Chromosome Res (2011) 19:625–633
DOI 10.1007/s10577-011-9222-7

Presence of a functional $(TTAGG)_n$ telomere-telomerase system in aphids

Valentina Monti · Monica Giusti · Davide Bizzaro · Gian Carlo Manicardi · Mauro Mandrioli

Received: 15 March 2011 / Revised: 18 May 2011 / Accepted: 20 May 2011 / Published online: 11 June 2011

Abstract The structure of the telomeres of four aphid species (*Acyrthosiphon pisum*, *Megoura viciae*, *Myzus persicae* and *Rhopalosiphum padi*) was evaluated by Southern blotting and fluorescent in situ hybridization, revealing that each chromosomal end consists of a $(TTAGG)_n$ repeat. The presence of a telomerase coding gene has been verified successively in the *A. pisum* genome, revealing that aphid telomerase shares sequence identity ranging from 12% to 18% with invertebrate and vertebrate homologues, and possesses the two main domains involved in telomerase activity. Interestingly, telomerase expression has been verified in different somatic tissues suggesting that, in aphids, telomerase activity is not as restricted as in human cells. The study of telomeres in a *M. persicae* strain with a variable chromosome number showed that aphid telomerase can initiate the de novo synthesis of telomere sequences at internal breakpoints, resulting in the stabilization of chromosomal fragments.

Keywords aphid · holocentric chromosomes · telomere · telomerase

Abbreviations

FITC	Fluorescein isothiocyanate
RACE	Rapid amplification of cDNA ends
TERT	Telomerase reverse transcriptase

Introduction

Telomeres are specialized DNA–protein structures constituting the end of chromosomes (Blackburn 1991). They are essential to protect chromosomal ends from erosion by exonucleases, to avoid chromosome stickiness and to mediate the attachment of chromosomes to the nuclear envelope before chromatin remodelling at cell division (Blackburn 1991; Zakian 1995). Furthermore, telomeres have been shown to be involved in the regulation of cell senescence and carcinogenesis in many organisms (Kim et al. 1994; Krupp et al. 2000).

Telomeres are partially lost at each replication cycle in most somatic cells due to incomplete replication of the DNA molecule end (Blackburn 1991). This loss can be avoided by telomere elongation mediated by reverse transcription due to telomerases—ribonucleoprotein enzymes that are highly conserved from unicellular organisms to flowering plants and

Responsible Editor: Walther Traut.

V. Monti · M. Giusti · M. Mandrioli (✉)
Dipartimento di Biologia,
Università di Modena e Reggio Emilia,
Via Campi 213/D,
41125 Modena, Italy
e-mail: mauro.mandrioli@unimo.it

V. Monti · G. C. Manicardi
Dipartimento di Scienze Agrarie e degli Alimenti,
Università di Modena e Reggio Emilia,
Reggio Emilia, Italy

D. Bizzaro
Dipartimento di Biochimica, Biologia e Genetica,
Università Politecnica delle Marche,
Ancona, Italy

vertebrates (Krupp et al. 2000). Nevertheless, not all eukaryotes possess telomerase and some organisms compensate for telomere loss by different mechanisms, including the insertion of mobile elements and unequal recombination between long tandem repeats (Biessmann and Mason 2003; Pardue and DeBaryshe 2003).

Although telomeric sequences may vary in composition in eukaryotes, their conservation is strict in some taxonomic groups so that the hexameric $(TTAGGG)_n$ repeat is typical of vertebrates (Meyne et al. 1989) and the sequence $(TTTAGGG)_n$ is typical of plants (Fajkus and Zentgraf 2002), whereas the $(TTAGG)_n$ telomeric repeat has been reported in many of the main lineages of insects and other invertebrates (Okazaki et al. 1993; Sahara et al. 1999; Bizzaro et al. 2000; Mandrioli 2002; Frydrychová et al. 2004; Vitkova et al. 2005; Lukhtanov and Kuznetsova 2010). In particular, the $(TTAGG)_n$ repeat is widespread in insects (Hymenoptera, Lepidoptera, Trichoptera and Megaloptera) with exceptions observed in Ephemeroptera, Odonata, Dermaptera and Heteroptera. Moreover, telomeric repeats are absent in the clade Antliophora (Diptera, Siphonaptera and Mecoptera), where long repeated sequences (as in the non-biting midge *Chironomus pallidivittatus*, Zhang et al. 1994) or retrotransposable elements (as reported in the fruit fly *Drosophila melanogaster*, Biessmann et al. 2002) are present in place of the classical telomere-telomerase system, thus indicating that telomere elongation could be telomerase-independent in insects (Biessmann and Mason 2003; Pardue and DeBaryshe 2003).

