Phylogenetic distribution of TTAGG telomeric repeats in insects

Radmila Frydrychová, Petr Grossmann, Pavel Trubač, Magda Vítková, and František Marec

Abstract: We examined the presence of TTAGG telomeric repeats in 22 species from 20 insect orders with no or inconclusive information on the telomere composition by single-primer polymerase chain reaction with $(TTAGG)_6$ primers, Southern hybridization of genomic DNAs, and fluorescence in situ hybridization of chromosomes with $(TTAGG)_n$ probes. The $(TTAGG)_n$ sequence was present in 15 species and absent in 7 species. In a compilation of new and published data, we combined the distribution of $(TTAGG)_n$ telomere motif with the insect phylogenetic tree. The pattern of phylogenetic distribution of the TTAGG repeats clearly supported a hypothesis that the sequence was an ancestral motif of insect telomeres but was lost repeatedly during insect evolution. The motif was conserved in the "primitive" apterous insect orders, the Archaeognatha and Zygentoma, in the "lower" Neoptera (Plecoptera, Phasmida, Orthoptera, Blattaria, Mantodea, and Isoptera) with the exception of Dermaptera, and in Paraneoptera (Psocoptera, Thysanoptera, Auchenorrhyncha, and Sternorrhyncha) with the exception of Heteroptera. Surprisingly, the $(TTAGG)_n$ motif was not found in the "primitive" pterygotes, the Palaeoptera (Ephemeroptera and Odonata). The Endopterygota were heterogeneous for the occurrence of TTAGG repeats. The motif was conserved in Hymenoptera, Lepidoptera, and Trichoptera but was lost in one clade formed by Diptera, Siphonaptera, and Mecoptera. It was also lost in Raphidioptera, whereas it was present in Megaloptera. In contrast with previous authors, we did not find the motif in Neuroptera. Finally, both TTAGG-positive and TTAGG-negative species were reported in Coleoptera. The repeated losses of TTAGG in different branches of the insect phylogenetic tree and, in particular, in the most successful lineage of insect evolution, the Endopterygota, suggest a backup mechanism in the genome of insects that enabled them frequent evolutionary changes in telomere composition.

Key words: chromosomes, fluorescence in situ hybridization, FISH, insects, phylogeny, single primer PCR, Southern hybridization, telomere, telomeric repeats.

Résumé : Les auteurs ont sondé l'ADN de 22 espèces d'insectes appartenant à 20 ordres pour la présence de répétitions TTAGG à l'aide d'une amplification PCR faisant appel à une seule amorce $(TTAGG)_6$, d'hybridations Southern et d'hybridations in situ en fluorescence (FISH) au moyen de sondes $(TTAGG)_n$. Avant ce travail, rien ou rien de concluant n'était connu au sujet de la composition des télomères chez ces espèces. La séquence $(TTAGG)_n$ était présente chez 15 espèces et absente chez les sept autres. En compilant de nouvelles données ainsi que d'autres déjà publiées, les auteurs comparent la distribution des motifs télomériques $(TTAGG)_n$ et l'arbre phylogénétique des insectes. La distribution des insectes à motif télomérique TTAGG au sein de l'arbre phylogénétique supporte nettement l'hypothèse selon laquelle cette séquence constitue le motif ancestral des télomères chez les insectes, motif qui aurait été perdu à plusieurs occasions au cours de l'évolution des insectes. Le motif chez les ordres 'primitifs' d'insectes aptères, les Archaeognatha et les Zygentoma, chez les Neoptera 'inférieurs' (Plecoptera, Phasmida, Orthoptera, Blattaria, Mantodea et Isoptera), à l'exception des Dermaptera, et chez les Paraneoptera (Psocoptera, Thysanoptera, Auchenorrhyncha et Sternorrhyncha), à l'exception des Heteroptera. Étonnement, le motif $(TTAGG)_n$ n'a pas été observé chez les ptérygotes 'primitifs', les Paleoptera (Ephemeroptera et Odonata). Les Endopterygota étaient hétérogènes en ce qui a trait à la présence de répétitions TTAGG. Le motif était conservé chez les Hymenoptera, les Lepidoptera et les Trichoptera, mais il avait été perdu au sein d'un clade formé par les Diptera + Siphonaptera + Mecoptera. Il avait aussi été perdu chez les Raphidioptera tandis qu'il était toujours présent chez les Megaloptera. Contrairement à ce qui a été rapporté par d'autres auteurs, ce motif n'a pas été observé chez les Neuroptera. Finalement, chez les Coleoptera, tant des espèces avec que sans le motif TTAGG ont été rapportées. La perte répétée du motif TTAGG dans différentes branches de l'arbre phylogénétique des insectes, en particulier chez les Endopterygota (la branche ayant connu le plus de succès au

Received 11 June 2003. Accepted 26 August 2003. Published on the NRC Research Press Web site at http://genome.nrc.ca on 30 January 2004.

Corresponding Editor: W. Traut.

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doi: 10.1139/G03-100

cours de l'évolution des insectes), suggère un mécanisme de rechange dans le génome des insectes ce qui rend possible de fréquents changements dans la composition des télomères au cours de l'évolution.

Mots clés : chromosomes, hybridation in situ en fluorescence, FISH, insectes, phylogénie, PCR à amorce unique, hybridation Southern, télomère, répétitions télomériques.

[Traduit par la Rédaction]

Introduction

Telomeres, the protective protein–DNA end caps of linear eukaryotic chromosomes, play a vital role in securing chromosome stability. They prevent the chromosomes from end-to-end fusions, protect their ends from degradation, and compensate for nucleotide losses resulting from incomplete replication at the end of DNA molecules (Blackburn 1991). Other proposed telomere functions are related to cell cycle arrest, gene repression, association of chromosomes to nuclear periphery, and meiotic chromosome pairing (reviewed in Zakian 1995). In mammals, telomeres are also involved in cell proliferation, aging, and carcinogenesis (reviewed in Krupp et al. 2000).

Telomeric DNA of most eukaryotes is composed of lengthy stretches of a repeated simple sequence. These stretches are maintained at the DNA ends by a special reverse transcriptase, telomerase (reviewed in Greider 1996). Different organisms may differ in the composition of telomeric DNA. However, some telomeric repeats are characteristic for whole taxonomic groups (reviewed in Krupp et al. 2000). This also concerns insects. In this most numerous animal group, a pentanucleotide sequence repeat, $(TTAGG)_n$ (5′→3′ at the 3′ end), has been described (Okazaki et al. 1993). Fluorescence in situ hybridization (FISH) of insect chromosomes with $(TTAGG)_n$ probes proved that the $(TTAGG)_n$ motif is conserved in telomeres of three large insect orders, Hymenoptera, Lepidoptera, and Orthoptera (Meyne et al. 1995; Sahara et al. 1999; Kojima et al. 2002; Lorite et al. 2002; Mandrioli 2002), and in aphids (Hemiptera: Sternorrhyncha) (Spence et al. 1998; Bizzaro et al. 2000). However, beetles (Coleoptera) were heterogeneous for the presence of TTAGG telomeric repeats (Sahara et al. 1999; Frydrychová and Marec 2002), and the motif was absent in two insect orders, Diptera and Heteroptera (see table 1 in Sahara et al. 1999). In addition, the Southern hybridization data of Okazaki et al. (1993) indicated the absence of TTAGG repeats in several other insect taxa. Nevertheless, because the $(TTAGG)_n$ sequence was found in noninsect arthropods, Crustacea, Sahara et al. (1999) suggested that it is an ancestral motif of insect telomeres but was lost independently in some insect taxa, being replaced with another motif.

Two alternative mechanisms of the telomere maintenance have been demonstrated in the Diptera (reviewed in Biessmann and Mason 1997; Biessmann et al. 2002). In *Drosophila melanogaster*, telomeres are composed of *Het-A* and *TART* transposable elements (Biessmann et al. 1990; Levis et al. 1993) that are added to chromosome termini by transposition (Mason and Biessmann 1995; Pardue and DeBaryshe 1999). The other mechanism of telomere elongation is based on unequal recombination between long tandem repeats, found at chromosome termini in the midge genus *Chironomus* (Nielsen and Edström 1993; Zhang et al. 1994) and a mosquito (*Anopheles gambiae*) (Roth et al. 1997). A similar telomeric satellite sequence was reported for the *Drosophila virilis* species group (Biessmann et al. 2000). However, a recent study of Casacuberta and Pardue (2003) revealed homologues of *D. melanogaster TART* retrotransposons in telomeres of distantly related *D. virilis* and *Drosophila americana*, suggesting that the telomeric satellite sequence is, in fact, a telomere-associated sequence. The authors proposed the transposition-based model as an ancestral mechanism of telomere elongation in the entire genus *Drosophila*.

