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Short Communication

Distribution and conservation of the transposable element *gypsy* in Drosophilid species

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Abstract

In an attempt to understand the dynamics of transposable elements (T'S) in the genome of host species, we investigated the distribution, representativeness and conservation of DNA sequences homologous to the *Drosophila melanogaster gypsy* retrotransposon in 42 drosophilid species. Our results extended the knowledge about the wide distribution of *gypsy* in the genus *Drosophila*, including several Neotropical species not previously studied. The *gypsy*-like sequences showed high divergence compared to the *D. melanogaster gypsy* element. Furthermore, the conservation of the restriction sites between *gypsy* sequences from phylogenetically unrelated species pointed to a more complex evolutionary picture, which includes the possibility of the horizontal transfer events already described for this retrotransposon.

Key words: Drosophila, retrotransposon, *gypsy*, *Southern blot*, PCR.

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The superfamily of retrotransposons *Ty3/gypsy* is widely distributed among living organisms (Miller *et al.*, 1999; Marin & Lloréns 2000), and its relationship with retroviruses has been inferred in several studies (Xiong and Eickbush 1990; Kim *et al.*, 1994; Pelisson *et al.*, 1997 and Lerat & Capy 1999).

The *gypsy* retroelement (also known as *mdg4*) was first described in *Drosophila melanogaster* as a 7.5 kb sequence with 482 bp-long terminal repeats (LTRs) (Georgiev *et al.*, 1981; Bayev *et al.*, 1984). Kim *et al.* (1994) published evidence that culminated in the characterization of *gypsy* as the first retrovirus in invertebrates. As in other retroviruses, the *gypsy* retrotransposon has three open reading frames (ORFs) called *gag*, *pol* and *env*, encoding proteins responsible for its replication and infectivity. Later, *gypsy* was isolated and sequenced from the genomes of *Drosophila virilis* and *Drosophila subobscura* (Mizrokhi and Mazo 1991; Alberola and De Frutos 1996).

Southern- and dot-blot screenings have shown that the *gypsy* retrotransposon is widely distributed in the genus *Drosophila* (Stacey *et al.*, 1986; Alberola *et al.*, 1997; Loreto *et al.*, 1998). However, close inspection of these data revealed that the different hybridization signals using a *D. melanogaster gypsy* probe do not strictly follow the traditional phylogeny of the genus (hybridization signals being indicative of homology between sequences).

Lambertsson *et al.* (1989) used restriction-site polymorphism analysis to demonstrate the coexistence of several *gypsy* subfamilies in the *D. melanogaster* genome and that the majority of *gypsy* copies are defective and greatly divergent, while Chalvet *et al.* (1998) used the same approach and the presence of discrete *Hind*III and *Xba*I restriction sites to discover and characterize an active *D. melanogaster gypsy* subfamily.

The widespread presence of *gypsy* homologues in *Drosophila* was initially thought to be because the *gypsy* retrotransposon was present in the ancestral genome before the main radiation branches separated, with subsequent expansion occurring by vertical transmission (Alberola & De Frutos 1996). Nevertheless, Southern-blot and phylogenetic studies of *gypsy* sequences within groups of Drosophila species has pointed to a more complex evolutionary picture, including the possibility of horizontal transfer events (Stacey *et al.*, 1986; De Frutos *et al.*, 1992; Alberola & De Frutos 1993[a,b], 1996; Terzian *et al.*, 2000; Vázquez-Manrique *et al.*, 2000). Our recent findings suggest that multiple horizontal transfer events have indeed occurred during the recent evolutionary history of *gypsy* (Herédia *et al.*, 2004).

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This scenario is supported by the work of Mejlumian *et al.* (2002), who have reported the existence of DNA sequences putatively encoding full-length and functional *env* proteins in the genome of Drosophila species closely related to *D. melanogaster* and more distant species such as *D. virilis* and *D. subobscura*. These data strengthen the hypothesis that these sequences are potentially infectious *gypsy* copies that are able to spread between sexually isolated species.