At present, the occurrence of the $(TTAGG)_n$ repeat has been reported also in Hemiptera and, in particular, in the aphids *Myzus persicae*, *Myzus antirrhinii* and *Acyrthosiphon pisum* (Aphididae) (Spence et al. 1998; Bizzaro et al. 2000). Considering that heterogeneity in the presence of the TTAGG repeats has been reported in Coleoptera and Neuroptera (Sahara et al. 1999; Frydrychová and Marec 2002; Frydrychová et al. 2004), it could be very interesting to verify the presence of this repeat in other aphid species. Moreover, although telomere sequence has been identified in several insects, telomerase coding genes have been sequenced in only a few species.

In the present paper, we performed experiments to verify the presence of the $(TTAGG)_n$ sequence at the telomeres of four aphid species. We subsequently identified the telomerase (TERT) coding gene and analyzed its expression pattern in different tissues and developmental stages in order to verify if TERT transcription in aphids is highly regulated as reported in humans (Krupp et al. 2000), or diffuse as observed in some insects (Sasaki and Fujiwara 2000; Honey Bee Genome Sequencing Consortium 2006). Lastly, the presence of mechanisms involved in de novo telomere synthesis is evaluated and discussed.

Materials and methods

The specimens of *A. pisum* used for this research were obtained from the LSR1 laboratory lineage, kindly supplied by M. Plantagenest (INRA, France), and maintained on broad bean (*Vicia faba*) plants. *Megoura viciae* aphids belong to a strain reared in our department since 1999, kindly provided by S. Ganassi (University of Modena and Reggio Emilia, Italy), and maintained on *Vicia faba* plants. *Myzus persicae* specimens were obtained from two lineages (1 with a canonical karyotype $2n=12$ and 33 H with karyotype varying from $2n=12$ to $2n=14$), kindly provided by E. Mazzoni (Università Cattolica di Piacenza, Italy), and maintained on pea (*Pisum sativum*) plants. *Rhopalosiphum padi* aphids belong to a laboratory strain reared in our department since 1964 and maintained on maize (*Zea mays*) plants. All aphids were reared in our department at 19°C with 16 h light, 8 h darkness.

Chromosome preparations from parthenogenetic females were made by spreading embryo cells as previously described by Manicardi et al. (1996). Chromosome dimensions (expressed as relative percentage of the total complement length) in *M. persicae* strains were evaluated using the software MicroMeasure, which is freely available at the Biology Department at Colorado State University website (http://rydberg.biology.colostate.edu/MicroMeasure). We evaluated 30 metaphasic plates for both 1 and 33 H specimens.

DNA was extracted from aphid embryos as described in Mandrioli et al. (1999). In order to test for the presence of the telomeric $(TTAGG)_n$ repeat, a probe was obtained by PCR amplification using the two primers F $(TTAGG)_5$ and R $(CCTAA)_5$ in the absence of template, as described by Ijdo et al. (1991). Random priming probe biotin-labeling was performed with the Biotin High Prime (Roche, Basel, Switzerland), whereas PCR digoxigenin labelling was performed using the Dig High Prime kit (Roche).

Both types of labelling were done according to Roche protocols. Southern blotting and fluorescence in situ hybridization (FISH) were performed as described by Mandrioli et al. (1999).

