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Characterization of Non-LTR Retrotransposable TRAS Elements in the Aphids *Acyrthosiphon pisum* and *Myzus persicae* (Aphididae, Hemiptera)

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Abstract

A non-LTR TRAS retrotransposon (identified as TRASAp1) has been amplified in the pea aphid *Acyrthosiphon pisum* and its presence has been assessed also in the peach potato aphid *Myzus persicae*. This TRAS element possesses 2 overlapping ORFs (a *gag*-ORF1 and a *pol*-ORF2 containing the reverse transcriptase and the endonuclease domains) that show a similarity ranging from 40% to 48% to proteins coded by other TRAS elements identified in insects (including the beetle *Tribolium castaneum* and the moth *Bombyx mori*). The study of the TRAS chromosomal insertion sites, performed by standard fluorescent *in situ* hybridization (FISH) and fiber FISH, showed that TRAS elements were located in a subtelomeric position, just before the telomeric $(TTAGG)_n$ repeats. In both the aphid species, TRAS elements were present at all termini of autosomes, but the 2 X chromosome telomeres show a clear-cut structural difference. Indeed, cromomycin A_3 staining, together with FISH using a TRAS probe, revealed that TRAS signals only occur at the telomere opposite to the NOR-bearing one. Lastly, the analysis of the distribution of TRAS retrotransposons in a *M. persicae* strain possessing spontaneous fragmentations of the X chromosomes assessed that TRAS elements were not involved in the healing of *de novo* telomeres.

Key words: *aphid, fiber FISH, holocentric chromosomes, non-LTR retrotransposable elements, subtelomeric regions, TRAS*

Telomeres are specialized nucleoprotein structures constituting the end of chromosomes (Blackburn 1991). Although eukaryotic telomeric sequences may vary in composition, they are strictly conserved in some taxonomic groups, so that the hexameric $(TTAGGG)_n$ repeat is typical of vertebrates (Meyne et al. 1989) and the $(TTTAGGG)_n$ sequence was found in plants (Fajkus and Zentgraf 2002), whereas the $(TTAGG)_n$ telomeric repeat was reported in many invertebrate species, including the main lineages of insects (Okazaki et al. 1993; Sahara et al. 1999; Bizzaro et al. 2000; Frydrychová and Marec 2002; Mandrioli 2002; Frydrychová et al. 2004; Vítková et al. 2005; Lukhtanov and Kuznetsova 2010; Monti et al. 2011).

Differently from the conservation of telomeres, subtelomeric regions are more polymorphic and variable in composition (Mandrioli et al. 2013). In this regard, repetitive telomere-associated sequences have been commonly found in the subtelomeric region of various species, such as the 169-bp MpR satellite DNA sequence in the aphid *Myzus persicae* (Spence et al. 1998) and the highly conserved 9-kb-long terminal unit identified in the Taiwan cricket *Teleogryllus taiwanemma* (Kojima et al. 2002). Both these repetitive sequences were located in the subtelomeric regions and they were species-specific or, at most, present in few phylogenetically highly related species. Indeed, the 169-bp MpR subtelomeric satellite has been found in *M. persicae*, *M. antirrhinii*, and *M. certus*, but absent in other aphid species (Spence et al. 1998). Similarly, *T. taiwanemma* sequence is absent in other crickets, including the Japanese field cricket *Teleogryllus emma* that is thought to be one of the species closest to *T. taiwanemma* (Kojima et al. 2002).

The telomere-associated sequences identified up till now bear a structural resemblance to *Chironomus* TA repeats (Mason et al. 2011), which evolved from telomeric repeat sequences and truncated retrotransposons (Zhang et al. 1994; Martinez et al. 2001; Mason et al. 2011) suggesting that retrotransposons could be commonly present below telomeres shaping the structure of the subtelomeric regions (Mandrioli et al. 2013).