To gain a more comprehensive insight into the evolutionary history of *gypsy* in the genus *Drosophila*, we carried out a broad Southern blot and polymerase chain reaction (PCR) analysis of 42 Drosophila species belonging to different species groups and subgenera (several of which had not hitherto been investigated for the *gypsy* retrotransposon) together with samples of the Drosophilid *Zaprionus indianus* which has recently been introduced into South America (Vilela, 1999).

Isofemale lines of all the species studied were established and reared in cornmeal medium (Marques *et al.*, 1966) at constant temperature and humidity (17 ° C ± 1 °C; 60% relative humidity). A list of all the species investigated and the number of populations employed is given in Table 1. Approximately 100 adult flies per sample were macerated with liquid nitrogen in a 1.5 mL microcentrifuge tube and the genomic DNA extracted using the method of Jowett (1986).

Table 1 - List of species and Southern blot and polymerase chain reaction (PCR) results plus the estimated copy number of *gypsy* retroelements (estimated using *Bam*HI) and the number of fragments larger than 7 kb (based on *Bgl*II), probably representing complete *gypsy* copies. The banding patterns were compared separately to *D. melanogaster*, *D. virilis* (both used as controls) and *D. subobscura* restriction maps and classified as being positive or negative for the *melanogaster* (M), *virilis* (V) or *subobscura* (S) expected fragment or having unexpectedly high signal bands (H). Species that did not produce fragments of the expected length (M, V or S) or a high signal band are indicated by an 'X'. Species with the *melanogaster* fragment are indicated by 'M' and with the *subobscura* fragment by 'S'. The abbreviation 'na' indicates that data was not available for this species.

Subgenus, group and species (number of populations analyzed)	Southern blot analysis			PCR analysis primers (positive (+) or negative (-) for the specified primers)			Gypsy retroelement data	
	*Hind*III	*Bgl*II	*Bam*HI	GYP1S/ GYP1AS	GYP31S/ GYP3AS	GYP32S/ GYP3AS2	Gypsy copy number	Number of fragments < 7 kb
Drosophila subgenus								
guarani group								
D. maculifrons (1)	X	M	H	+	-	+	9	4
D. griseolineata (1)	H	M, H	X	-	-	+	12	4
D. ornatifrons (1)	X	H	X	-	-	+	6	3
cardini group								
D. polymorpha (3)	X	X	X	-	-	+	16	2
D. cardinoides (1)	X	X	X	-	-	-	12	4
D. neocardini (1)	X	X	X	-	-	-	16	3
immigrans group								
D. immigrans (3)	X	H	X	-	-	-	10	1
pallidipennis group								
D. pallidipennis (1)	X	H	H	+	+	+	15	3
tripuntacta group								
D. bandeirantorum (2)	X	X	X	+	+	+	12	1
D. angustibucca (1)	X	X	H	-	-	-	13	4
D. mediopunctata (1)	X	H	X	+	-	+	10	2
D. mediosignata (1)	na	H	X	-	-	+	12	2
D. mediopicta (1)	X	na	X	+	-	+	15	3
D. mediostriata (1)	M	X	X	+	-	+	12	3
virilis group								
D. virilis (1)	Control	Control	Control	Control	Control	Control	na	na
repleta group								
D. hydei (1)	H	H	X	+	+	+	10	3
D. zotti (1)	na	na	na	+	+	+	na	na
D. mercatorum (3)	H	H	H	-	-	-	7	2
*bromelioide*s group								
D. bromelioides (1)	na	na	na	-	+	-	na	na

Table 1 (cont.)