RNA extraction and RT-PCR experiments were performed using the "SV Total RNA Isolation System" (Promega, Madison, WI) and with the "Access RT-PCR System" (Promega) respectively, according to the supplier's recommendations. RACE amplification of the telomerase was done with the "5′/3′ RACE Kit" (Roche), according to the supplier's datasheet using primers F-TERT1200 (5′-ACAACGT ATGCCGGGTGT) and R-TERT1200 (5′-AACCCCA AAAACTTGACCATC).

Results

The presence of the (TTAGG)$_n$ repeat was evaluated in four aphid species (*A. pisum*, *Megoura viciae*, *Myzus persicae* and *R. padi*) by Southern blotting and FISH. Southern blotting revealed a diffuse smear of hybridization, together with several well recognizable electrophoretic bands in all lanes (Fig. 1), whereas FISH experiments with the telomeric (TTAGG)$_n$ probe showed bright FITC-fluorescent spots at the ends of all chromosomes (Fig. 2a–d). In the inter-

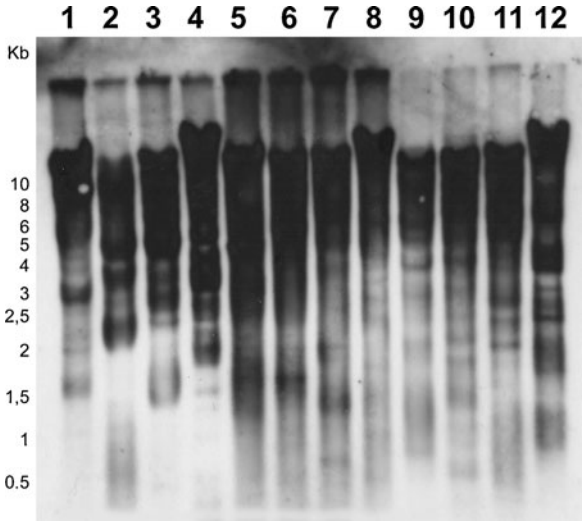


Fig. 1 Genomic DNAs of *Megoura viciae* (lanes *1–4*), *Myzus persicae* (lanes *5–8*) and *Rhopalosiphum padi* (*9–12*) digested with *Hind*III (lanes *1*, *5*, *9*), *Eco*RI (lanes *2*, *6*, *10*), *Sca*I (lanes *3*, *7*, *11*) and *Xho*I (*4*, *8*, *12*) and hybridized with the telomeric probe (TTAGG)$_n$ showing a diffuse and intense labelling of aphid DNAs

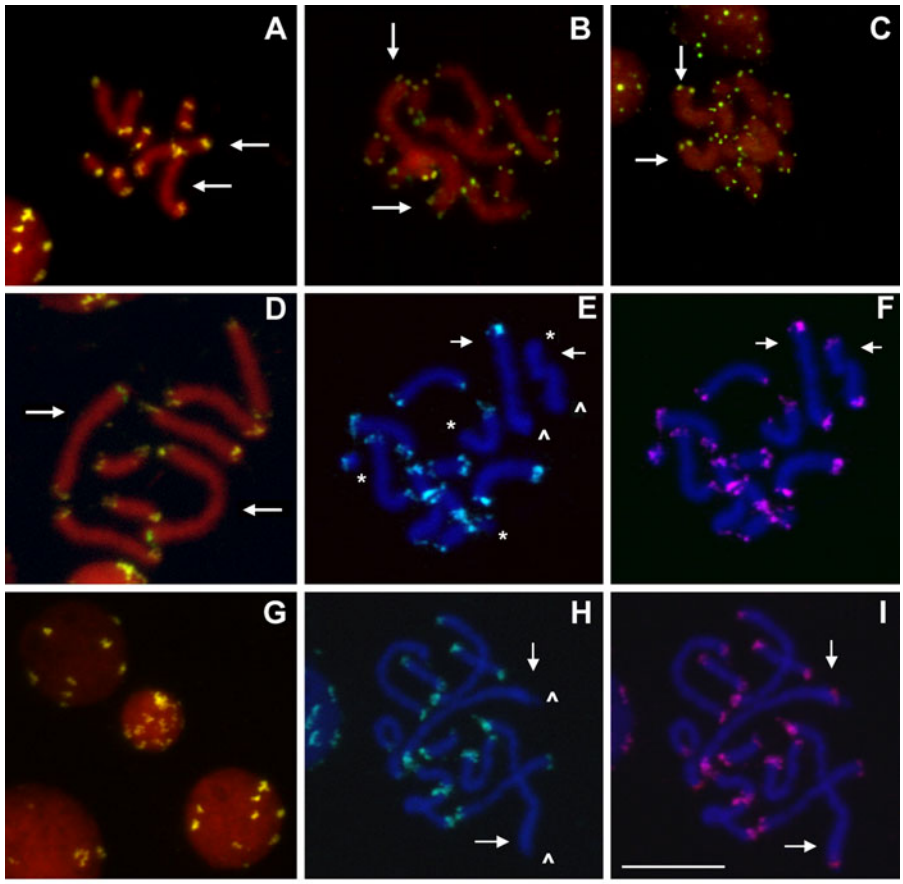
phase nuclei of *M. persicae*, telomeres clustered in few highly fluorescent foci (Fig. 2g).