Aphids have been frequently studied at a chromosomal level because they possess holocentric chromosomes, where spindle fibers attach along the entire length of the chromosome, dragging the chromosome broadside toward the poles at anaphase (Hughes-Schrader and Schrader 1961; Mandrioli and Manicardi 2012). Chromosome fragments that would be acentric and consequently lost in an organism with localized centromeres may be properly inherited in aphids favouring rapid changes in their karyotype structure (Monti et al. 2012). Despite such an interest, the structure of subtelomeric regions in aphids has been to date studied in the peach potato aphid *M. persicae* only, where attention has been limited to the presence of satellite DNAs (Spence et al. 1998). Interestingly, Southern blot experiments with a telomeric probe showed that aphid telomeres are composed of the $(TTAGG)_n$ repeat that is occasionally interrupted by other repeated sequences still not identified (Monti et al. 2011). At the same time, partial sequences of the non-LTR retrotransposon TRAS have been annotated in the genome project of the pea aphid *Acyrthosiphon pisum*, but these elements have been not analyzed neither located on chromosomes (International Aphid Genomic Consortium 2010).

The presence of non-LTR retrotransposons has been frequently reported in Lepidoptera, and TRAS retrotransposons have been isolated from the subtelomeric regions of the moths *Bombyx mori*, *Dictyoploca japonica*, *Samia cynthia ricini*, and *Mamestra brassicae* (Okazaki et al. 1995; Kubo et al. 2001; Mandrioli 2002; Fujiwara et al. 2005). Furthermore, the analysis of the different families of the non-LTR retrotransposons TRAS in *B. mori* revealed that they are highly transcribed and actively retrotransposed into the $(TTAGG)_n$ telomeric repeats in a highly sequence-specific manner (Okazaki et al. 1993; Kubo et al. 2001; Fujiwara et al. 2005).

In order to better understand the structure of aphid subtelomeric regions, we amplified, sequenced, and localized copies of the non-LTR retrotransposon TRAS in the aphids *A. pisum* and *M. persicae*, 2 of the most studied species at a cytogenetic level due to their relevance as experimental models and crop pests.

Materials and Methods

The specimens of the pea aphid *A. pisum* used for this research were obtained from the LSR1 laboratory lineage, kindly furnished by Manuel Plantagenest (INRA, France), and maintained on broad bean *Vicia faba* plants at 19 °C with a light–dark regime of 16 h of light and 8 h of darkness.

Specimens of peach potato aphid *M. persicae* were obtained from laboratory lineages 1 and 33H, maintained on pea (*Pisum sativum*) plants at 19 °C with a light–dark regime of 16 h of light and 8 h of darkness. Both clones were kindly supplied by Emanuele Mazzoni, Università Cattolica di Piacenza, Italy. *Myzus persicae* clone 33H is derived from a single specimen of the US1L strain, originally provided by A.L. Devonshire, Rothamsted Research, Harpenden, Hertfordshire, UK.

DNA extraction followed a standard phenol-chloroform protocol (Mandrioli et al. 1999), whereas the *M. persicae* subtelomeric DNA probe has been amplified by PCR using the oligonucleotide primers MpR-F (5'-TCAAAGTTCTCGTTCTCC-3') and MpR-R (5'-GTTT TAACA GAGTGCTGG-3'), designed according the subtelomeric repeat sequence available in the literature (Spence et al. 1998). The reaction conditions were 94 °C for 90 s, and a total of 25 cycles of 94 °C for 30 s, 51 °C for 30 s, and 72 °C for 30 s.

The presence of TRAS retrotransposons has been preliminary evaluated using the BLAST algorithm at *Aphidbase* (http://www.aphidbase.com/aphidbase/). Successively, a 801-bp-long portion of the TRAS ORF coding for reverse transcriptase (RT) has been amplified using the primers F-TRAS (5-ATGAGTACCCCCACCATCAA) and R-TRAS (5'- CCTCTCCGAGA TGACCTGAA), designed according the *A. pisum* partial TRAS3 sequence available in GenBank (XM_001942587). The amplification mix contained 100 ng of genomic DNA, 1 μM of each primer, 200 μM dNTPs, and 2 U of DyNAZyme II polymerase (Finnzymes Oy). Amplification was performed with a Hybaid thermalcycler at an annealing temperature of 51 °C for 1 min and making extension at 72 °C for 1 min.