Subgenus, group and species (number of populations analyzed)	Southern blot analysis			PCR analysis primers (positive (+) or negative (-) for the specified primers)			Gypsy retroelement data	
	*Hind*III	*Bgl*II	*Bam*HI	GYP1S/ GYP1AS	GYP31S/ GYP3AS	GYP32S/ GYP3AS2	Gypsy copy number	Number of fragments < 7 kb
annulimana group								
D. annulimana (1)	na	na	na	+	+	+	na	na
Sophophora subgenus								
willistoni group								
D. willistoni (2)	X	X	X	-	-	+	7	1
D. nebulosa (1)	X	H	na	-	+	+	na	na
D. fumipennis (1)	na	X	na	-	-	-	na	na
D. paulistorum (1)	X	X	X	-	-	+	6	0
D. bocainensis (1)	X	X	X	-	-	-	8	2
D. capricorni (1)	X	na	na	-	+	-	na	na
D. equinoxialis (1)	na	na	na	+	-	-	na	na
D. tropicalis (1)	H	X	X	-	-	-	13	4
D. sucinea (1)	X	X	na	-	-	-	na	na
D. insularis (1)	X	X	X	-	-	-	10	2
saltans group								
D. prosaltans (1)	na	H	X	-	+	-	8	2
D. sturtevanti (7)	X	na	na	+	+	-	na	na
melanogaster group								
D. kikkawai (1)	X	X	X	+	+	-	11	3
D. melanogaster (1)	Control	Control	Control	Control	Control	Control	23	6
D. ananassae (1)	X	H	X	-	-	-	16	2
D. malerkotliana (1)	X	H	X	+	+	-	11	2
D. simulans (3)	M, H	X	X	+	+	+	4	0
Scaptodrosophila subgenus								
D. lebanonensis (1)	X	X	X	-	+	-	13	3
D. galloi (1)	X	X	X	-	-	-	13	3
D. latifasciaeformis (1)	H	X	X	-	-	+	10	1
Dorsilopha subgenus								
D. busckii (2)	H, S	H	X	+	+	+	4	3
Zaprionus subgenus								
armatus group								
Z. indianus (1)	NA	M	X	+	+	+	14	2

Southern blotting was carried out using the pGGHS plasmid as the probe, this plasmid containing the complete *D. melanogaster gypsy* retroelement (Dorsett *et al.*, 1989). Figure 1 shows the molecular structure of the *D. melanogaster gypsy* retrotransposon, highlighting the ORFs, LTRs and the restriction sites for the endonucleases used in our study (*Bgl*II, *Hind*III and *Bam*HI, all from Invitrogen). Figure 1 also shows the restriction polymorphism between the complete elements described in the literature for *D. virilis* and *D. subobscura*, the maps constructed using the pDRAw 32 1.0 program (Kjeld Olesen, freeware). The number of copies of the retrotransposon per genome was estimated using *Bam*HI, for which there are no internal restriction sites in the *D. melanogaster gypsy* transposon. The *Bgl*II endonucleases was used to recognize sequences located in the *gypsy* LTRs, 7-kb fragments indicating the presence of probable complete *gypsy* retrotransposons. We cleaved 10 µg of each DNA sample with the restriction enzymes cited above according to the manufacturers' instructions. The fragments produced were fractionated on 0.8% (w/v) agarose gels, transferred to a Hybond N+ membrane (Amersham Biosciences) and hybridized with the probe according to the Gene Images® kit protocol (Amersham Biosciences). The probe was labeled with a random primer and hybridized at 60 °C in a solution containing 0.1% (w/v) sodium dodecyl sulfate (SDS), 5%