The identification of telomeric TTAGG sequence at the ends of chromosomes implies that the aphid genome should encode a telomerase reverse transcriptase (TERT). Using sequences of other insect telomerases (isolated in the honey bee, *Apis mellifera* and in the flour beetle, *Tribolium castaneum*), we identified a candidate gene encoding a putative telomerase (predicted protein XP_001946970) in the recently sequenced genome of the pea aphid *A. pisum* (International Aphid Genomic Consortium 2010). The identified telomerase gene encodes a predicted protein with an N-terminal region typical of a telomerase containing the telomerase RNA binding domain (TRBD) made up of alpha helices and two short beta sheets, and a TERT domain corresponding to the catalytic subunit of all telomerase reverse transcriptases. Unusually for a telomerase, the C-terminal region of peptide XP_001946970 contains a ICMT domain identified in a number of bacterial and eukaryotic proteins of unknown function that are approximately 300 residues long. In order to clarify the real structure of pea aphid telomerase, a TERT cDNA was amplified by 5′ and 3′ RACE. The resultant sequence was 2,521 bp in length and encoded a 840 amino acid telomerase.

Interestingly, the ICMT domain was not present in the *A. pisum* TERT cDNA. *A. pisum* TERT exhibited 18% identity and 39% similarity with the 801 amino acids of honey bee telomerase, and similar values were obtained upon comparing aphid TERT with the homologous genes in *Bombyx mori* and *Tribolium castaneum* (Table 1). At a structural level, aphid telomerase possesses the two main TERT functional domains reported previously in *A. mellifera* (Fig. 3).

RT-PCR experiments performed using RNA samples extracted from embryos, head, gut and the whole aphid body (but without embryos, gut and head) showed that TERT expression is present both in adults and embryo tissues of all aphid species analyzed (Fig. 4).

Chromosomes of *M. persicae* strains 1 and 33 H were hybridized simultaneously with a TRITC-telomeric (TTAGG)$_n$ probe and with an FITC-labelled subtelomeric 169 bp satellite DNA (Spence et al. 1998). In strain 1, which has the standard *M. persicae* karyotype with $2n=12$, the 169 bp satellite DNA probe labelled the subtelomeric regions of all chromosomes, with the exception of the NOR-bearing telomeres of the X chromosomes (Fig. 2h), in