Aphid TRAS sequence was completed by inverse PCR with the primers F-TRAS-i (5'-CACTCTCACCCACCCTTCAT) and R-TRAS-i (5'-AAAAGAGTGCCCGTAACCCT), according to Martin and Mohn (1999).

All the amplified fragments were cloned using the pGEM T-easy cloning kit (Promega). Amplified fragments have been sequenced at BMR Genomics, whereas sequence alignments and search for internal repeats were done using the GCG software (GCG Computer Group, MA).

Chromosome preparations were made from parthenogenetic female embryos by spreading, as previously described (Monti et al. 2011), whereas chromomycin A_3 (CMA_3) staining was performed as described by Mandrioli et al. (1999).

PCR digoxigenin labelling of the 801-bp-long *A. pisum* TRAS probe was performed with the "PCR DIG labelling mix" according to the Roche protocol.

Fluorescent in situ hybridization (FISH) was done according to Mandrioli et al. (1999) with a slight modification regarding DNA denaturation. In particular, chromosome preparations were pretreated for 1 h at 37 °C with RNase A (200 μg/ml in 2× SSC), for 5 min at 37 °C with pepsin (5 μg/mL in 0.01 M HCl), and for 15 min at RT with freshly depolymerized paraformaldehyde (4% in phosphate buffer 0.1 M). The hybridization mixture contained 1 ng/μL of labelled TRAS probe, 60% formamide, 0.25% SDS, 2× SSC, and 10% dextran sulphate. The probe was denatured by boiling for 3 min and cooled on ice immediately before use. After denaturation at 72 °C for 7 min in 70% formamide in 2× SSC, slides were dehydrated through an alcohol series and air-dried. Slides were successively covered with 25 μL of denaturated hybridization mixture (sealed under a coverslip) and placed in a humid chamber for overnight hybridization at 37 °C. After hybridization, slides were washed twice, for 15 min, in 0.1× SSC at room temperature, twice in 0.1× SSC for 15 min at 37 °C, and finally in PBS pH 7.2–7.4 at room temperature for 5 min. Pre-incubation was carried out in PBS plus 0.5% blocking reagent (provided along with the

Boerhinger digoxigenin revelation kit) at 37 °C for 30 min. Detection was carried out at 37 °C with anti-digoxigenin antibody fluorescein isothiocyanate conjugate (FITC) for 30 min. Slides were then washed twice in PBS for 10 min. After FISH, chromosomes were counterstained with 100 ng/mL propidium iodide and mounted in buffered glycerol.

FISH slides were observed using a Zeiss Axioplan epifluorescence microscope equipped with a 100-W mercury light source. Photographs of the fluorescent images were taken using a CCD camera (Spot; Digital Instrument, MA) and using the Spot software supplied with the camera and processed using Adobe Photoshop (Adobe Systems, Mountain View, CA).

Slides with DNA fibers have been obtained from chromosomes fixed onto slides by spreading. Successively, as soon as an iridescent halo appeared on the slide drying surface, slides have been washed in 1× PBS pH 7.4 for 2 min before dropping onto the slides 60 µL of a NaOH/ethanol solution (0.07 N NaOH/absolute ethanol 5:2) that has been smeared on the slide with a cover slip. Successively, 2 drops of methanol have been added and, after 20 s, 2 further methanol drops have been added before placing the slide in vertical position for draining them. Successively, further 4 drops of methanol have been added. When dried, slides have been examined by phase-contrast microscopy, dehydrated in an alcohol hydration series (alcohol 70%, alcohol 90%, ethyl alcohol 95%, alcohol 100%) before FISH experiments. Fiber FISH hybridization has been performed according to previously reported protocol, but slides have been observed using a laser-scanning confocal microscope SP2-AOBS (Leica).

Results

According to the results of the *A. pisum* genome project, the sequence XM_001942587 has been annotated in GenBank as partial TRAS sequence similar to TRAS3. This sequence corresponded to the typical RT domain of elements belonging to the TRAS families. Starting from this annotated sequence, we designed 2 couples of primers which allowed us to: 1) amplify a 801-bp-long fragment of this ORF; 2) complete the sequence of the *A. pisum* TRAS elements by inverse PCR (Figure 1).

