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The vanishing clone: karyotypic evidence for extensive intraclonal genetic variation in the peach potato aphid, *Myzus persicae* (Hemiptera: Aphididae)

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Analysis of holocentric mitotic metaphase chromosomes of the peach-potato aphid *Myzus persicae* (Sulzer) clone 33H revealed different chromosome numbers, ranging from 12 to 17 within each embryo, in contrast to the standard karyotype of this species ($2n = 12$). Chromosome length measurements revealed that the observed chromosomal mosaicism is the result of recurrent fragmentations of chromosomes X, 1 and 3 because of fragile sites or hot spots of recombination. Fluorescent *in situ* hybridization experiments showed that X chromosomes were frequently involved in recurrent fragmentations, in particular their telomeric end opposite to the nucleolar organizer region. Experiments to induce males showed that *M. persicae* clone 33H is obligately parthenogenetic. The reproduction by apomictic parthenogenesis, together with a high telomerase expression that stabilized the chromosomes involved in the fragmentations observed in the *M. persicae* clone 33H, appears to favour the stabilization of the observed chromosome instability. © 2011 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2012, **105**, 350–358.

ADDITIONAL KEYWORDS: fragile sites – holocentric chromosomes – rearrangements – repetitive DNAs – telomerase.

INTRODUCTION

The chromosome number and the structure of the karyotypes of aphids (Hemiptera: Aphididae) are generally stable within genera, although karyotype variations are relatively common within some species and may perhaps be associated with host plant specialization, as reported in the corn leaf aphid *Rhopalosiphoum maidis* (Fitch) feeding in barley and sorghum (Brown & Blackman, 1988). A considerable increase in the chromosome number has been found to occur as a consequence of autosome fissions in *Amphorophora* spp., which showed host-associated forms that have evolved to the point of speciation (i.e. feeding on raspberry and blackberry, respectively) (Blackman, 1980, 1987).

Aphids show more variation in karyotype than other organisms because they have holocentric chromosomes with kinetic activity spread along the whole chromosome axis (Blackman, 1980). Hence, chromosomal fragments can contact the microtubules and move properly in the daughter cells during cell division. By contrast, fragments of monocentric chromosomes may be lost during mitosis and meiosis in the absence of centromeric activity in the chromosome fragment.

In the peach potato aphid *Myzus persicae* (Sulzer), variations in the chromosome number and structure have been observed. These are mainly the result of chromosomal translocations and, occasionally, fragmentations that give rise to an increased chromosome

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number (Blackman, 1980; Lauritzen, 1982). For example, several populations of *M. persicae* were heterozygous for a translocation between autosomes 1 and 3, and this particular rearrangement has been shown to be involved in resistance to organophosphate and carbamate insecticides (Blackman, Takada & Kawakami, 1978; Spence & Blackman, 1998).

Myzus persicae populations with 13 chromosomes have also been identified in different countries as a result of autosome 3 fission (Blackman, 1980). Interestingly, at least two independent and diverse fragmentations of the autosome 3 were reported (Blackman, 1980; Lauritzen, 1982), suggesting that different naturally occurring rearrangements of the same chromosome may be observed in this aphid's karyotype (Blackman, 1980; Lauritzen, 1982). In some *M. persicae* populations, a further fission of autosome 2 give raise to karyotype consisting of $2n = 14$ chromosomes (Blackman, 1980; Lauritzen, 1982), making this species a good experimental model for the study of chromosome rearrangements in aphids (Spence & Blackman, 1998).

We recently showed (Monti *et al*., 2011) that *M. persicae* clone 33H individual aphids could contain cells with $2n = 12$ or $2n = 14$. In the present study, the different karyotypes observed in this aphid strain have been analyzed in detail using two chromosomal markers (an X-chromosome specific Hind200 satellite and the subtelomeric repeat). Monti *et al*. (2011) also demonstrated the presence of active telomerase in the *M. persicae* clone 33H. Therefore, in the present study, we compared the expression level of the telomerase-encoding gene in the embryo of the *M. persicae* clone 33H with that in a control strain as a possible explanation for the observed chromosomal mosaicism.