Alternative mechanisms of telomere maintenance can also be expected in other insect taxa, namely the Coleoptera. Recently documented repeated losses of the $(TTAGG)_n$ motif in different lineages of the Coleoptera phylogenetic tree suggest a predisposition or a backup mechanism of telomere maintenance in the genome that enabled beetles to make frequent evolutionary changes in telomere composition (Frydrychová and Marec 2002).

Although the aforementioned studies were restricted to only several insect orders, they allow us to predict dramatic changes in telomere composition during the evolution of insects. Therefore, the present study aims to extend the survey of the $(TTAGG)_n$ motif to all main branches of the insect phylogenetic tree and thus better introspect a process of phylogeny of the insect telomere motif. We examined the presence–absence of TTAGG telomeric repeats in 22 species of 20 insect orders with no or inconclusive information by using three different approaches, single-primer PCR amplification of genomic DNAs with $(TTAGG)_6$ primers, Southern hybridization of genomic DNAs with $(TTAGG)_n$ probes, and FISH of chromosomes with $(TTAGG)_n$ probes. The results obtained, together with the previously reported data on the distribution of the $(TTAGG)_n$ telomere motif, were then combined with the insect phylogenetic tree.

Materials and methods

Specimens

We studied the occurrence of TTAGG telomeric repeats in 22 species of 20 orders representing all main lineages of the insect phylogenetic tree (see Kristensen 1991). These included six species of holometabolous orders (the group Endopterygota), four species of hemipteroid insects (Paraneoptera), seven species of the "lower" Neoptera, two species of the "primitive" lineages of Pterygota, the Odonata and Ephemeroptera, altogether referred to as Palaeoptera, and three "primarily apterous insects" of the orders Zygentoma and Archaeognatha. The species examined along with their origin are given in Table 1.

Table 1. List of insect species used in the present study.

Higher taxa	Species studied	Source or locality
Neoptera: Endopterygota (Holometabola)		
Trichoptera	*Limnephilus decipiens* (caddisfly)	Collected by authors in "Domín" Pond near České Budějovice
Siphonaptera	*Ctenocephalides canis* (dog flea)	Collected by authors in the dog refuge, České Budějovice
Mecoptera	*Panorpa communis* (scorpion fly)	Collected by A. Bezděk near Nové Hrady, South Bohemia
Neuroptera	*Chrysoperla carnea* (green lacewing)	From cultures at Inst. Entomol., provided by M. Červenská and Z. Růžička
Raphidioptera	*Magnoraphidia major* (snakefly)	Collected by authors in "Vltava" River near České Budějovice
Megaloptera	*Sialis lutaria* (alderfly)	Collected by T. Soldán in "Čertovo" Lake, "Šumava" Mts., South Bohemia
Neoptera: Paraneoptera (hemipteroid assemblage)		
Hemiptera: Auchenorrhyncha	*Calligypona pellucida* (leafhopper)	Collected by authors in environs of České Budějovice
Hemiptera: Sternorrhyncha	*Trialeurodes vaporarium* (greenhouse whitefly)	Collected by authors in greenhouses at Inst. Mol. Biol. Plants ASCR, České Budějovice
Thysanoptera	*Parthenothrips dracenae* (thrip)	Collected by authors on an indoor plant (*Monstera* sp.)
Psocoptera	*Stenopsocus lachlani* (bark lice)	Collected by J. Havelka in "Šumava" Mts., South Bohemia
"Lower" Neoptera		
Dermaptera	*Forficula auricularia* (earwig)	Collected by authors in "Liblín" Village, West Bohemia
Dictyoptera: Isoptera	*Reticulitermes santonensis* (French subterranean termite)	From cultures at Inst. Org. Chem. Biochem. ASCR, Prague, provided by J. Šobotník
Dictyoptera: Mantodea	*Creobroter pictipennis* (mantis)	From authors' cultures
	Hierodula sp. (mantid)	From authors' cultures
Dictyoptera: Blattaria	*Periplaneta americana* (American cockroach)	From cultures at Inst. Entomol., provided by H. Sehadová
Phasmida	*Extratosoma tiaratum* (walking stick)	From cultures at Inst. Entomol., provided by B. Konopová
Plecoptera	*Perla burmeisteriana* (stonefly)	Collected by authors in "Vltava" River near České Budějovice
Palaeoptera		
Odonata	*Ischnura elegans* (damselfly)	Collected by authors in "Stromovka" parkland in České Budějovice
Ephemeroptera	*Cloeon dipterum* (mayfly)	Collected by authors in "Vrbenské" ponds near České Budějovice
"Primarily apterous insects"		
Zygentoma	*Thermobia domestica* (firebrat)	From cultures at Inst. Entomol., provided by D. Doležel
	Lepisma saccharina (silverfish)	Home-collected by authors
Archaeognatha	*Lepismachilis* sp. (bristletail)	Collected by F. Šťáhlavský in environs of Komořany near Prague

$(TTAGG)_n$ telomere probe

Reverse and forward 20-mer primers, devised by Sahara et al. (1999) for TTAGG telomere repeats and custom made by Generi Biotech (Hradec Králové, Czech Republic), were used for producing unlabeled $(TTAGG)_n$ probes by the non-template PCR method of Ijdo et al. (1991). PCRs were carried out in 25-μL reaction volumes containing 0.5 μM of each primer, 200 μM of each dNTP, 1.5 mM $MgCl_2$, and 2 units of *Taq* DNA polymerase in 1× PCR buffer (GibcoBRL, Life Technologies Inc., Karlsruhe, Germany). An initial period of 90 s at 94 °C was followed by 30 cycles each of 45 s at 94 °C, 30 s at 52 °C, and 60 s at 72 °C and concluded with a final extension step of 10 min at 72 °C. The PCR-generated probes were purified by precipitation.

For nonradioactive Southern hybridization, the probes were labeled by random-primed labeling with digoxigenin 11-dUTP using the DIG DNA Labeling and Detection Kit (Roche Diagnostics GmbH, Roche Molecular Biochemicals,

Mannheim, Germany). For FISH, the probes were labeled by nick translation with biotin-14-dATP using the BioNick Labeling System (GibcoBRL, Life Technologies Inc.).

DNA isolation

Total genomic DNA of each species was extracted from larvae or adults. Three different methods were used depending on the body size. Standard phenol – chloroform – isoamyl alcohol extraction was used in relatively large species with a minimum amount of 10 mg of tissues. DNAs of species, whose tiny body size prevents the extraction of a sufficient amount of DNA, were extracted either using an AquaPure Genomic DNA Kit (Bio-Rad Laboratories, Hercules, Calif.) or by phenol – chloroform – isoamyl alcohol extraction combined with chitinase treatment (Sakagami et al. 1997). In the latter DNA extraction method, ready-frozen samples (using liquid nitrogen) were homogenized with a rounded tip glass rod in a 1.5-mL tube. The homogenate was diluted in 500 µL of extraction buffer (150 mM Tris–HCl, pH 6.8), and chitinase solution (10 mg/mL in 150 mM Tris–HCl, pH 6.8) (Wako Pure Chemicals Industries Ltd., Osaka, Japan) was added to a final concentration of 2 mg/mL. Then the sample was incubated for 1 h at 37 °C. Afterwards, a 1% solution of sodium dodecyl sulfate (Sigma-Aldrich, St. Louis, Mo.) and proteinase K (100 µg/mL) (Sigma-Aldrich) was added and the sample was incubated for 2 h at 50 °C. The final incubation with RNase A (1 µg/mL) (Sigma-Aldrich) for 1 h at 37 °C was followed by standard phenol – chloroform – isoamyl alcohol extraction.

Single-primer PCR

We devised a 30-mer primer, 5′-TTAGGTTAGGTTAGG-TTAGGTTAGGTTAGG-3′ (custom made by Generi Biotech, with a phosphorylated 5′-end). The PCR procedure was performed essentially as described in Weiss and Scherthan (2002) with slight modifications. A reaction mix (25 µL) contained 100 ng of template DNA, 0.1 µM of the primer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, and 0.125 unit of *Taq* DNA polymerase in 1× PCR buffer (Invitrogen, Life Technologies, San Diego, Calif.). An initial period of 90 s at 94 °C was followed by 35 cycles each of 60 s at 94 °C, 40 s at 63 °C, 150 s at 72 °C, and one final step of 10 min at 72 °C. The amplified products were separated on 1% agarose gel.