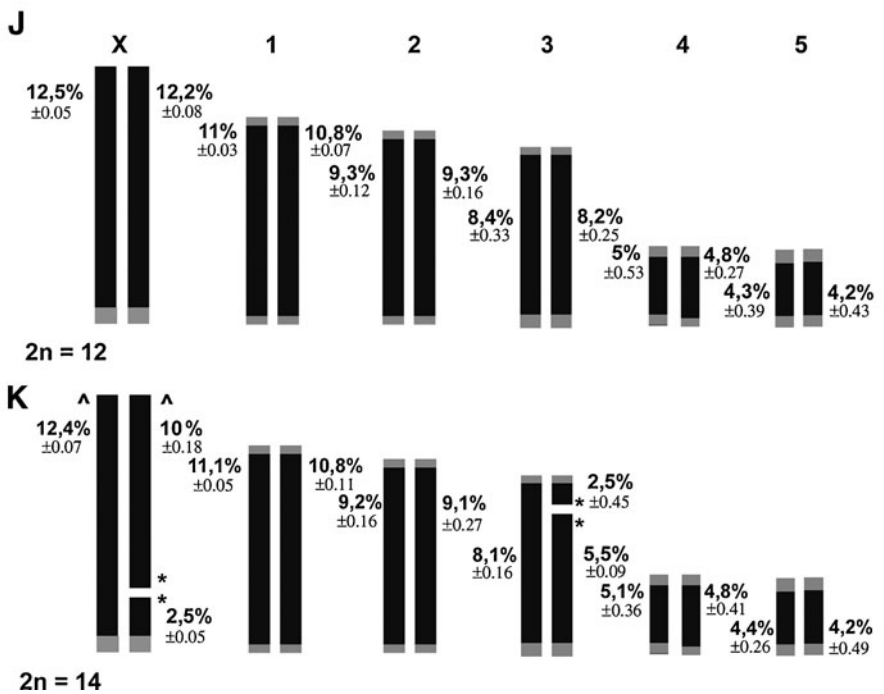


Fig. 2a–i Fluorescence in situ hybridization (FISH) analysis of insect chromosomes. *R. padi* (**a**) and *M. viciae* (**b**), *M. persicae* (**c**) and *A. pisum* (**d**) chromosomes showed bright FITC fluorescent signals at both ends of all chromosomes with telomeric $(TTAGG)_n$ probes. Bright spots were also observed in several foci in *M. persicae* interphase nuclei (**g**). Signal comparison after hybridization with the FITC-labelled 169 bp subtelomeric probe (**e**, **h**) and the TRITC-labelled telomeric probe (**f**, **i**) on *M. persicae* chromosomes of strains 33 H (**e**–**f**) and 1 (**h**, **i**) allowed identification of de novo synthesized telomeres. Comparison between the karyotypes of strains 1 and clone 33 H (*lower panel*: J and K) shows labelling after hybridization with a subtelomeric probe (*gray*) with the chromosomes (*black*). The percentages indicate the length of each chromosome expressed as a percentage of the total complement length. *Arrows* X chromosomes, *asterisks* chromosomal ends involved in de novo telomere synthesis (i.e., positive labelling with telomeric probe and absence of labelling with subtelomeric probe). The symbol ^ indicates the NOR-bearing telomeres of the X chromosomes. *Bar* 10 μm

Table 1 Similarity / identity percentages of *Apis mellifera* (GenBank NP_001035771), *Tribolium castaneum* (GenBank NP_001035796), *Bombyx mori* (GenBank NP_001037666) and *Acyrthosiphon pisum* (GenBank JF897623) TERT amino acidic sequences

	A. melifera	*B. mori*	*T. castaneum*
A. pisum	18% / 39%	13% / 27%	12% / 23%
A. mellifera		16% /31%	12% / 25%
B. mori			16% / 34%

agreement with published results (Spence et al. 1998). Hybridization of the same plate with the telomeric (TTAGG)$_n$ repeat showed bright spots at the ends of all chromosomes (Fig. 2i).

Strain 33 H exhibited intra-individual chromosome mosaicism so that several plates revealed 14 chromosomes in place of the standard $2n=12$ chromosome number. Accordingly to estimates of relative chromosome length, the observed variations in chromosome number were due to chromosomal fragmentations (Fig. 2j–k). In view of the recurrent fragmentation observed in *M. persicae* strain 33 H, plates with $2n=14$ were very interesting in order to evaluate if a new telomere has been synthesized at the broken end. After hybridization with the 169-bp satellite DNA probe, six telomeres were negative for labelling: two corresponding to the NOR-bearing X telomeres and four due to chromosomal fragmentations (Fig. 2f). On the contrary, all the telomeres were labelled with the telomeric probe, including both termini of the two broken chromosomes, indicating that de novo (TTAGG)$_n$ telomere synthesis occurred after chromosomal fragmentation.