MATERIAL AND METHODS

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

Male induction was evaluated by exposing parthenogenetic females to short photoperiods (8 h light: 16 h dark) according to Crema at 18 °C (1979).

Chromosome preparations from parthenogenetic females were made by spreading embryo cells and by squash preparation of single embryos as reported previously by Mandrioli *et al*. (1999a), whereas chromomycin A_3 (CMA_3) staining was performed *sensu* Manicardi *et al*. (1996).

DNA extraction, performed in accordance with a standard phenol–chloroform protocol, and restriction enzyme digestion were both carried out as described previously (Mandrioli *et al*., 1999a). The Hind200 probe was isolated by digesting *M. persicae* genomic DNA with the restriction enzyme *Hind*III at 37 °C for 16 h and eluting from the 1.2% agarose gel the band corresponding to the Hind200 satellite monomers (GenBank AF161255) *sensu* Mandrioli *et al*. (1999c). The eluted DNA was labelled using a random priming DIG labelling kit in accordance with the manufacturer's instructions (Roche Diagnostics).

Polymerase chain reaction (PCR) digoxigenin labelling of the subtelomeric repeat was performed with a PCR DIG labelling kit in according with the manufacturer's instructions (Roche) using the specific oligonucleotide primers MpR-F (5′-TCAAAGTTCT CGTTCTCC-3′) and MpR-R (5′-GTTTTAACAGAGTG CTGG-3′), designed in accordance with 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.

Fluorescent *in situ* hybridization (FISH) was performed as described by Mandrioli *et al*. (2011). 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 charge-coupled device camera (Spot Imaging Solutions, a division of Diagnostic Instruments, Inc.) and the SPOT software supplied with the camera and processed using ADOBE PHOTOSHOP (Adobe Systems Inc.).

For each different karyotype observed in clone 33H, measurements of chromosome lengths were performed on 30 metaphases using the MICROMEASURE (http://rydberg.biology.colostate.edu/MicroMeasure). Evaluation of clone 1 was performed on 100 metaphases and always revealed a standard karyotype.

RNA extraction and reverse transcriptase (RT)-PCR were performed using the SV Total RNA Isolation System (Promega) and the Access RT-PCR System (Promega), respectively, in accordance with the manufacturer's instructions. Amplification of the telomerase gene was performed using a Hybaid thermocycler with the primers F-TERT1200 (5′-ACAACGTATGCCGGGTGT-3′) and R-TERT1200 (5′-AACCCCAAAAACTTGACCATC-3′) at an annealing temperature of 60 °C for 1 min and an extension step at 72 °C for 1 min. Cytoplasmic actin (primers F 5′-AGCAGGAGATGGCCACC-3′ and R 5′-TCCACATC TGCTGGAAGG-3′) was amplified as a loading control in RT-PCR experiments. For the cytoplasmic actin PCR reactions, 25 cycles were performed, with

annealing for 40 s at 58 °C and elongation for 45 s at 72 °C. Both the primer sets have been designed according to the orthologous genes identified in the pea aphid *Acyrthosiphon pisum* genome.

RESULTS

The *M. persicae* standard karyotype consisted of 12 chromosomes comprising two homologous X chromosomes and five homologous sets of autosomes (Fig. 1A). Analysis of mitotic metaphases of the *M. persicae* clone 33H (observed after both spreading and squashing) revealed the occurrence of different chromosome numbers in the range 12–17 also within each analyzed embryo, hence revealing the occurrence of an intra-individual chromosomal mosaicism (Fig. 1A, B, C, D, E, F). Approximately 18% of the observed plates showed the $2n = 12$ standard chromosome number (Fig. 1G) and approximately 21% had 13 chromosomes, whereas 46% of the observed metaphases had 14 chromosomes. Metaphases with more than 15 chromosomes were scarcely represented and, as a whole, constituted approximately 14% of the observed plates (Fig. 1G).