Southern hybridization

For Southern experiments, 1.5 µg of genomic DNA was digested with a *Hin*fI–*Rsa*I enzyme mixture (1:1) and separated on 0.8% agarose gel in TAE buffer (0.04 M Tris–acetate, 0.001 M EDTA) using standard horizontal electrophoresis. Then, the gel was blotted onto a positively charged nylon membrane (Hybond-N^+; Amersham Int. plc., Buckinghamshire, U.K.). Hybridization of DNAs with DIG-labeled telomere probe and subsequent chemiluminescent detection were performed using the Telo TAGGG Telomere Length Assay (Roche Diagnostics GmbH, Roche Molecular Biochemicals, Mannheim, Germany) as described in Frydrychová and Marec (2002).

Chromosome preparations and FISH

Spread chromosome preparations were made from gonads (preferably from testes) of grownup larvae or adults as described in Frydrychová and Marec (2002). The preparations were dehydrated in an ethanol series (70%, 80%, and 100%, 30 s each) and stored at –20 °C until further use.

FISH with biotinylated $(TTAGG)_n$ probe was performed following the protocol of Sahara et al. (1999). Hybridization signals were detected with Cy3-conjugated streptavidin (Jackson ImmunoRes. Labs. Inc., West Grove, Pa.) and one round of amplification with biotinylated antistreptavidin (Vector Labs. Inc., Burlingame, Calif.) and Cy3-conjugated streptavidin. The preparations were counterstained with 0.5 µg 4′,6-diamidino-2-phenylindole (DAPI)/mL (Sigma-Aldrich) and mounted in antifade based on DABCO (Sigma-Aldrich).

The preparations were observed in a Zeiss Axioplan 2 microscope. Black-and-white images of chromosomes were recorded with a CCD camera (F-View; Soft Imaging System GmbH, Münster, Germany) separately for each fluorescent dye. Images were pseudo-colored (light blue for DAPI and red for Cy3) and superimposed with Adobe Photoshop version 5.0.

Results

Single-primer PCR analysis of genomic DNAs

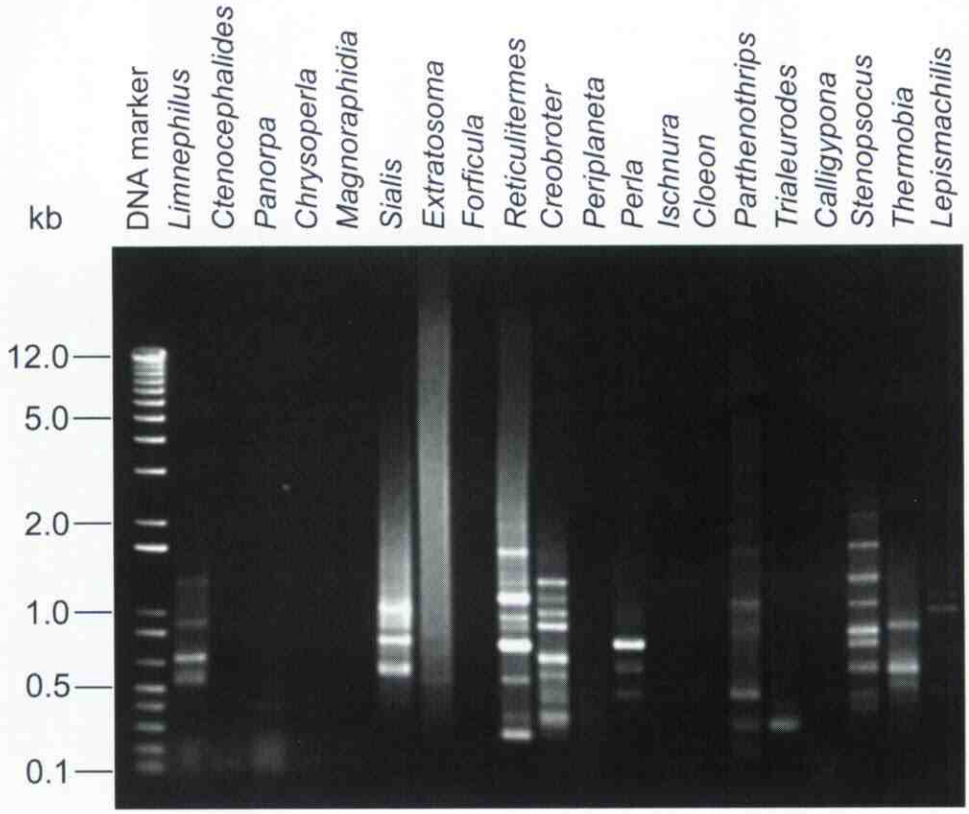
Genomic DNAs of 20 species were used as templates for single-primer PCR with $(TTAGG)_6$ primers (Fig. 1). In 15 species, PCR amplification yielded products of different sizes and different patterns on the electrophoretic gel. Most products consisted of several bands in the range of 200 bp to 2 kb and a smear. The pattern of bands most probably represented double-stranded products generated by inverted TTAGG repeats that could occur scattered in the genome, whereas the smear most likely represented single-stranded products generated by direct repeats because the primers could bind at any position across the long tandem array of telomeric repeats in the template DNA. Such a smear was absent in the amplification product of the damselfly *Ischnura elegans*. In this species, PCR amplified a single band of a size slightly above 1 kb. We failed to detect any amplification products in five species, the dog flea *Ctenocephalides canis*, the green lacewing *Chrysoperla carnea*, the snakefly *Magnoraphidia major*, the earwig *Forficula auricularia*, and the mayfly *Cloeon dipterum*.

Southern hybridization

The $(TTAGG)_n$ probe was hybridized to *Hin*fI–*Rsa*I-digested genomic DNAs of 21 species (Fig. 2). DNAs of 14 species cross-reacted with the probe, while no hybridization signals were observed in seven species examined. The experiments were repeated two to four times for each particular species, always with the same results. All TTAGG-positive species were those in which amplification products were obtained by single-primer PCR, whereas TTAGG-negative species included one species (*Panorpa*) that showed a faint but clear PCR product and one species that showed a single band but no smear after PCR (*Ischnura*), in addition to five species that yielded no PCR product.

Three "primarily apterous insects", the bristletail *Lepismachilis* sp. (Archaeognatha), the firebrat *Thermobia domestica*, and the silverfish *Lepisma saccharina* (Zygentoma), displayed a diffuse smear of hybridization sig-

Fig. 1. Single-primer PCR of genomic DNAs of 20 insect species with the $(TTAGG)_6$ primer. Full species names are given in Table 1.