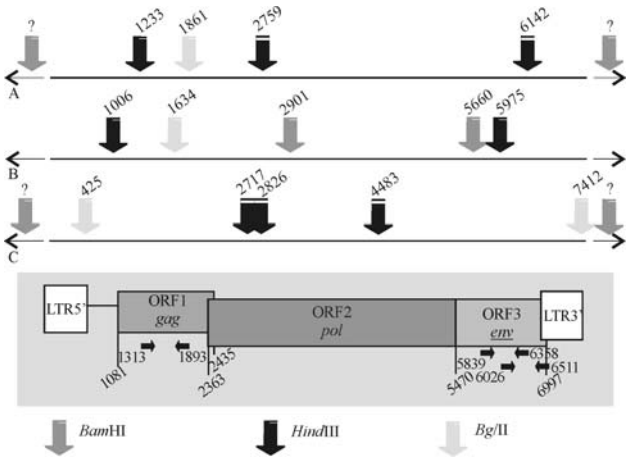

Figure 1 - The molecular structure of the *gypsy* retrotransposon. The numbers inside the smaller box indicate the nucleotide position of the open reading frame (ORF) limits and the location of the primer alignments. Above the smaller box are the restriction maps for the *Drosophila* species *subobscura* (A), *virilis* (B) and *melanogaster* (C) with the restriction sites for enzymes used in this work indicated by arrows, with the numbers showing the exact positions of the restriction sites.

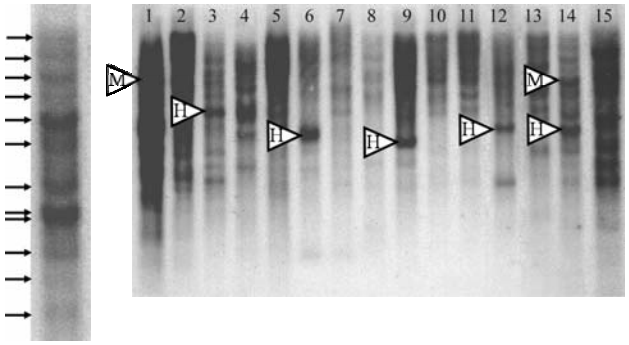

Figure 2 - A) An example of the estimation of the *gypsy* retrotransposon copy number using the *Bam*HI restriction pattern. Southern blot analysis (12 bands) of *Drosophila griseolineata* total genomic DNA isolated and digested with *Bam*HI. B) Southern blot analysis of total genomic DNA isolated from different Drosophila species and digested with the *Bgl*II restriction enzyme. The complete *D. melanogaster gypsy* sequence was used as a probe. Numbers correspond to the following *Drosophila* species: 1) *D. melanogaster* - Harwich; 2) *D. cardinoides*; 3*) D. pallidipennis*; 4) *D. mediopunctata*; 5) *D. mediostriata*; 6) *D. nebulosa*; 7) *D. tropicalis*; 8) *D. sucinea*; 9) *D. ananassae*; 10) *D. lebanonensis*; 11) *D. galloi*; 12) *D. malerkotliana*; 13) *D. polymorpha*; 14) *D. griseolineata*; 15) *D. neocardini*. Arrows indicate the restriction pattern classification. M = *melanogaster* expected fragment; H = high signal band. Molecular markers are shown in kb. All banding patterns were checked and confirmed using different exposure times.

(w/v) dextran sulfate and a blocking liquid (from the kit) diluted 20 times in 5 x saline-sodium citrate (SSC) buffer. The membrane was washed by shaking for 15 min at 60 °C in 1 x SSC and 0.1% (w/v) SDS and then in 0.5 x SSC and 0.1% (w/v) SDS and the fragments detected using the CPD-Star® kit (Amersham Biosciences). The molecular length of the fragments (in kb) detected was determined using a 1 kb Plus DNA ladder® (GIBCO/BRL) molecular weight marker as a control. The complex banding patterns obtained were compared separately to *D. melanogaster*, *D. subobscura*, and *D. virilis* restriction maps and classified as possessing, or not possessing, the *melanogaster* expected fragment (M), the *subobscura* expected fragment (S) or the *virilis* expected fragment (V), with a further category for those banding fragments with unexpectedly high signal bands (H-bands). Figure 2 shows the banding pattern obtained and illustrates the fragment classification.