Discussion

Three telomerases have been identified to date in insects (*A. mellifera*, *T. castaneum*, *B. mori*); these three telomerases share ~20% sequence identity among themselves and similar identity to their vertebrate homologues (Honeybee Genome Sequencing Consortium 2006; Osanai et al. 2006; Robertson and Gordon 2006). *A. pisum* TERT exhibits sequence identity to both invertebrate and vertebrate homologues ranging from 12% to 18% and possesses the two main domains involved in the telomerase activity, indicating that it is a functional enzyme.

Aphid TERT is expressed in different body parts, such as gut and head, in full agreement with data published by Sasaki and Fujiwara (2000) reporting telomerase activity in different organs and tissues of crickets and cockroaches. Overall, we have different evidence suggesting that, in insects, robust telomerase expression is present also in somatic tissues and not only in germ and pluripotent stem cells as observed in human tissues (Krupp et al. 2000; Donate and Blasco 2011). TERT expression was also reported in *A. mellifera* and *B. mori*, where telomerase mRNAs have been found in different tissues albeit in low amounts (Honey Bee Genome Sequencing Consortium

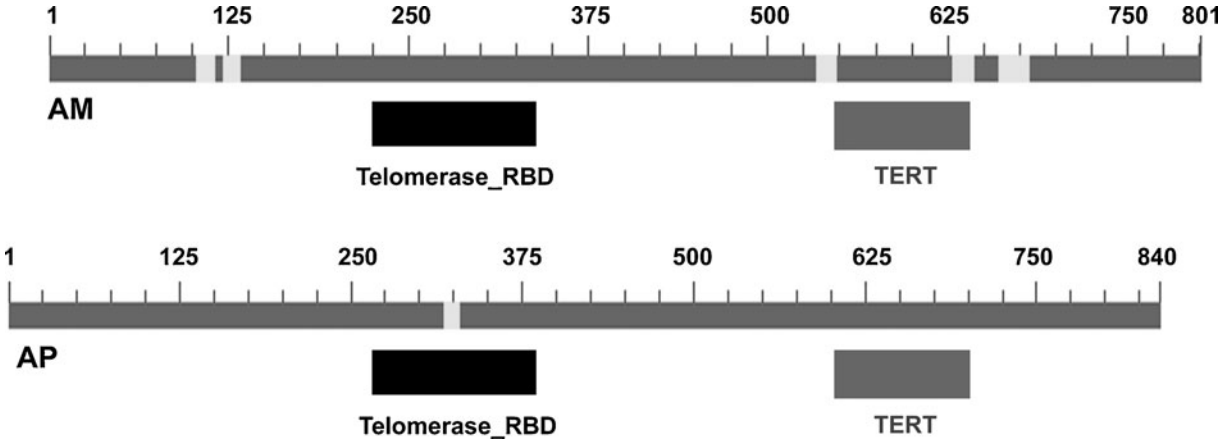


Fig. 3 Comparison of the telomerase functional domains identified in *Apis mellifera* (AM) and *Acyrthosiphon pisum* (AP). The two main domains are both conserved and spaced in the same way in the two insect TERTs. *Telomerase_RBD* Telomerase ribonucleoprotein complex–RNA binding domain, *TERT* telomerase reverse transcriptase domain

2006; Osanai et al. 2006). Interestingly, weak telomerase activity was observed in different adult human tissues, where it is insufficient to prevent telomere shortening. It could therefore be intriguing to delve deeper into the study of TERT activity in insects, and in particular that of in *A. mellifera* and *B. mori*, in order to better comprehend the role of telomerase expression in insect somatic tissues.