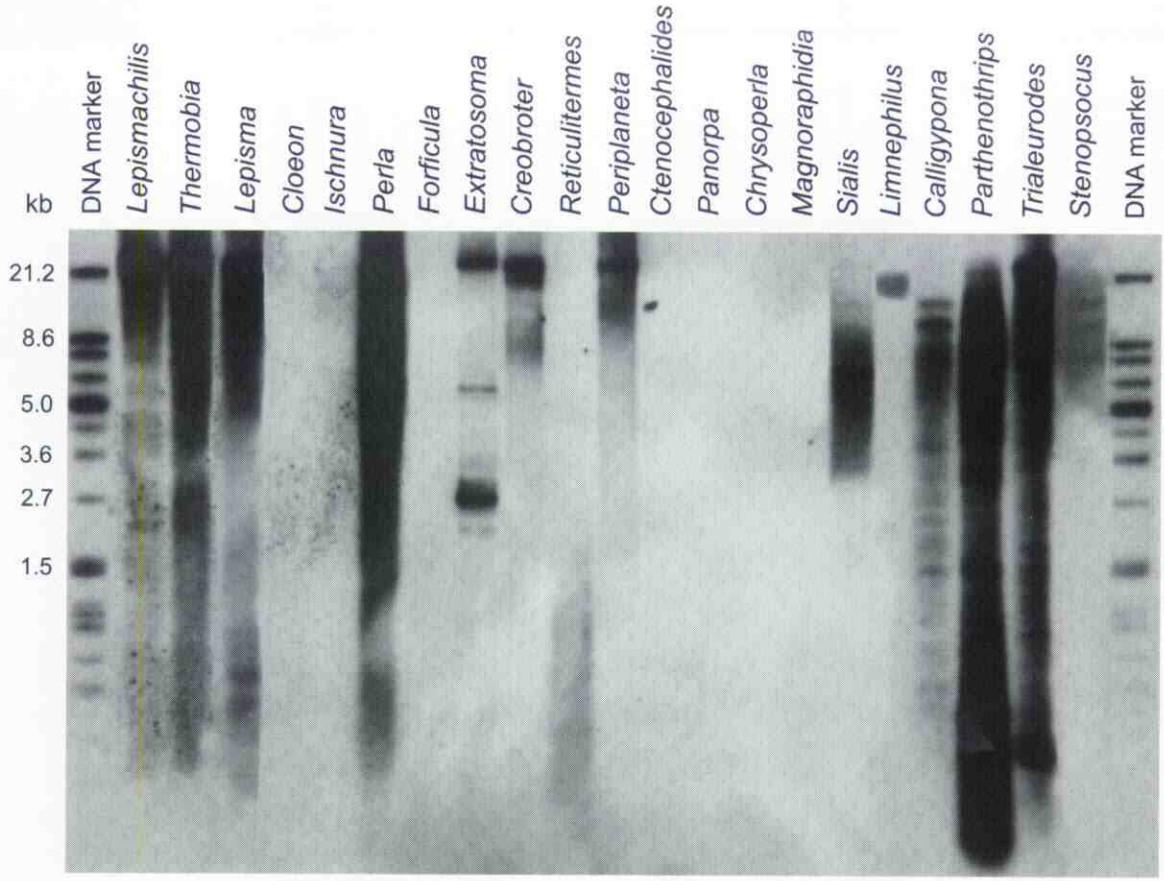


nals in the high molecular mass range (5–21 kb) with a superimposed strong band, indicating that a large fraction of restriction fragments was longer than 21 kb. In addition, *Lepisma* showed a few bands in the range below 1 kb. On the contrary, hybridization signals were absent in both representatives of "primitive" Pterygota, the mayfly *C. dipterum* (Ephemeroptera) and the damselfly *I. elegans* (Odonata).

Interestingly, representatives of three main lineages of the Neoptera, i.e., "lower" Neoptera, Endopterygota, and Paraneoptera, showed a high heterogeneity in both the $(TTAGG)_n$ presence and the pattern of hybridization signals. In "lower" Neoptera, hybridization signals were absent only in the earwig *F. auricularia* (Dermaptera). Hybridization signals in the stonefly *Perla burmeisteriana* (Plecoptera) resembled those in the "apterous insects", forming a continuous smear in the range of 1.5–21 kb. Three other species, the walking stick *Extratosoma tiaratum* (Phasmida), the mantis *Creobroter pictipennis* (Mantodea), and the American cockroach *Periplaneta americana* (Blattaria), displayed one or two distinct bands of about 21 kb. Besides, *Extratosoma* showed a few additional bands, particularly one faint band of about 6 kb and a strong band of about 2.7 kb. Finally, the termite *Reticulitermes santonensis* (Isoptera), although clearly TTAGG positive, exhibited only a weak smear of signals in the low molecular mass range. However, this hybridization pattern obviously arose from degraded DNA, as we failed repeatedly to extract the termite genomic DNA at a good quality (degradation was seen on a control electrophoretic gel before digestion).

In Endopterygota, the $(TTAGG)_n$ motif was present only in two species, the caddisfly *Limnephilus decipiens* (Trichoptera) and the alderfly *Sialis lutaria* (Megaloptera), whereas the other tested species, the dog flea *C. canis* (Siphonaptera), the scorpion fly *P. communis* (Mecoptera), the green lacewing *C. carnea* (Neuroptera), and the snakefly *M. major* (Raphidioptera), were TTAGG negative. In *Limnephilus*, a thick band of about 20 kb was observed. *Sialis* displayed a smear in the range of 3–10 kb with a faint band above this range. In contrast with Endopterygota, DNAs of all hemipteroid species (Paraneoptera) hybridized with the telomeric probe, but the hybridization pattern was remarkably different from that observed in other TTAGG-positive insects. In each species, hybridization signals formed a series of discernible bands at a different range of fragment lengths. The leafhopper *Calligypona pellucida* (Auchenorrhyncha) displayed hybridization signals in the range of less than 1 to about 15 kb. An extremely wide range of restriction fragments from less than 0.5 to 21 kb was found in the thrip *Parthenothrips dracenae* (Thysanoptera) and the greenhouse whitefly *Trialeurodes vaporarium* (Sternorrhyncha). The latter two species displayed a curious, mirror pattern of hybridization signals with particularly prominent series of superior and inferior bands. Finally, the restriction fragments ranged from 5 to 21 kb in the bark lice *Stenopsocus lachlani* (Psocoptera). A similar pattern of distinct wide-ranged bands was observed in TTAGG-positive beetles (Coleoptera) (Frydrychová and Marec 2002). We suggest that long restriction fragments in the hemipteroid insects could represent true telomeric DNA fragments, whereas shorter restriction fragments could represent proximal telomeric segments separated from distal segments by integrated nontelomeric DNA domains containing corresponding restriction sites. Such domains were found in telomeres and subtelomeric regions of the silkworm *Bombyx*

Fig. 2. Southern hybridization of the $(TTAGG)_n$ probe to *Hin*fI–*Rsa*I-digested genomic DNAs from 21 species of insects. Full species names are given in Table 1.



mori (Okazaki et al. 1995; Anzai et al. 2001) and several other Lepidoptera (Kubo et al. 2001; Mandrioli 2002). Finally, some restriction fragments might originate from nontelomeric sites and represent the so-called interstitial telomeric-like sequences (Azzalin et al. 2001).

FISH

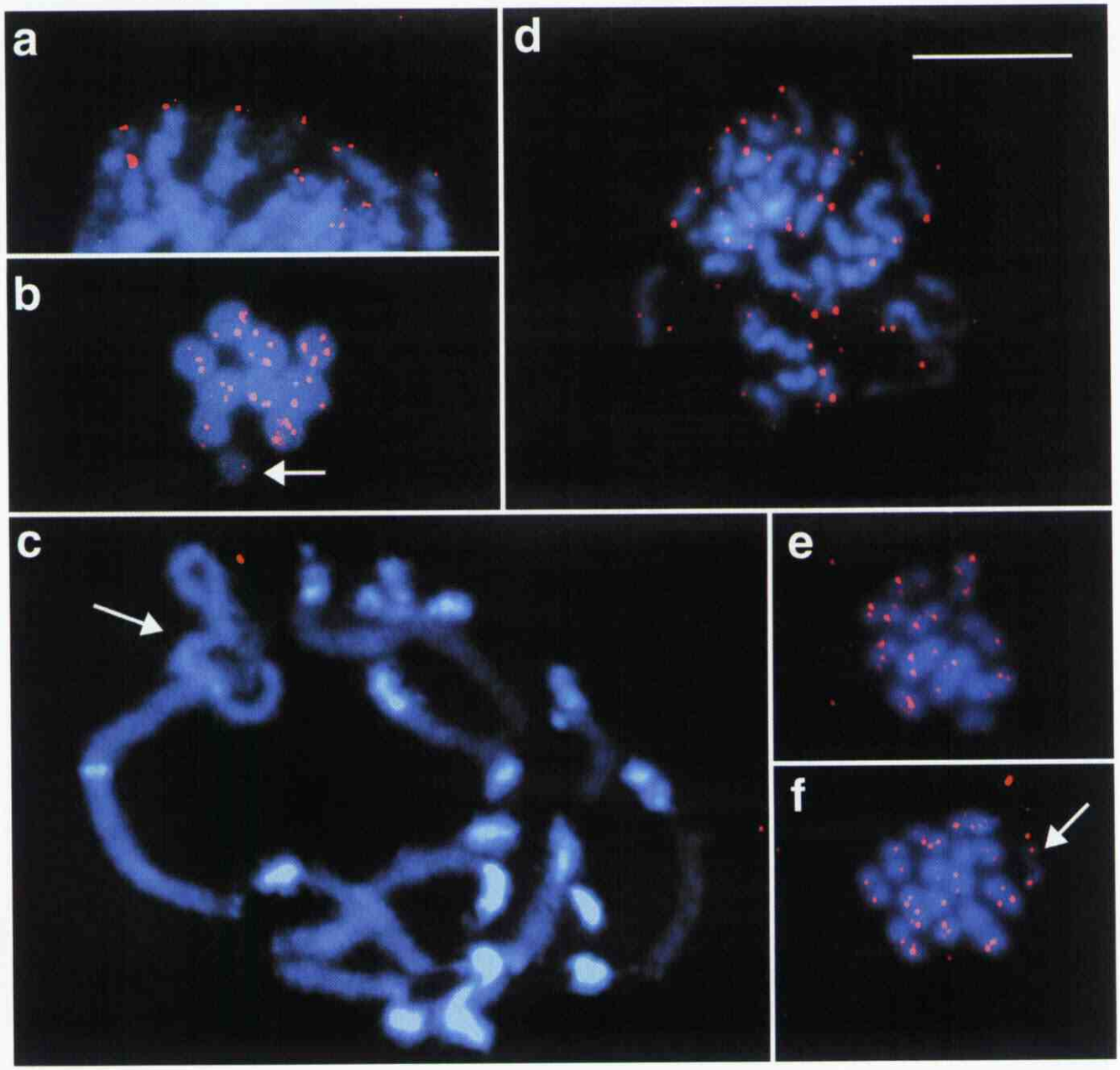
FISH with $(TTAGG)_n$ probe was made on chromosome spreads of 14 insect species. These included all species that were TTAGG positive in the single-primer PCR amplification and (or) Southern hybridization except three species of Paraneoptera, the leafhopper, the parthenogenetic thrip, and the greenhouse whitefly, in which we failed to find optimal stages for chromosome preparations. We also made FISH in species that showed controversial results and in two species that were clearly TTAGG negative, the mayfly and the earwig.