Analyzing the observed changes in the chromosome number, we focused our attention on the four most commonly observed karyotypes consisting of 12, 13, 14, and 15 chromosomes.

To identify the chromosomes involved in the fissions, we performed FISH experiments with the subtelomeric satellite DNA that labelled all the *M. persicae* chromosome ends with the exception of the rDNA telomere in each X chromosome (Spence *et al.*, 1998) (Fig. 2). Measurements revealed that the observed mosaicism is a result of fragmentations (Fig. 3). All the karyotypes with 12 chromosomes represented the standard complement consisting of five couples of autosomes with both the telomeres labelled with the subtelomeric probes and two X chromosomes with a single-labelled end (Figs 2A, 3A).

Karyotypes consisting of 13 chromosomes were the result of a single fission involving autosome 1 or 3 or, more frequently, the X chromosome, which was fragmented in more than half of the observed plates (Figs 2B, 3B). X chromosome identification was confirmed by *in situ* hybridization with the Hind200 satellite DNA highlighting several intercalary bands on both the X chromosomes (Mandrioli *et al.*, 1999a) (Fig. 4A). One X chromosome of *M. persicae* clone 33H showed the standard banding pattern after FISH, whereas the second one showed an unusual telomeric Hind200 band (Fig. 4B, C), indicating that an X chromosome fragmentation occurred at this site. CMA_3 staining (Fig. 4D) further showed that the X chromosome fission involved the X telomere opposite to the rDNA-bearing one (Fig. 4E). Interestingly, the presence of heteromorphism in the size of the CMA_3 stained-telomeres revealed that the fission always occurred in the X chromosome possessing a smaller CMA_3-positive telomere (Fig. 4F).

Metaphase plates consisting of 14 chromosomes were the result of fissions involving chromosomes 1,

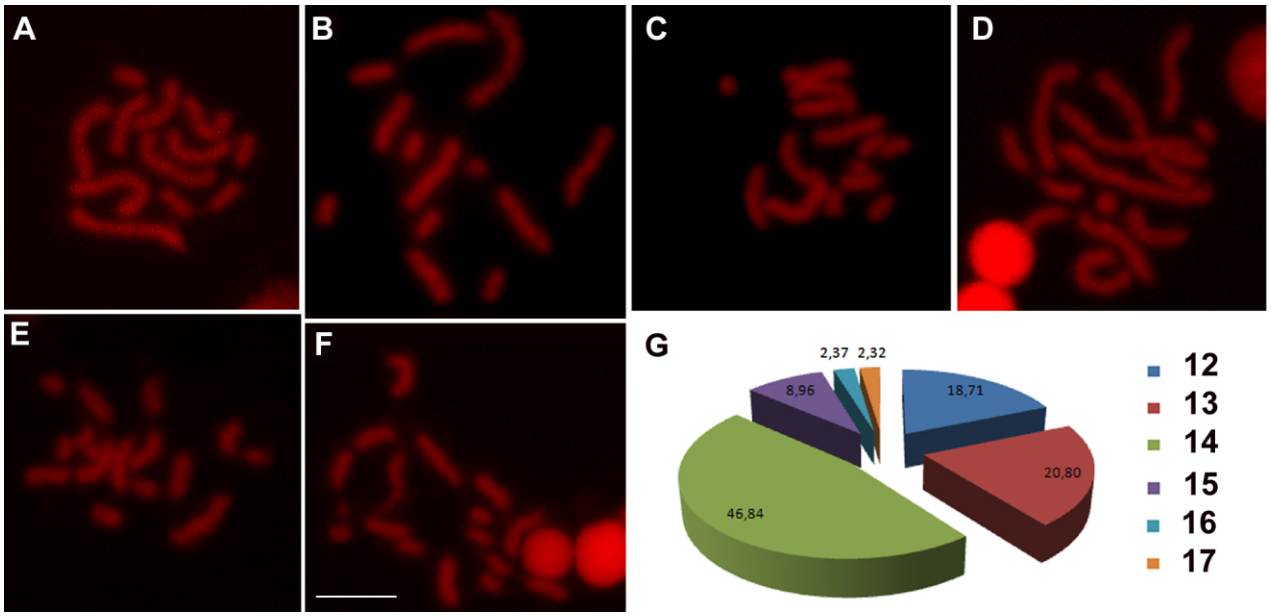

Figure 1. Propidium iodide staining of *Myzus persicae* 33H metaphases gave plates with 12 (A), 13 (B), 14 (C), 15 (D), 16 (E), and 17 (F) chromosomes within each single aphid embryo. G, the frequencies of the different complements in the analyzed embryos. Scale bar = 10 μm.