An evident band has also been amplified by direct PCR in the *M. persicae* genome (Figure 1A). In both species, electrophoresis evidenced several other smaller PCR bands that sequencing identified as truncated copies of the TRAS elements (reported in the Supplementary Material). In view of the unusual presence of truncated TRAS copies, we verified the presence of deleted copies of TRAS elements in *Aphidbase* evidencing several scaffolds containing portion of this retrotransposon; all the deleted copies were not flanked by the canonical telomeric TTAGG sequence.

Inverse PCR generated several electrophoretic bands (Figure 1B) that have been sequenced in order to assemble a complete copy of the *A. pisum* TRAS retrotransposon. The exhaustive search of the *Aphidbase* database allowed us to identify 2 scaffolds (identified in Genbank as GL349650.1 and 186642550, respectively) containing portion of the TRAS elements. This approach allowed us to: 1) confirm our sequencing results; 2) assemble a 7.762-bp TRAS element identified as TRASAp1 (GenBank ID JX875955).

TRASAp1 elements possessed the typical CA repeat in the 5' portion and 2 overlapping ORF regions with a *gag*-like ORF1 and a *pol*-ORF2 containing the RT and the endonuclase domains. Alignments of the TRAS amino acidic sequence deduced by the ORF2 sequence showed that *A. pisum* TRAS possessed a similarity ranging from 40% to 48% with TRAS elements available in GenBank and identified in the beetle *Tribolium castaneum*, as well as in the moths *B. mori*, *Saturnia japonica*, and. *S. cynthia*.

The investigation of the TRAS chromosomal insertion sites, performed by FISH, evidenced bright hybridization signals at both the termini of all *A. pisum* and *M. persicae* autosomes. Differently, the X chromosome telomeres showed a clear-cut structural dimorphism because TRAS hybridization signals were present only at the telomere opposite to the NOR-bearing one, as evidenced by the CMA_3 staining of the NOR regions (Figure 2). In the interphase nuclei, TRAS elements were clustered in several small discrete spots in both the species, according to the previously reported distribution of telomeres in the aphid nucleus (Monti et al. 2011).

In view of the presence of a satellite DNA sequence in *M. persicae* subtelomeric regions (Spence et al. 1998), we evaluated by fiber FISH the precise organization of its telomeres (Figure 3). According to our results, TRAS elements were located between the inner portion of the telomeric $(TTAGG)_n$ array and the outer part of the subtelomeric satellite DNA cluster.

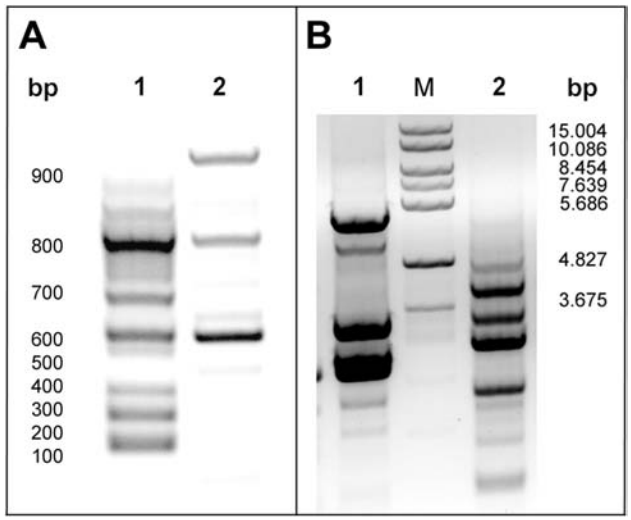

Figure 1. Amplification of the TRAS portions in *A. pisum* (1) and *M. persicae* (2) by direct (**A**) and inverse (**B**) PCR amplifications. Molecular weight has been evaluated using the markers 100-kb ladder (in panel A) and widerange genladder (in panel B, lane M).