Endopterygota

In the caddisfly *L. decipiens* (Trichoptera), chromosome preparations were done from testes of the last-instar larvae, which contained different stages of meiotic spermatocytes with the predominating pachytene stage. FISH confirmed the positive results of the single-primer PCR amplification and Southern hybridization. In pachytene spermatocytes, twin hybridization signals of the $(TTAGG)_n$ probe were typically located at the ends of all bivalents; the twin signals represented two ends of the synapsed homologous chromosomes (Fig. 3*a*). In metaphase I complements, 10 bivalents were observed; nine autosome bivalents were similar in their size, whereas the sex chromosome bivalent (ZZ) was much smaller than the others (cf. Marec and Novák 1998). The number of bivalents corresponded to the haploid chromosome number of $n = 10$ reported by Klingstedt (1931). The spherical metaphase I bivalent displayed up to four strong hybridization signals each. The signals were regularly observed inside the highly condensed chromatin mass, indicating the position of actual chromosomal ends (Fig. 3*b*). A similar location of hybridization signals was observed in metaphase I bivalents of the silkworm *B. mori* (see Sahara et al. 1999).

The scorpion fly *P. communis* (Mecoptera) was positive in single-primer PCR amplification, but Southern hybridization failed to detect the $(TTAGG)_n$ motif in the genomic DNA. In chromosome preparations from adult testes, no hybridization signals, either at the telomeres or at nontelomeric sites, were observed (Fig. 3*c*). Thus, FISH confirmed the negative data of Southern hybridization. The chromosome number was not assessed because we examined mainly pachytene nuclei. Pachytene bivalents displayed conspicuous blocks of pericentric heterochromatin that were strongly highlighted with DAPI. In addition, a univalent chromosome was identified in favorable nuclei (see arrow in Fig. 3*c*). The univalent was most probably the sex chromosome X, suggesting that

Fig. 3. FISH of the $(TTAGG)_n$ telomere probe (red signals) to chromosome spreads (counterstained with DAPI) of "higher" Neoptera. (*a* and *b*) *Limnephilus decipiens* male. (*a*) A part of the pachytene nucleus with twin telomeric signals at the ends of bivalents. (*b*) Metaphase I complement with hybridization signals inside bivalents; the arrow indicates the sex chromosome pair ZZ. (*c*) *Panorpa communis* male. Pachytene bivalents without hybridization signals; the arrow indicates the presumable sex chromosome univalent X. (*d*) *Sialis lutaria* male. Mitotic prometaphase chromosomes with $(TTAGG)_n$ telomeric signals in most but not all chromosome ends. (*e* and *f*) *Stenopsocus lachlani* male. Metaphase I chromosomes with strong hybridization signals inside bivalents. (*e*) Metaphase I showing 11 bivalents. (*f*) Metaphase I showing 11 bivalents plus one small element (arrow), most probably representing the univalent X chromosome. Bar = 10 μm.

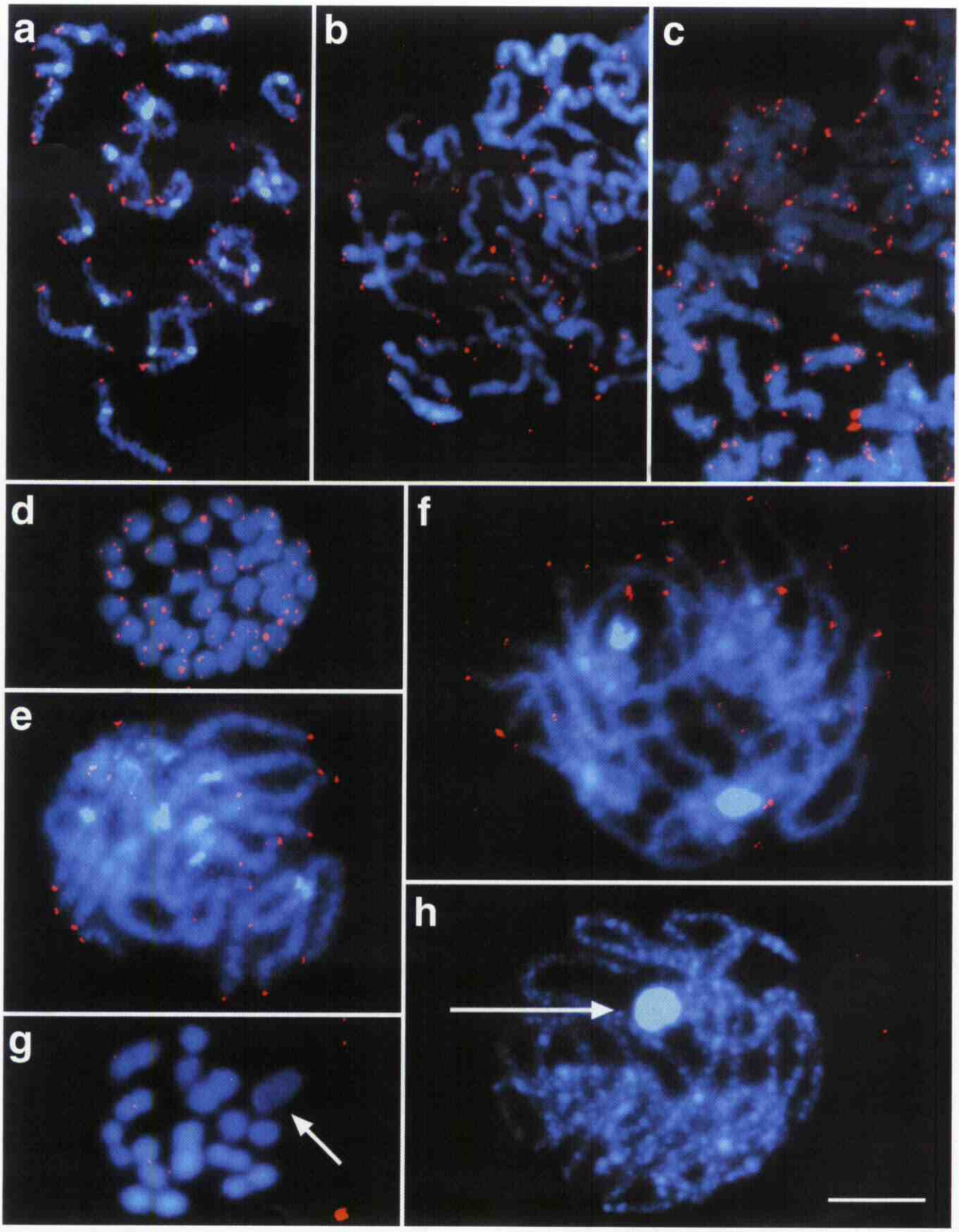


this species has an X0/XX (males/females) sex chromosome system (cf. Traut 1999).