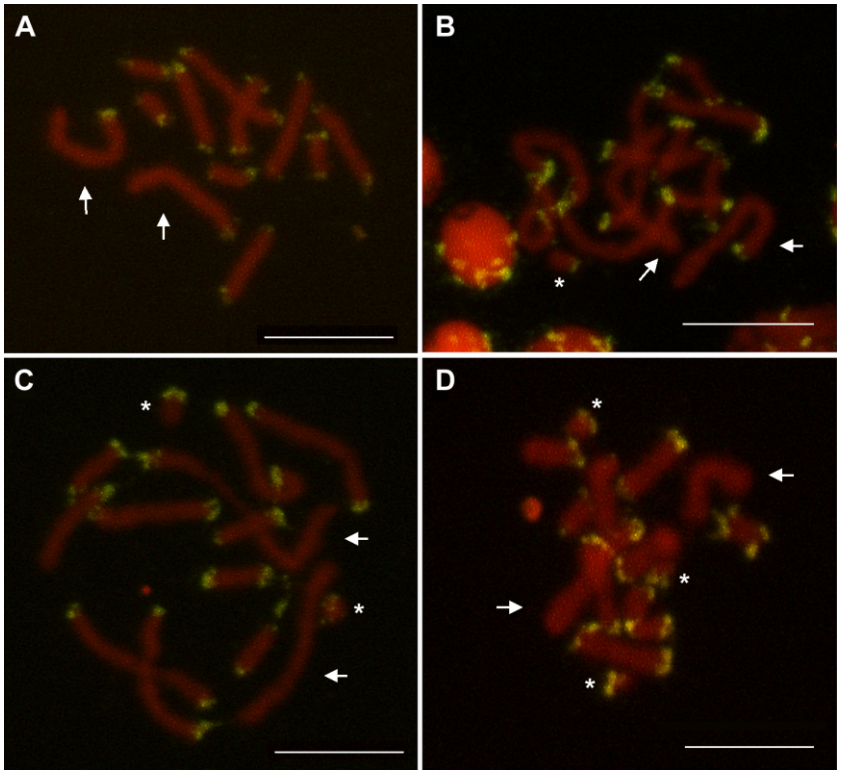

Figure 2. Fluorescent *in situ* hybridization with the subtelomeric probes allowed the identification of the chromosomes involved in the fragmentations resulting in changes in the chromosome number of the *Myzus persicae* 33H metaphases with 12 (A), 13 (B), 14 (C), and 15 (D) chromosomes. Arrows indicate X chromosomes. Asterisks indicate chromosome fragments. Scale bars = 10 μm.

3, and X, although simultaneous fragmentations occurring on one X chromosome and one autosome 3 were most frequent (Figs 2C, 3C).

Karyotypes consisting of 15 chromosomes involved simultaneously the fission of chromosomes 1, 3, and X, thus producing three small chromosomes (Figs 2D, 3D).

Male induction revealed that the *M. persicae* clone 33H is anholocyclic because it was not possible to induce the sexual generation, in contrast to what was obtained under the same experimental conditions with the holocyclic *M. persicae* strain 1 used as a control.

Evaluation of the expression of the telomerase gene (TERT) showed that *M. persicae* clone 33H embryos presented a higher TERT expression level than embryos from the control strain 1 (Fig. 5).

DISCUSSION

Chromosome instability within individuals is an almost unique finding in the animal kingdom except in malignant cells. Among insects, it has reported only in ants (Imai, Crozier & Taylor, 1977; Imai, Taylor & Crozier, 1994; Karnik *et al*., 2010).