For PCR analysis we designed three pairs of degenerate primers based on GenBank (NCBI) *gyp*sy sequences alignments for *D. melanogaster* (GenBank M12927), *D. virilis* (GenBank M38438) and *D. subobscura* (GenBank X72390) and using the *D. melanogaster* sequence as reference, the primers being: GYP1S (sense GAGTTTGCAGG TGGARGCRCC, ORF1 region 1313-1333) and GYP1AS (antisense GCRAACARGCTTCTCTCWATGCTWGC, ORF1 region 1869-1893) coding for a 580-nt fragment; GYP3S1 (sense YCTMGATTTCTTAGGYACWGC, ORF3 region 5839-5859) and GYP3AS1 (antisense GTCYTCGTCGAGKCGCARRAT, ORF3 region 6336-6358) coding for a 519-nt fragment; and GYP3S2 (sense AAAGGCGAYTTGGTTGACACTCC, ORF3 region 6026-6048) and GYP3AS2 (antisense CARGTGGCTRGG TTGRGTGTG, ORF3 region 6491-6511) coding for a 485-nt fragment. The letters R, W and Y represent degenerate positions in the primers.

The reaction mixture consisted of 50 ng of sample DNA, 1 unit of Taq polymerase (Invitrogen), 50 mM of each nucleotide, 20 pmol of each primer and 1.5 mM of $MgCl_2$ in a volume of 50 µL. Amplification was for 2 min at 96 °C followed by 35 cycles of 15 s at 96 °C, 30 s at 55 °C and 90 s at 72 °C, with a final extension for 5 min at 72 °C. Products were separated on 1% agarose gels.

We evaluated the distribution of *gypsy*-homologous sequences in different *Drosophila* species and compared the conservation of their restriction sites in relation to those found in *D. melanogaster* by Southern blot. However, some of the species were difficult to rear in the laboratory and did not provide enough flies to extract DNA for all of the restriction site analyses.

We detected *gypsy*-homologous sequences in all the species investigated, indicating that this retrotransposon is ubiquitous in this genus. However, the hybridization patterns of the different species were very diverse and characterized by a high number of bands and weak hybridization signals. The bands observed were mostly weak as compared with those of the *D. melanogaster* control DNA.

As outlined above, the complex banding patterns were classified as the *melanogaster* expected fragment (M), the *subobscura* expected fragment (S) or the *virilis* expected fragment (V), with a further category for those banding fragments with unexpectedly high signal bands (H-bands) probably reflecting the products of a new internal restriction pattern involving more than one restriction site in the *gypsy* sequence (Table 1, Figure 2b).

In the majority of the species we investigated, the *Hind*III and *Bgl*II restriction fragments did not correspond to those normally seen in the *D. melanogaster* reference map, indicating that the *gypsy* restriction sites in the species studied diverged from those of *D. melanogaster*.

Although the *gypsy* elements in *Drosophila mediostriata* and *D. simulans* showed a similar 1.7-kb fragment as *D. melanogaster* after *Hind*III digestion, those in *Drosophila busckii* had fragments of about 3.4 and 1.5 kb, possibly corresponding to those expected for the *Drosophila subobscura gypsy* element (Figure 1). High signal bands (H) were seen in other *Drosophila* species (*griseolineata*, *hydei*, *mercatorum*, *busckii*, *simulans*, *tropicalis* and *latifasciaeformis*), indicating the diversity of restriction sites in the different species.

In the blots in which the genomic DNAs of the species studied were cleaved with *Bgl*II endonuclease, the approximately 7-kb *gypsy* fragment expected for *D. melanogaster* was also observed in *D. griseolineata*, *Drosophila maculifrons* and *Z. indianus*. Unexpectedly high signal bands (H-bands) were found in some *Drosophila* species (*immigrans*, *mediosignata*, *hydei*, *mercatorum*, *prosaltans*, *busckii*, *pallidipennis*, *mediopunctata*, *nebulosa*, *ananassae*, *malerkotliana*, *ornatifrons* and *griseolineata*). The fact that the same enzymes did not show homologous H-bands in all species investigated suggests that there are different restriction patterns among the different species (Figure 2b).

The estimated the copy number of *gypsy* homologous sequences as exemplified in Figure 2a varied from 4 to 16 in the 32 species assessed (Table 1). In most of the species studied the *Bam*HI bands were estimated as being in excess of 7.5 kb and probably represented complete copies of the *gypsy* retrotransposon because *Bam*HI does not recognize internal *gypsy* restriction sites. However, these estimates should nevertheless be regarded as preliminary because our results showed restriction polymorphism in the *gypsy* sequences of these species.