The presence of a telomerase in aphids is coupled to the presence of the $(TTAGG)_n$ repeats that are located in all the telomeres of the four aphid species studied here. This result could be controversial considering that data recently published in the pea aphid genome suggested that only five of the eight *A. pisum* telomeres possessed the classical TTAGG sequence, whereas the remaining three telomeres were regulated by non-LTR retrotransposon insertions as reported in *T. castaneum* (Tribolium Genome Sequencing Consortium 2008; International Aphid Genomic Consortium 2010). The unusual structure of the latter three telomeres has been suggested during attempts to determine their structure using sequences not assembled into scaffolds. This strategy could lead to the assembly of sequences that are near the telomeres but that do not constitute true chromosomal ends (International Aphid Genomic Consortium 2010). On the basis of our data, we suggest that all the aphid telomeres consist of the $(TTAGG)_n$ sequence.

Southern blot experiments with the $(TTAGG)_n$ probe showed both smears and bands in all four aphid species, suggesting that aphid telomeres are composed of TTAGG repeats that are occasionally interrupted by other repeated sequences. Future experiments on the study of aphid telomeric regions will thus be of great interest as these regions seem to be very complex and enriched in repetitive sequences belonging to different families.

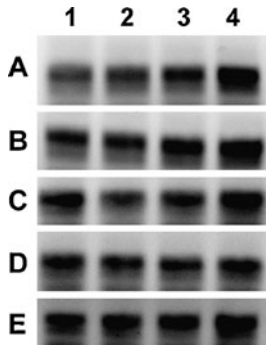


Fig. 4 RT-PCR of RNA isolated from different tissues of *A. pisum* (**a**), *M. viciae* (**b**), *R. padi* (**c**), *M. persicae* strain 1 (**d**) and *M. persicae* strain 33 H (**e**) to detect telomerase expression. Lanes: *1* Embryos, *2* gut, *3* head, *4* aphid body devoid of embryos

Considering that *A. pisum*, *Megoura viciae* and *Myzus persicae* belong to the tribe Macrosiphini, whereas *R. padi* belongs to the tribe Aphidini, it may be inferred that aphids have telomeres with a conserved composition in the different subfamilies. However, Macrosiphini and Aphidini are phylogenetically related (Ortiz-Rivas and Martinezez-Torres 2010), so it would also be interesting to look at other aphid tribes considering that, in some insect orders (such as Coleoptera and Neuroptera), heterogeneity in terms of the actual presence of the $(TTAGG)_n$ repeats has been observed (Sahara et al. 1999; Frydrychová and Marec 2002; Frydrychová et al. 2004).

FISH experiments showed a clear hybridization signal on each telomere of all aphid chromosomes and there was no evidence of any interstitial labelling. Our data demonstrate that TTAGG repeats are restricted to the terminal regions of all aphid chromosomes, as previously hypothesized by Bizzaro et al. (2000).

In the interphase nuclei of most organisms, the telomeric regions are arranged in an ordered fashion with an association to the nuclear matrix and clustering at least at some stages of cell life (Palladino et al. 1993; Luderus et al. 1996; Pryde et al. 1997). In aphid nuclei, telomeres appeared to be clustered into a few foci and were not located predominantly near the nuclear periphery as reported in other insects such as *D. melanogaster* (Hochstrasser et al. 1986) and the cabbage moth *Mamestra brassicae* (Mandrioli 2002). In view of such findings, it appears evident that, even if the telomeric structure and function are conserved in insects, the positioning of telomeres in interphase nuclei could, and probably does, vary among different species.

Aphid karyotypes have been described frequently in the literature as highly variable in view of the holocentric/holokinetic nature of their chromosomes, which stabilize rearrangements and fragmentations (Blackman 1980; Blackman et al. 2000). Indeed, holocentric/holokinetic chromosomes show that centromeric activity spread along the whole chromosomal axis so that chromosomal fragments can be properly segregated. In fact, the presence of a diffuse centromeric activity would be not be sufficient to stabilize chromosome fragments since, when chromosomes are

broken, the breakpoints become highly unstable and have a propensity to fuse with other broken ends. The breakpoints need therefore to be stabilized before transmission of chromosomal fragments to the daughter cells (Vermeesch and Price 1994; Hug and Lingner 2006; Pennaneach et al. 2006). This phenomenon, known as "healing of breakpoints" or "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 signaling (Lydall 2003). Hence, the addition of telomere repeats results in the stabilization of the new chromosome end and allows resumption of cell cycling (Vermeesch and Price 1994; Hug and Lingner 2006; Pennaneach et al. 2006). In the absence of healing, irreparable double-strand breaks lead to programmed cell death, as reported in yeast (Sandell and Zakian 1993), or to the activation of proto-oncogenes, as described in mammals (Lee and Myung 2009).