The *M. persicae* clone 33H shows a highly variable karyotype resulting in the presence of different chromosome numbers (range 12–17), between embryos from different individuals within the same asexual lineage, between single embryos from the same individual, and within embryos. The variant karyotypes result from fissions involving predominantly the X chromosomes, as well also autosomes 1 and 3. These data support the earlier molecular evidence indicating that intraclonal genetic variation occurs in nature and is a potentially important force for generating variation in asexual lines (Loxdale & Lushai, 2003; Lushai, Loxdale & Allen, 2003).

Previous data have shown that autosomes 3 and, more rarely, 1 are the chromosomes mostly involved in changes in the *M. persicae* karyotype (Spence & Blackman, 1998). By contrast, fragmentations associated with the X chromosomes are extremely rare in aphids not only in natural populations, where only one case has been reported in *Schoutedenia lutea* (van der Goot) (Hemiptera, Aphididae, Greenidinae) (Hales, 1989), but also in X-ray irradiated aphids

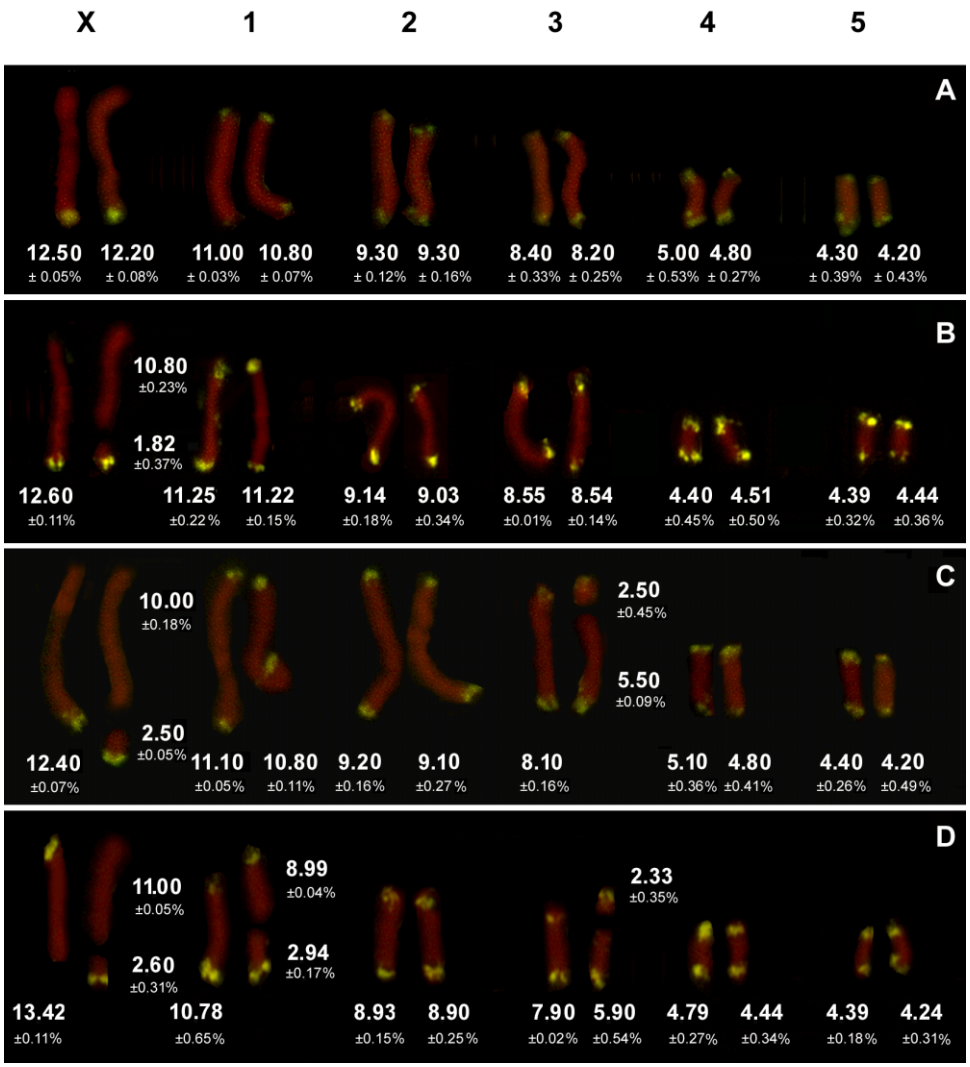

Figure 3. Comparing standard karyotype (A) with the variable ones, the most frequent fissions involved chromosome X in plates with 13 chromosomes (B), chromosomes X and 3 in metaphases with 14 chromosomes (C), and chromosomes X, 1, and 3 in plates with 15 chromosomes (D). The percentages near each chromosome indicate the relative length of each chromosome (i.e. expressed as a percentage of the total complement length).