In the alderfly *S. lutaria* (Megaloptera), chromosome preparations were made from testes of mature larvae, which contained mainly mitotic spermatogonia. In mitotic prometaphase chromosomes, clear hybridization signals were seen at the chromosomal ends (Fig. 3*d*) in accordance with the results of single-primer PCR and Southern hybridization. However, not all chromosomal ends were labeled with the telomeric probe, perhaps as a result of a low copy number of telomeric repeats at the end in combination with highly stringent conditions during the FISH procedure used.

Paraneoptera

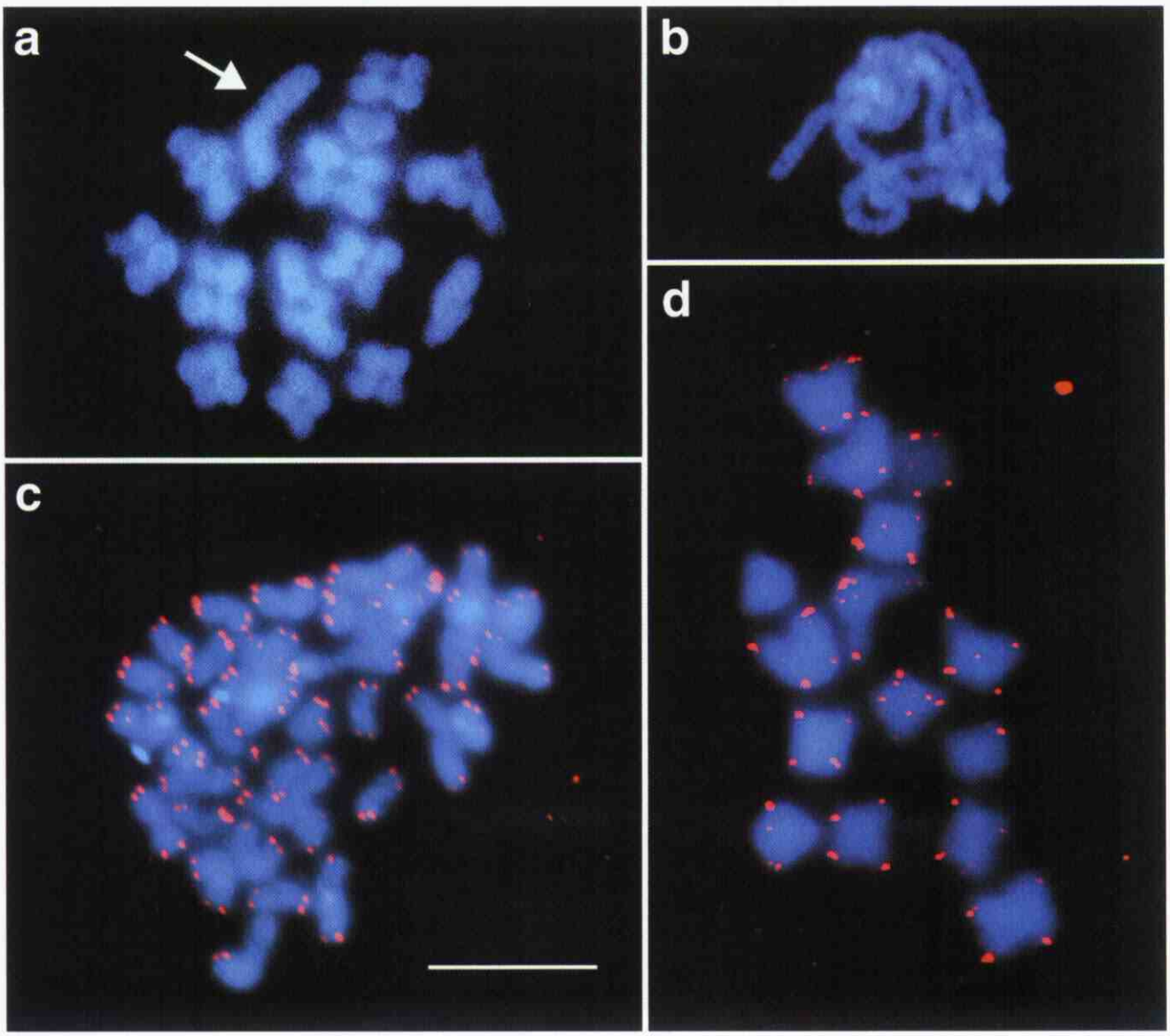
FISH was carried out only in the bark lice *S. lachlani* (Psocoptera). Spread preparations of adult testes displayed



apparent hybridization signals. Twin signals were typically observed at the ends of pachytene bivalents (not shown), whereas metaphase I bivalents showed usually four signals inside the chromatin mass, two in each chromosome homologue (Figs. 3*e* and 3*f*). Most metaphase I complements consisted of 11 bivalents of similar sizes (Fig. 3*e*); however, some complements showed 11 bivalents plus a tiny bivalent-like element (Fig. 3*f*). We suggest that the

Fig. 4. FISH of the $(TTAGG)_n$ telomere probe (red signals) to chromosome spreads (counterstained with DAPI) of "lower" Neoptera. (*a*) *Reticulitermes santonensis* nymfoid female. Pachytene oocyte nucleus with twin hybridization signals at the end of bivalents; note the DAPI-positive blocks of pericentric heterochromatin. (*b*) *Extratosoma tiaratum* female. A part of the mitotic prometaphase complement with strong hybridization signals at the chromosome ends. (*c*) *Perla burmeisteriana* male. A part of the mitotic metaphase complement with strong telomeric signals. (*d* and *e*) *Periplaneta americana* male. (*d*) Mitotic metaphase complement with strong hybridization signals. (*e*) Pachytene nucleus with $(TTAGG)_n$ signals at the end of bivalents. (*f*) *Hierodula* sp. male. Pachytene nucleus with hybridization signals at the ends of bivalents. (*g* and *h*) *Forficula auricularia* male. No hybridization signals are seen. (*g*) Metaphase I complement showing 11 autosome bivalents and the X chromosome univalent (arrow). (*h*) Pachytene nucleus showing the conspicuous heterochromatin body of the presumable X chromosome (arrow). Bar = 10 μm.

Fig. 5. FISH of the $(TTAGG)_n$ telomere probe (red signals) to chromosome spreads (counterstained with DAPI) of "primitive" insects. (*a*) *Ischnura elegans* male. Diplotene bivalents without hybridization signals; note the large univalent of the X chromosome (arrow). (*b*) *Cloeon dipterum* male. Pachytene nucleus without hybridization signals. (*c*) *Thermobia domestica* male. Mitotic metaphase chromosomes with strong telomeric signals. (*d*) *Lepismachilis* sp. male. Diplotene/diakinesis bivalents with $(TTAGG)_n$ signals. Bar = 10 μm.



small element was the X chromosome, forming a pseudobivalent in metaphase I, that was lost in many complements during the spreading procedure. Thus, the diploid chromosome number of *S. lachlani* males is likely $2n = 23$, with an X0 sex chromosome system typical for most Psocoptera (cf. Golub and Nokkala 2001).

"Lower" Neoptera

Extraordinary strong hybridization signals were observed at the ends of pachytene bivalents prepared from ovaries of nymphoid replacement reproductives of the termite *R. santonensis* (Isoptera). Pachytene complements consisted

Table 2. List of insect species tested for the presence of the $(TTAGG)_n$ telomere sequence.