On the basis of our results in *M. persicae* strain 33 H, we demonstrate that aphid telomerase initiates the de novo synthesis of telomere sequences resulting in the stabilization of chromosomal fragments, as evidenced both by finding TERT expression in all aphid tissues analyzed and by FISH with telomeric sequences.

Stabilization of broken chromosome ends by telomere sequence addition has been observed in many organisms, from yeast to man (Vermeesch and Price 1994; Hug and Lingner 2006; Pennaneach et al. 2006), but until now, in only two insect species (*D. melanogaster* and the coccid, *Planococcus lilacinus*) (Biessmann et al. 1990; Mohan et al. 2011). Considering that *Drosophila* exhibits a non-canonical telomere-telomerase system, aphids and coccids are therefore the first insect models to be shown experimentally to exhibit de novo telomere synthesis.

Loxdale and Lushai suggested several times that, in aphids, sexual reproduction was related not only to genetic recombination and variability, but perhaps also to the resetting of telomere length (Loxdale and Lushai 2003; Lushai and Loxdale 2007). In the absence of sexual reproduction, asexual aphid generations will thus shorten telomere length consecutively, resulting in a short persistence of obligate parthenogenetic strains/generations (Lushai and Loxdale 2007).

Whilst this suggestion is intriguing, our experimental data do not support this hypothesis, since, as we have demonstrated, telomerase expression is not highly regulated as it has been shown to be in vertebrates (particularly in mammals), but telomerase mRNAs have been observed in different tissues in both adults and embryos. Telomere length can therefore be seemingly regulated also during parthenogenetic generations. Moreover, FISH experiments performed in different aphid species showed bright telomeres also, for instance, in *R. padi* specimens reared in our laboratory for more than 40 years that reproduce themselves by clonal reproduction only. If the Lushai and Loxdale hypothesis is correct, strains reproduced in our laboratory from more than 40 years should have very short telomeres, but FISH experiments do not seem to support this assertion, and RT-PCR revealed telomerase activity in clonal generations, although no quantitative assays have yet been performed.

In aphids, therefore, telomere length seems to be regulated by telomerase not only during sexual generations, but also in parthenogenetic females, leaving open the question of the presence and purpose of sexual generations in aphids. Considering that aphids can stabilize chromosomal fragmentation by combining the holocentric nature of their chromosomes with the de novo synthesis of telomeres, we hypothesize that maintenance of meiosis is important not only for the production of variable offspring, but also for the stability of both karyotype and chromosome structure. Indeed, chromosomes not involved in pairing and crossing over can quickly differentiate so that, in the absence of sexual reproduction, chromosomal rearrangements could well become fixed in the aphid karyotype, facilitating one-way, potentially detrimental karyotypic changes and genomic instability. Interestingly, similar trends have been observed in aphids of the genus *Trama*, in which males have rarely been found (Blackman et al. 2000; Blackman and Eastop 2000; Blackman et al. 2001). Indeed Blackman and colleagues reported decay in the structure of *Trama* chromosomes resulting, among other things, in the loss of rDNA arrays (Blackman et al. 2000).

Acknowledgments We are greatly indebted to Emanuele Mazzoni, Manuel Plantagenest and Sonia Ganassi for sending us their aphid strains. This work was supported by the grant "F. A.R." from the University of Modena and Reggio Emilia (M. M.), Italy, and by the grant "Experimental approach to the study of evolution" from the Department of Animal Biology of the University of Modena and Reggio Emilia (M.M.).

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