(Khuda-Bukhsh & Pal, 1985). Irradiation of *Aphis gossypii* (Glover) and *Aphis nerii* (Boyer de Fonscolombe) specimens produced several breaks occurring mainly on autosome 1 and never on the X chromosome, suggesting that X fragmentations are less viable than autosome ones (Khuda-Bukhsh & Pal, 1985). Studies on natural populations of the same species confirmed these results, showing fragmentations of autosomes 1 and 4 but never at X chromosomes (Khuda-Bukhsh & Pal, 1985).

The fragmentation of the X chromosome observed in *M. persicae* 33H specimens occurred near (or within) a heterochromatic band enriched in satellite DNAs. The presence of chromosome breakpoints occurring within constitutive heterochromatin is well established in the scientific literature and, for example, much of the evolution of mammals has involved pericentromeric heterochromatin that is known to be particularly variable, as is also known in some insects, such as grasshoppers (Orthoptera: Acrididae) (John, 1983; Blackman, Spence & Normark, 2000).

Changes in heterochromatin are presumably largely selectively neutral and are therefore unlikely to lead to any significant genetic, and hence phenotypic, change (Blackman *et al*., 2000). On the other hand, fragmentations that move genes from the proximity of heterochromatic areas of the X chromosomes to a new chromosome could affect the expression of genes by position-effect variegation, making these