Group	Species	FISH	Southern hybridization	TRAP	Other telomere structures
Endopterygota					
Hymenoptera	*Apis mellifera*	Yes[a]	Yes[a]		
	Myrmecia spp.	Yes[b]			
	Manica yessensis		Yes[c]		
	Tapinoma nigerrimum	Yes[d]	Yes[d]		
	19 species of ants		Yes[d]		
Lepidoptera	*Antheraea yamamai*		Yes[c]		
	Antheraea pernyi		Yes[c]		
	Bombyx mandarina		Yes[c]		
	Bombyx mori	Yes[a,c]	Yes[a,c]	No[e]	
	Ephestia kuehniella	Yes[a]	Yes[a]		
	Galleria mellonella	Yes[a]	Yes[a]		
	Samia cynthia ricini		Yes[c]		
	Mamestra brassicae	Yes[f]	Yes[f]		
	Agrius convolvuli			Yes[e]	
	Papilio xuthus			Yes[e]	
Trichoptera	*Limnephilus decipiens*	Yes[g]	Yes[g]		
	Stenopsyche japonica		Yes[c]		
Siphonaptera	*Ctenocephalides canis*		No[g]		
Mecoptera	*Panorpa communis*	No[g]	No[g]		
Diptera	*Chironomus* spp.		No[h]		satellite sequence[h]
	Drosophila melanogaster	No[a]	No[a,c]	No[e]	retrotransposons[i]
	Drosophila virilis sp.				retrotransposons[j] satellite sequence[k]
	Eristalomya tenax		No[c]		
	Megaselia scalaris	No[a]	No[a]		
	Neoitamus angusticornis		No[c]		
	Sphyximorphoides pleuralis		No[c]		
	Sarcophaga peregrina			No[e]	
	Tabanus trigonus		No[c]		
Coleoptera	*Silpha obscura*	Yes[l]	Yes[l]		
	Geotrupes stercorarius	No[l]	No[l]		
	Agrilus viridis	Yes[l]	Yes[l]		
	Ampedus sanguineus	Yes[l]	Yes[l]		
	Stegobium paniceum	Yes[l]	Yes[l]		
	Thanasimus formicarius	No[l]	No[l]		
	Oryzaephilus surinamensis	Yes[l]	Yes[l]		
	Tenebrio molitor	No[a]	No[a]		
	Leptinotarsa decemlineata	Yes[l]	Yes[l]		
	Sitophilus granarius	No[l]	No[l]		
	Ips typographus	Yes[a]	Yes[a]		
	Orectochilus vilosus	No[l]	No[l]		
	Graphoderus cinereus	Yes[l]	Yes[l]		
	Pterostichus oblongopunctatus	No[l]	No[l]		
	Anomala cuprea		No[c]		
	Arhopalus coreanus		Yes[c]		
	Diacanthous undosus		Yes[c]		
	Gonocephalum sexuale		No[c]		
	Hydrochara affinis		Yes/no[c]		
	Melanotus legatus		Yes[c]		
	Sipalinus gigas		No[c]		
	Spondylis buprestoides		Yes[c]		
Neuroptera	*Chrysoperla carnea*		No[g]		
	Protidricerus japonicus		Yes[c]		
Raphidioptera	*Magnoraphidia major*		No[g]		
Megaloptera	*Sialis lutaria*	Yes[g]	Yes[g]		

Table 2 (*concluded*).

Group	Species	FISH	Southern hybridization	TRAP	Other telomere structures
Paraneoptera					
Thysanoptera	*Parthenothrips dracenae*		Yes[g]		
Sternorrhyncha	*Trialeurodes vaporarium*		Yes[g]		
	Acyrthosiphon pisum		Yes[m]		
	Myzus persicae		Yes[n]		
	Terpnosia nigricosta		Yes/no[c]		
Auchenorrhyncha	*Calligypona pellucida*		Yes[g]		
Psocoptera	*Stenopsocus lachlani*	Yes[g]	Yes[g]		
Heteroptera	*Halyomorpha mista*		No[c]		
	Pyrrhocoris apterus	No[a]	No[a]		
"Lower" Neoptera					
Dermaptera	*Forficula auricularia*	No[g]	No[g]		
	Forficula scudderi		No[c]		
Blattaria	*Periplaneta americana*	Yes[g]	Yes[g]	Yes[e]	
	Periplaneta fuliginosa		Yes[c]		
	Blattella germanica	Yes[o]			
	Blaberus craniifer	Yes[o]			
Mantodea	*Creobroter pictipennis*		Yes[g]		
	Hierodula sp.	Yes[g]			
Isoptera	*Reticulitermes santonensis*	Yes[g]	Yes[g]		
	Hodotermopsis japonicus		Yes[c]		
Orthoptera	*Diestrammena japonica*		Yes[c]		
	Locusta migratoria	Yes[a]	Yes[a,c]		
	Teleogryllus taiwanemma	Yes[p]	Yes[p]	Yes[e]	
Phasmida	*Extratosoma tieratum*	Yes[g]	Yes[g]		
Plecoptera	*Perla burmeisteriana*	Yes[g]	Yes[g]		
Palaeoptera					
Odonata	*Ischnura elegans*	No[g]	No[g]		
Ephemeroptera	*Cloeon dipterum*	No[g]	No[g]		
"Apterous insects"					
Zygentoma	*Thermobia domestica*	Yes[g]	Yes[g]		
	Lepisma saccharina		Yes[g]		
Archeognatha	*Lepismachilis* sp.	Yes[g]	Yes[g]		

Note: *Protidricerus japonicus* was classified erroneously to Mecoptera by Okazaki et al. (1993). TRAP, telomere repeat amplification protocol assay.
[a]Sahara et al. (1999).
[b]Meyne et al. (1995).
[c]Okazaki et al. (1993).
[d]Lorite et al. (2002).
[e]Sasaki and Fujiwara (2000).
[f]Mandrioli (2002).
[g]This study.
[h]López et al. (1996), Nielsen et Edström (1993), and Zhang et al. (1994).
[i]Biessmann et al. (1990).
[j]Casacuberta and Pardue (2003).
[k]Biessmann et al. (2000).
[l]Frydrychová and Marec (2002).
[m]Bizzaro et al. (2000).
[n]Spence et al. (1998).
[o]Marziliano (1999).
[p]Kojima et al. (2002).

of 21 bivalents. Each bivalent exhibited a small block of pericentric heterochromatin, indicating the position of the centromere (Fig. 4*a*). The observed haploid chromosome number of $n = 21$ corresponds to those reported for other termites (Martins 1999).

In the walking stick *E. tiaratum* (Phasmida), FISH preparations of ovaries from subadult specimens showed nuclei with a large number of relatively long chromosomes with strong twin or single telomeric signals at most chromosome ends (Fig. 4*b*). Taking into account the general appearance of the chromosomes, their large number (around 40), and a large genome size of Phasmida in comparison with other insects (Gregory 2002), we suppose that these were prometaphase chromosomes of mitotic oogonia and the twin signals represented the telomeres of two sister chromatids.

Preparations of larval testes from the stonefly *P. burmeisteriana* (Plecoptera) showed mitotic spermatogonia with large numbers of chromosomes. Very strong hybridization

signals, colocalized with the ends of metaphase chromosomes, proved unambiguously the presence of the TTAGG sequence in the stonefly telomeres (Fig. 4*c*).

Preparations of testes from adult males of the American cockroach *P. americana* (Blattaria) contained various stages of spermatogenesis, from mitotic spermatogonia to mature sperm, each showing clear hybridization signals. In metaphase plates of mitotic spermatogonia ($2n = 33$), two to four hybridization signals were observed (Fig. 4*d*). The odd chromosome number indicated an X0 system of the male sex determination (cf. Traut 1999). Pachytene nuclei displayed typically twin signals at the ends of long bivalents (Fig. 4*e*).

In the mantis *Hierodula* sp. (Mantodea), we used chromosome preparations made from testes of 5-day-old adult males. They contained numbers of pachytene spermatocytes with bivalents forming clumps that were difficult to spread. Nevertheless, the ends of bivalents at the margins of the pachytene nuclei were decorated with red labels (Fig. 4*f*), clearly confirming that the TTAGG sequence is a component of the mantis telomeres.

FISH confirmed the absence of the $(TTAGG)_n$ motif in the earwig *F. auricularia* (Dermaptera). No hybridization signals were found in preparations of meiotic chromosomes from adult testes. In each pachytene spermatocyte, a large heterochromatin body was observed in the clump of bivalents (Fig. 4*h*). This body most probably represented the univalent X chromosome, indicating an X0 sex chromosome system (cf. Traut 1999). Accordingly, metaphase I complements showed 12 elements, 11 autosome bivalents and the large X chromosome (Fig. 4*g*).

Palaeoptera

In chromosome preparations from adult testes of the damselfly *I. elegans* (Odonata), no hybridization signals, either at the telomeres or at nontelomeric sites, were observed (Fig. 5*a*). Thus, FISH did not explain the controversial result of PCR amplification but approved the negative data of Southern hybridization. Diplotene and diakinesis nuclei, which were most frequent on the preparations, showed regularly 13 bivalents and the large X-chromosome univalent (Fig. 5*a*). The observed chromosome number of $2n = 27$ with the X0 sex chromosome system is in agreement with the published data of Arefyev and Devyatkin (1988).

On chromosome preparations made from testes of late-instar larvae of the mayfly *C. dipterum* (Ephemeroptera), only pachytene spermatocytes were observed. Well-spread nuclei showed five bivalents of a similar size. The XY sex chromosome pair was not recognizable from autosome pairs. This finding including the chromosome number of $2n = 10$ coincides with the report of Kiauta and Mol (1977). In accordance with the results of PCR and Southern hybridization, no $(TTAGG)_n$ signals were observed (Fig. 5*b*).

