be one manifestation of the activity of mobile elements. For example, many breakpoints in P-M dysgenesis-induced chromosomal rearrangements have been shown to occur at, or very near to, the sites of *P* element insertions (Engels and Preston 1984). Similar observations have been made concerning the *hobo* element (Lim 1988; Gelbart and Blackman 1989; Lyttle and Haymer 1992; Ladeveze et al. 1998). There are several possible mechanisms by which a chromosomal inversion might be formed. Ectopic recombination, occurring between two inverted TE copies, has been associated with inversion formation. Alternatively, inversions may be formed by breakage at or near the sites of two elements, inserted in the same orientation, with subsequent inversion of the intervening sequences (Engels and Preston 1984; Boeke and Corces 1989; Lim and Simmons 1994). Little is known about the distribution of TE inversion breakpoints in natural populations. One exception is a study of endemic inversions in wild populations of *D. melanogaster* (Lyttle and Haymer 1992). These authors found four endemic inversions with *hobo* elements present at seven out of eight of their breakpoints. The recent origin of these inversions was consistent with the hypothesized recent introduction of *hobo* into this species. In addition, a Brazilian study (Regner et al. 1996) has identified *P* elements at, or near, the breakpoints of inversions found in natural populations of *D. willistoni.*

A recent finding in Anopheline mosquitoes is also relevant to an understanding of the origins of the multiplicity of inversions in the *virilis* group. Following the analysis of the first inversion breakpoint to be cloned and sequenced in any Dipteran, a transposable element, named *Odysseus*, was discovered at the distal breakpoint of 2Rd', a polymorphic inversion in *Anopheles arabiensis* (Mathiopoulos et al. 1998). As noted by these authors, and by Engels and Preston (1984), TEs are more likely to be found associated with endemic inversions than cosmopolitan ones, because of the tendency of TEs to be lost over time from inversion breakpoints. Perusal of Fig. 5A and B shows that the association of *Penelope* and *Ulysses* insertion sites with inversion breakpoints is indeed more common for endemic than cosmopolitan inversions. A further point to be emphasized in this respect is that any future detailed molecular analysis of inversion breakpoints will be expected to underestimate the frequency of TE involvement in their formation because of the expected high frequency of element loss or divergence from such sites (Engels and Preston 1984). Also, for the same reason, the degree of underestimation of TE involvement would be expected to increase with the age of the inversions examined.

In conclusion, the *virilis* species group of *Drosophila*, with its well-known phylogeny based on chromosomal

inversions and fusions, together with a unique system of hybrid dysgenesis in which several transposons are simultaneously mobilized, provides a unique opportunity for studying the role of mobile elements in the evolution and restructuring of host genomes. Further detailed genetic and evolutionary investigations are necessary, however, to evaluate the role of these elements in the production of gross genomic rearrangements and to determine whether these have played a role in sexual isolation and speciation. Such studies are now in progress, including the cloning and sequencing of *Penelope* and other TEs activated in hybrid dysgenesis from different species of the group, and detailed characterization of the interrelationships of the TEs with each other and their host genomes.

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Appendix

Let N be the total number of chromosomal subsections among which there are distributed N_1 mobile elements of a certain family and N_2 breakpoints of chromosomal inversions. We consider a mobile element to coincide with an inversion breakpoint only if they are observed at the same subsection. Assume that all the mobile elements and inversion breakpoints are independently distributed among the chromosomal subsections. Then the number of coincidences of mobile elements and breakpoints, C is proportional to the binomial coefficient $\binom{N_2}{C}$. Consequently the distribution of mobile elements that do not coincide with breakpoints, N_1–C, is proportional to $\binom{N-N_2}{N_1-C}$. Therefore the probability of having a certain number of common subsections is proportional to the product of the above binomials. Since the sum of the products taken over all possible Cs equals $\binom{N}{N_1}$, the probability that the number of common bands is equal to or larger than the observed number of common subsections, C_{obs}, is as follows:

$$P = 1 - \sum_{C=0}^{C_{\text{obs}}-1} \binom{N}{N_1}^{-1} \binom{N_2}{C} \binom{N-N_2}{N_1-C}$$

This is exactly Fisher's 2×2 test, for which the expected number of coincidences of mobile elements with inversion breakpoints, C_{exp}, equals $N_1\ N_2/N$. We calculated P directly from Eq. (1), using the computer package Mathematica (Wolfram 1993). Testing shows highly significant deviations from independence of the distributions of *Penelope*, *Ulysses* and the inversion breakpoints (see Table 5).

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