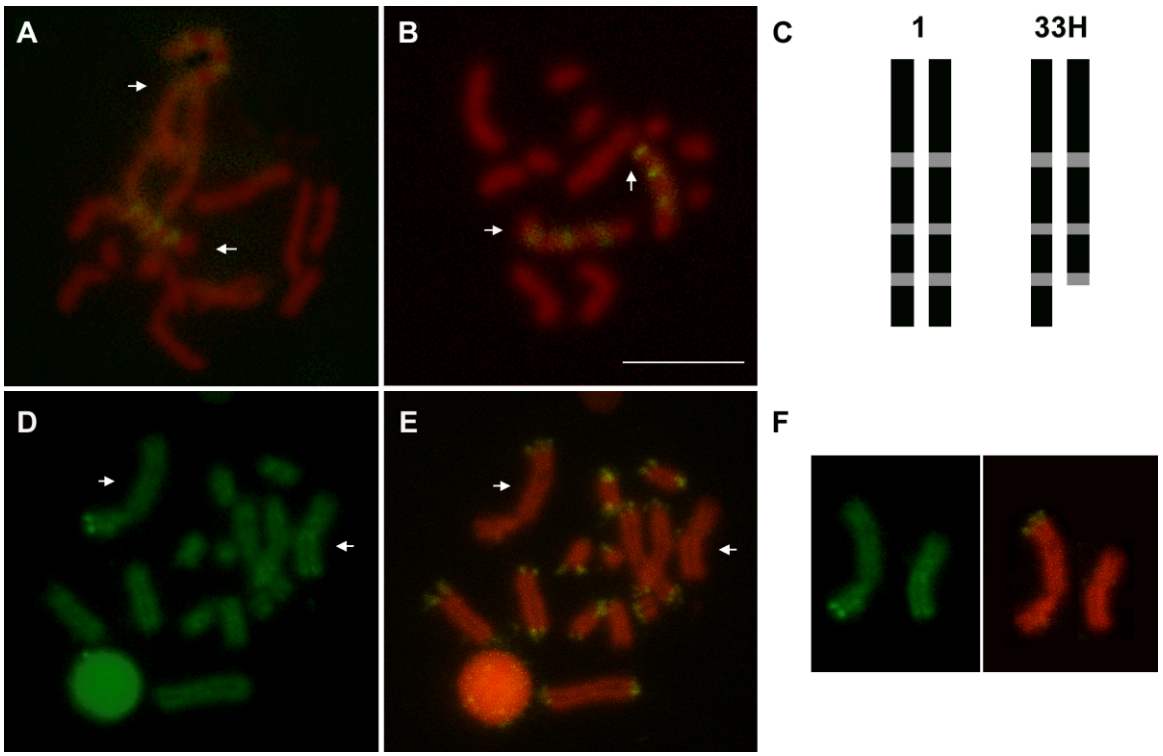

Figure 4. Fluorescent *in situ* hybridization (FISH) with the Hind200 satellite DNA allowed the identification of the two X chromosomes in the metaphases of *M. persicae* aphids of strain 1 (A) and clone 33H (B). Comparison of the hybridization signals allowed the reconstruction of karyograms (C) showing the fragmentation of an X chromosome telomere below a Hind200 band (in grey). CMA_3 staining (D) identified the nucleolar organizer region-bearing X telomeres that were not involved in X chromosome fragmentation, as indicated by FISH of the same plate with the subtelomeric repeat (E). Comparison of X chromosomes after chromomycin A_3 staining and FISH with the subtelomeric repeat is shown in (F). Arrows indicate the X chromosomes. Scale bar = 10 μm.

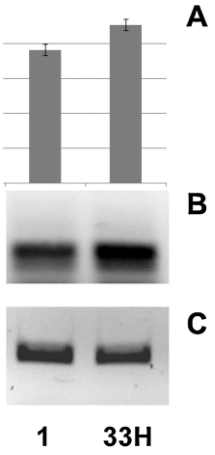

Figure 5. Reverse transcriptase-polymerase chain reaction (RT-PCR) amplification of the TERT gene in strain 1 and clone 33H (B) showed a higher telomerase expression level in the 33H embryos with a variable chromosome number than in strain 1 with a stable karyotype, as also shown by densitometric analysis of the amplified bands (A). Cytoplasmic actin has been amplified by RT-PCR as a loading control (C).

changes not neutral at a selective level. For example, in *M. persicae*, translocation of the E4 esterase genes enhanced their expression so that aphids with this rearrangement produce a greater amount of the enzyme, thereby becoming resistant to a range of insecticides (i.e. organophosphates, carbamates and pyrethroids) (Blackman *et al*., 1978; Foster & Devonshire, 2007; Loxdale, 2009). This effect is very interesting considering that the E4 array is generally located near the subtelomeric region of the *M. persicae* chromosome 3 that is made by the tandem repetition of the 169-bp satellite and that, as shown in *Drosophila melanogaster* Meigen (Eberl, Duyf & Hilliker, 1993), the presence of a gene near repetitive DNAs influences its expression through position-effect variegation. Translocations may therefore turn on and off the E4 genes modifying their expression and, consequently, the level of resistance of aphids to insecticides. Further analysis of the *M. persicae* karyotype could therefore prove very interesting in view of the relationship between chromosomal rearrangements and the insecticide resistance level.

Fragmentation of chromosomes 1 and 3 involved euchromatic areas and it has been hypothesized that these chromosomal rearrangements could affect some complex phenotypic traits, such as host choice (Blackman, 1987; Ffrench-Constant *et al*., 1988; Margaritopoulos *et al*., 2000; Vargas *et al*., 2005).