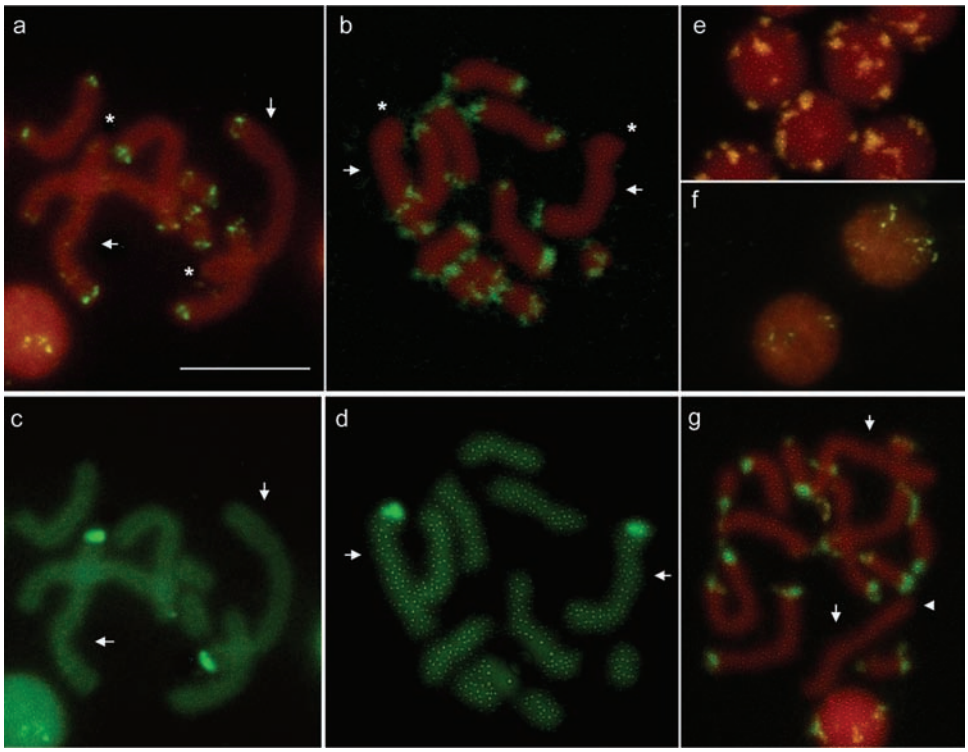

Figure 2. FISH with the *A. pisum* TRAS probe showed that TRAS elements are located at both telomere of the autosomes and in a single telomere in the 2 X chromosomes in *A. pisum* (**a**) and *M. persicae* (**b**). CMA_3 staining of the same plates showed that the subtelomeric regions of the NOR-bearing end of the 2 X chromosomes do not contain TRAS elements both in *A. pisum* (**c**) and *M. persicae* (**d**). In interphase *M. persicae* (**e**) and *A. pisum* (**f**) nuclei, FISH with the TRAS probe showed several small discrete spots. FISH experiments, carried out in the *M. persicae* strain 33H possessing spontaneous fragmentations of the X chromosomes, evidenced that TRAS elements were not involved in the healing of de novo chromosomal ends (**g**). Asterisks indicate X telomere devoid of TRAS elements. Arrow head indicates fragmented X chromosome without any TRAS hybridization signal. Arrows indicate X chromosomes. Bar corresponds to 10 μm.

FISH experiments, carried out in the *M. persicae* strain 33H possessing spontaneous fragmentations of the X chromosomes (Monti et al. 2012), allowed us to assess that TRAS elements were not involved in the *de novo* healing of chromosomal ends (Figure 2g).

Discussion

Highly conserved telomere-specific non-LTR retrotransposons have been found for the first time in the moth *B. mori* (Kubo et al. 2001; Fujiwara et al. 2005). Among them, the first identified telomere-associated mobile DNA is the TRAS1 element consisting of a stretch of 7.8 kb and encoding 2 overlapping ORFs that make this retrotransposon an autonomously transposing element (Kubo et al. 2001; Fujiwara et al. 2005). Further screening for telomeric-repeat associated retrotransposons revealed different TRAS families, so that in the silkworm there are at least 8 TRAS families (TRAS1, TRAS3, TRAS4, TRAS5, TRAS6, TRASY, TRASZ, and TRASW), which have the same telomeric target sequence, but they can be distinguished into different groups on the basis of their sequences (Kubo et al. 2001; Fujiwara et al. 2005).