The divergence and probable activity of the *gypsy*-homologous sequences at the population level were evaluated for some of the species by studying the *Hind*III digests of different populations (Table 1). Six of the seven *Drosophila* species showed no variation in *gypsy Hind*III banding, the exception being *Drosophila sturtevanti.*

Of the 42 species examined with the 3 different primer pairs (Table 1) 28 species produced PCR products for at least one pair. The fact that 12 species were negative although the *D. melanogaster gypsy* probe had produced positive hybridization signals in Southern blotting suggests that nucleotide substitutions may have occurred in the annealing regions of the primers. Some *Drosophila* species (*pallidipennis*, *bandeirantorum*, *hydei*, *zotti*, *annulimana*, *simulans* and *busckii*) and *Z. indianus* showed the expected PCR fragments with the three pairs of primers employed and the PCR bands were equivalent to those obtained for the controls (*D. melanogaster* and *D. virilis*).

Our results confirm and extend the finding that the *gypsy* retrotransposon is widely distributed within the genus *Drosophila* and that there are heterogeneous banding patterns, restriction polymorphism, and both complete and deleted copies of this transposon in the investigated genomes (Alberola and De Frutos, 1993[b]; Alberola *et al.*, 1997; De Frutos *et al.*, 1992; Loreto *et al.*, 1998; Stacey *et al.*, 1986). Our previous study on the same drosophilid species has shown that different *gypsy* subfamilies can coexist in the same genome and that *gypsy* exhibits a complex evolutionary pattern in which multiple invasion of the host genome can occur (Herédia *et al.*, 2004). Taking together, these aspects may explain the Southern blot banding patterns that we obtained in our present study. We propose that the divergence in restriction sites reflects the deterioration of ancient *gypsy* sequences, whereas the conserved restriction pattern in distant species indicates the introduction of new *gypsy* sequences as a result of recent invasion of the host genome by the *gypsy* retrotransposon.

Our *Bgl*II Southern blot results show that although *D. griseolineata*, *D. maculifrons* and *Z. indianus* are phylogenetically very distant from *D. melanogaster*, they appear to have complete copies of the *gypsy* retrotransposon with homologous sequences in which the *Bgl*II cleavage pattern is maintained. We also found that the typical *D. melanogaster* restriction pattern was neither observed in the remaining species of the *melanogaster* group nor in other closely related species such as those belonging to the subgenus *Sophophora* (except for *D. simulans*). Inconsistencies of this type were previously pointed out by Stacey *et al.* (1986). Furthermore, we found that the *Hind*III melanogaster expected fragment (M) was only detected in *D.* simulans and *D. mediostriata,* the latter species being only very distantly related to *D. melanogaster.* The same case was observed for the subobscura expected fragment (S) in *D. busckii*. Interestingly, horizontal transmission events have been described between *D. busckii* and *D. subobscura* and between *D. simulans* (a sibling species of *D. melanogaster*) and *Z. Indianus* (Herédia *et al.*, 2004). The PCR results also demonstrated the high homology among *gypsy* sequences of these species.

It is important to point out that in our study the variability of *gypsy* sequences at the inter-specific level did not occur at the inter-population level where we observed genomic stability in the *gypsy* profiles. These findings confirm the results described by Sassi *et al.* (2005) for different populations of *Drosophila willistoni*.

Both Southern blot and PCR were capable of detecting the complex evolutionary patterns, confirmed by the phylogenetic analysis (see Herédia *et al.*, 2004) and appear to be a good tool for the preliminary screenings of transposable elements in different Drosophila genomes. Southern blot methodology is particularly useful because it can be

used to assess the indirect variability of the complete sequence of the transposable element while other methods can only assess just a small part of the sequence, resulting in restricted conclusions about the presence, divergence and evolutionary aspects of these elements.

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