The random-breakage model of chromosome evolution has been the dominant paradigm for several years (Becker & Lenhard, 2007). Interestingly, several comparative mapping studies in a wide variety of closely-related eukaryotes showed a relationship between large-scale chromosomal rearrangement and repetitive DNA. The nature of the repetitive DNA within these breakpoint regions varies significantly, from clusters of rRNA and tRNA genes to various mobile elements (Caceres *et al*., 1999; Carlton *et al*., 2002; Coghlan & Wolfe, 2002; Kellis *et al*., 2003). Segmental duplications that distinguish the human and great-ape karyotypes have been, for example, related to breakpoints revealing that some human diseases may also be the result of chromosomal breakpoints because of hot spots of recombination (Bailey *et al*., 2004). In addition, computational analyses of breakpoints suggested that recurrent evolutionary breaks are found in fragile regions or hot spots, so that the random breakage model required substantial reassessment in favour of models that put the architecture of the chromosomes in a pivotal position for revealing the molecular basis of chromosomal evolution among species.

In view of the recurrent fission of the same chromosomes in the same region, the *M. persicae* genome appears therefore to have some fragile sites that could be the basis for the observed changes in the chromosome number. Hot spots of chromosomal recombination have already been identified in aphids within rDNA genes that contain specific sequences with high similarity to the consensus core region of human hypervariable minisatellites (Jeffreys, Wilson & Thein, 1985) and with the χ sequence of *Escherichia coli* (Smith, 1983). They are involved in rDNA pairing (Mandrioli *et al*., 1999b), as also previously reported in *D. melanogaster* (Ault, Lin & Church, 1982; Park & Yamamoto, 1995). As a consequence of such hot spots, the occurrence of X chromosomes paired at nucleolar organizer regions (NORs) has been observed in several aphid species (Mandrioli *et al*., 1999b), together with the occurrence of intra- and inter-individual NOR heteromorphism as a result of unequal crossing over between the two X chromosomes (Mandrioli *et al*., 1999a).

The holokinetic structure of aphid chromosomes that ensures the attachment of spindle microtubules along most of the chromosome length is itself not sufficient to stabilize chromosome fragments because chromosomal breakpoints may be highly unstable, displaying a facility to fuse with other broken ends. The breakpoints need therefore to be stabilized before the transmission of chromosomal fragments to the daughter cells (Vermeesch & Price, 1994; Hug & Lingner, 2006; Pennaneach, Putnam & Kolodner, 2006). This phenomenon, known as *de novo* telomere synthesis, generally involves the addition of repetitive telomeric sequences at the breakpoints by telomerase. Indeed, the essential function of telomeres is to protect chromosome ends from nucleolytic degradation, chromosome fusion, and the inappropriate engagement of checkpoint signalling (Lydall, 2003), so that the addition of telomere repeats results in the stabilization of the new chromosome ends, allowing the resumption of cell cycling (Vermeesch & Price, 1994; Hug & Lingner, 2006; Pennaneach *et al*., 2006). In the absence of healing, unrepairable double-strand breaks lead to programmed cell death, as reported in yeast (Sandell & Zakian, 1993), or to the activation of proto-oncogenes, as reported in mammals (Lee & Myung, 2009).

It has been suggested several times that, in aphids, sexual reproduction was not only related to genetic recombination, but also perhaps to the resetting of telomere length (Loxdale & Lushai, 2003; Lushai & Loxdale, 2007). In the absence of sexual reproduction, asexual aphid generations would thus shorten telomere length cumulatively, resulting in a finite persistence of obligately parthenogenetic strains/generations (Lushai & Loxdale, 2007). By contrast, recent molecular analyses in the peach potato aphid *M. persicae* revealed that aphids can initiate *de novo* synthesis of $(TTAGG)_n$ telomeric sequences at internal breakpoints, resulting in the stabilization of the chromosomal fragments (Monti *et al*., 2011).

The results of the present study have shown that telomerase is expressed at higher level in *M. persicae* clone 33H than in strain 1 (utilized as a control because it possesses a stable karyotype $2n = 12$), suggesting an active role in the stabilization of chromosome fragments.

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