Despite the presence of several insect genome projects, only partial copies of TRAS elements have been annotated outside lepidopteran species (Tribolium Genome Sequencing Consortium 2008; International Aphid Genomic Consortium 2010), making the pea aphid TRAS element here identified the first telomere-specific non-LTR element fully sequenced and studied in insects other than moths. *Acyrthosiphon pisum* TRAS retrotransposons present all the typical features of TRAS elements, suggesting that these retrotransposons are highly conserved not only among lepidopteran species (as previously suggested by Fujiwara et al. 2005) but also in other insect orders possessing $(TTAGG)_n$ telomeres.

Sequencing of the pea aphid TRASAp1 elements revealed the occurrence of several truncated copies of this retrotransposon, thus making the isolation of full-length copies more difficult than expected. This is not unusual for non-LTR retrotransposons that frequently lack the 5' end because reverse transcription often stops prematurely (Luan et al. 1993; Martin et al. 2005). Interestingly, in *B. mori* TRAS copies are always constituted by complete retrotransposons (Kubo et al. 2001; Fujiwara et al. 2005), suggesting that in aphids TRAS RT is more error-prone than in lepidopterans bringing to the

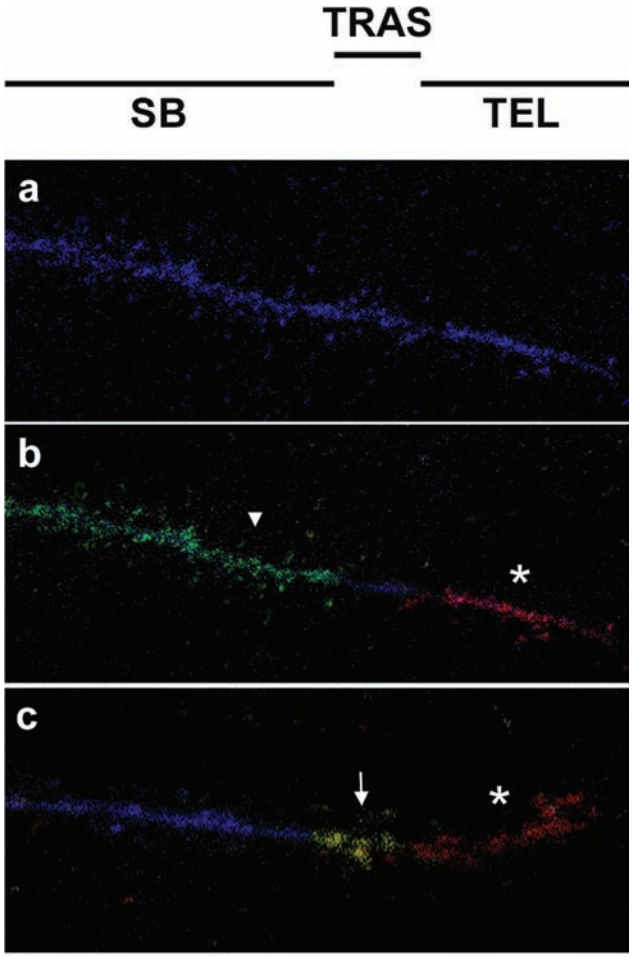

Figure 3. FISH on *M. persicae* DNA fiber, stained with DAPI (**a**), showed that TRAS elements (indicated by the arrow in panel **c** after hybridization with the *A. pisum* TRAS probe) are interspersed in the inner portion of the telomeric TTAGG array (indicated by the asterisk after the $TTAGG_n$ telomeric DNA probe labelling with TRITC, panels **b** and **c**) and not in the cluster of the subtelomeric satellite DNA (indicated by the arrow head in FISH experiments with the FITC-labelled subtelomeric DNA repeat as a probe, panel **b**).

synthesis of truncated copies of this retrotransposon. At the same time, a functional constraint could be at the basis of the structural difference of this mobile element in moths in respect to aphids. Indeed, it has been suggested that in the silkworm TRAS retrotransposons might compensate the repressed telomerase activity through the elongation of the subtelomeric regions so favouring the maintenance of a chromosomal capping (Fujiwara et al. 2005). On the contrary, the presence of an actively transcribed telomerase in aphids (Monti et al. 2011) could weaken the functional roles of TRAS elements in the aphids, making them less important for telomere maintenance and functioning.

Bioinformatic analyses on *A. pisum* assembled scaffolds revealed that several truncated TRAS copies were not inserted near a telomeric TTAGG sequence, and we were unable to identify any consensus sequence for these unusual TRAS insertions (data not shown). As recently revised by Mandrioli and Manicardi (2012), meiotic recombination seems to be much higher on the chromosome termini than in the central region of chromosomes in species possessing holocentric chromosomes. The presence of truncated TRAS copies could, therefore, be due to unequal recombination events occurring of the aphid telomeric regions. This suggestion is in accordance to Boissinot et al. (2001) that hypothesized that the high frequency of full-length L1 elements on the human Y chromosome could be due to the inability of the Y chromosome to recombine.

The investigation of the chromosomal insertion sites showed that TRASAp1 retrotransposons have been integrated near the telomeric end of all the autosomes and in a single telomere of the 2 X chromosomes in both the aphid species. In particular, fiber FISH in *M. persicae* revealed that TRAS copies are not located within the most external telomeric sequences, but between the subtelomeric region (constituted by the *M. persicae* subtelomeric satellite DNA) and the $(TTAGG)_n$ array. This localization is consistent with data obtained in *B. mori*, where the TRAS copies closest to the termini were found 6–8 kb away from the chromosome ends (Okazaki et al. 1995: Fujiwara et al. 2005), and strengthen the hypothesis that some telomere-binding proteins may protect telomeres preventing TRAS insertion in the most external portion of the chromosomal tip (Kubo et al. 2001; Fujiwara et al. 2005).

In both the aphid species analyzed, TRAS elements were not present in the subtelomeric region near the NOR-bearing telomeres of the 2 X chromosomes. These subtelomeric regions in *M. persicae* also do not have any telomere-associated satellite DNA (Spence et al. 1998), suggesting that the X telomeres may face different functional constraints.

Interestingly, the association of aphid X chromosomes at NORs has been frequently reported as occurring during the maturation division of the parthenogenetic oocytes (Schrader 1940; Orlando 1974; Hales and Mitler 1983; Blackman and Hales 1986). Indeed, male determination in aphids is based on the loss of one X chromosome because almost all aphid species present females with 2 X chromosomes (XX) and males with only one X chromosome (XO) (Blackman and Spence 1996). All the parthenogenetic eggs during the prophase present 2 X chromosomes linked by NORs (Schrader 1940; Orlando 1974; Hales and Mitler 1983; Blackman and Hales 1986). However, in eggs developing as females, the connection is quickly lost, but in male-generating eggs, the X chromosomes remain attached by sticky NORs and undergo a sort of non-canonical reductional division (Blackman and Hales 1986). At the end of this peculiar division, the egg has one X chromosome only and it is determined as a male. The absence of telomere-associated repeated sequences, such as satellite DNAs and transposable elements, at the NOR telomeres of the X chromosomes could be, therefore, due to the need of favouring rDNA genes pairing, which is at the basis of sex determination

in aphids. At the same time, the idea that subtelomeric regions may facilitate chromosome pairing in meiosis has been substantiated in various organisms (Scherthan et al. 1994; Bass et al. 1997). In view of this assumption, the high level of structural imbalance of the 2 telomeres of the X chromosomes could explain the lack of detectable genetic recombination observed on these chromosomes during the parthenogenetic production of female and male aphids (Hales et al. 2002).

On the basis of literature data, TRAS seems to be not directly involved in healing chromosomal breakages in *B. mori* (Okazaki et al. 1995; Fujiwara et al. 2005). FISH experiments in the *M. persicae* laboratory clone 33H confirmed the absence of TRAS retrotransposons in the *de novo* telomeres, whereas previous experiments showed the presence of a *de novo* synthesised $(TTAGG)_n$ telomeric array (Monti et al. 2011). As a whole, we have, therefore, further evidence assessing that telomere-specific TRAS retrotransposons are not involved in the healing and stabilization of the broken chromosomal ends.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

Acknowledgments

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