Primarily apterous insects

Particularly clear and regular hybridization signals were observed in mitotic metaphase chromosomes of the firebrat *T. domestica* (Zygentoma), prepared from testes of adult males. Each chromosome showed four signals, two at each end, each signal representing a telomere of one sister chromatid (Fig. 5*c*). Although metaphases were not optimally spread for chromosome counts, with the help of twin telomeric signals, we estimated that the karyotype consists of $2n = 36$ chromosomes.

Late meiotic prophase I spermatocytes predominated in preparations from adult testes of the bristletail *Lepismachilis* sp. (Archaeognatha). In the diplotene/diakinesis complements, 15 or 16 bivalents were regularly observed. The bivalents showed hybridization signals both at the ends of and inside the chromatin, indicating the position of actual chromosomal ends (Fig. 5*d*).

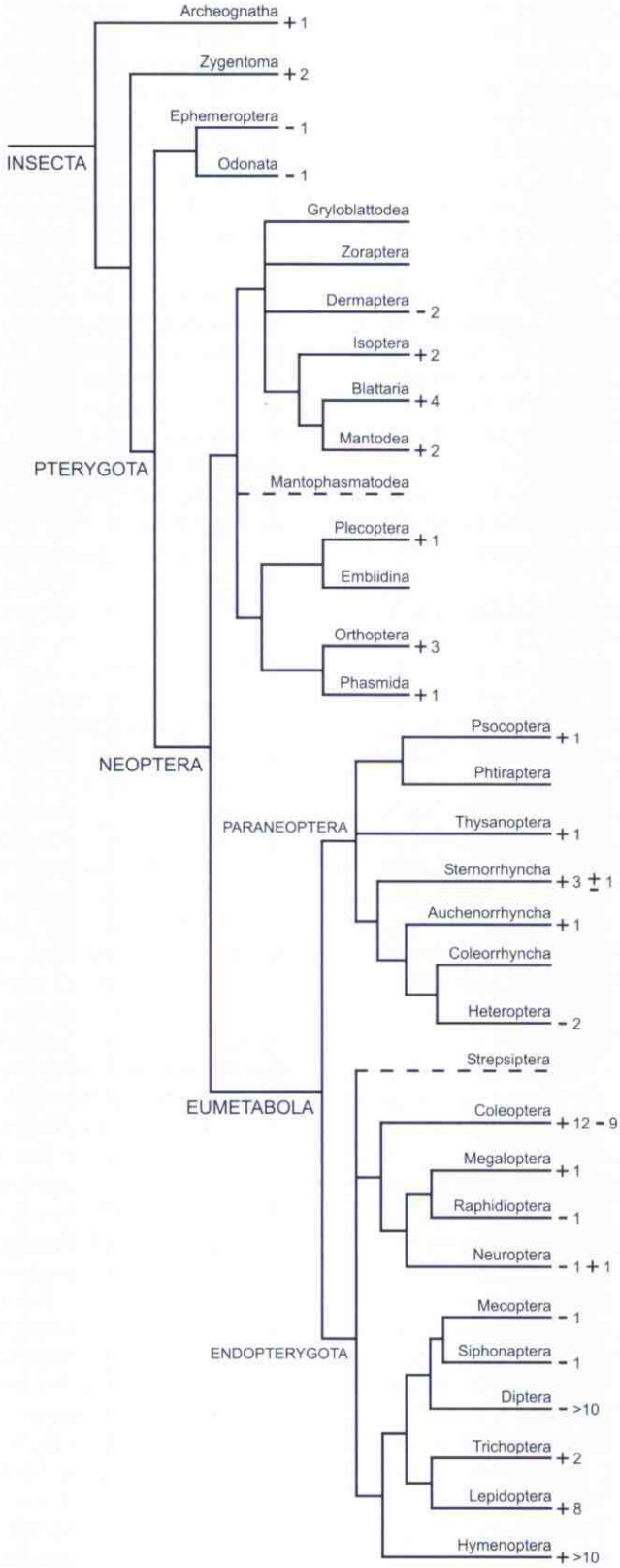
Discussion

By using single-primer PCR, Southern hybridization, and FISH, we screened 22 species of 20 different orders from a total of 33 insect orders for the presence of the TTAGG telomeric repeats. Our study involved representatives of all main lineages of the insect phylogeny. These were "primarily apterous insects", "primitive" winged insects, the Palaeoptera, and phylogenetically advanced pterygotes, the Neoptera. The latter involved the "lower" Neoptera, the hemipteroid assemblage (Paraneoptera), and the most successful and numerous lineage of insects, the Endopterygota (Holometabola). In total, the $(TTAGG)_n$ sequence was shown to be a component of the telomeres in 15 species, whereas it was absent in seven other species. Considering the sequence distribution, we confirmed the occurrence of TTAGG repeats in each of the main phylogenetic branches of insects with the exception of Palaeoptera. Taken together with previous reports (Table 2), the $(TTAGG)_n$ sequence appears conserved in 16 insect orders and absent in eight orders. Both the presence and absence were reported for two orders, the Coleoptera and Neuroptera. No data are yet available for seven minor orders. This overview supports the previous statement that insects as a group are heterogeneous with respect to the presence or absence of the $(TTAGG)_n$ telomere sequence, which is a widespread but not the only motif of insect telomeres (Okazaki et al. 1993; Sahara et al. 1999).

Comparing the three methods employed in this study, the single-primer PCR appeared to be the least reliable for evidence of telomeric repeats. The PCR product from a long telomeric TTAGG array is expected to be a high molecular mass smear. However, the heterogeneous DNA product is not a convincing indicator of TTAGG presence if it is not further characterized, e.g., by hybridization to a TTAGG probe. Since this was not done here, the PCR data were considered as preliminary and conclusions about the presence of TTAGG repeats were made according to the results of Southern hybridization and FISH. The latter two methods were done at highly stringent conditions, and thus, the risk of false-positive hybridization signals was negligible. It should also be mentioned that genomic DNAs for both PCR amplification and Southern hybridization were mostly extracted from whole insect bodies, and their contamination with a foreign DNA (e.g., from a TTAGG-positive endoparasite) could not be fully excluded. Thus, it is obvious that FISH, which yields hybridization signals directly at the chromosomal ends, is the most reliable method.

Our results in a compilation with previous data on the occurrence of the $(TTAGG)_n$ telomere motif are summarized in

Fig. 6. Phylogeny of insect orders examined for the presence (+), absence (–), or inconclusive occurrence (±) of the $(TTAGG)_n$ motif by FISH and (or) Southern hybridization (for details, see Table 2). Numbers of species examined are given. The cladogram is based on Kristensen (1991, 1999), Wheeler et al. (2001), and Hovmöller et al. (2002). Broken lines indicate groupings of uncertain phylogenetic position.



the cladogram (Fig. 6), which is modified according to recent knowledge of insect phylogeny. The pattern of phylogenetic distribution of the TTAGG repeats clearly supports the hypothesis of Sahara et al. (1999) that the sequence was an ancestral motif of insect telomeres but was lost several times in different branches of the insect phylogenetic tree, being probably replaced with other telomere motifs. The ancestral character of TTAGG is particularly obvious because of the sequence conservation in two lineages of "primarily apterous insects", the Archaeognatha and Zygentoma (this study).

Surprisingly, the $(TTAGG)_n$ telomeric repeats were absent in representatives of Ephemeroptera and Odonata (this study), indicating the loss of TTAGG in another "primitive" lineage of insects, the Palaeoptera (Fig. 6). TTAGG absence in both orders gives a supportive argument for the monophyletic origin of the Palaeoptera, recently suggested by Hovmöller et al. (2002). Providing the monophyly of Palaeoptera, the TTAGG type of telomeric sequence could be lost in a single evolutionary event some 300 million years ago (cf. the paleontological data in Carpenter 1992*a*, 1992*b*). However, independent losses of the sequence in each of the Ephemeroptera and Odonata cannot be excluded because the alternative mechanism(s) of telomere maintenance in these orders is not known.

In the Neoptera, there were apparent differences in the distribution and characteristics of TTAGG repeats between the "lower" Neoptera and "advanced" Neoptera, the so-called Eumetabola that consists of two sister groups, the Paraneoptera and Endopterygota. The $(TTAGG)_n$ motif appears to be well conserved in the telomeres of "lower" Neoptera. It was present in representatives of six orders (Isoptera, Blattaria, Mantodea, Plecoptera, Orthoptera, and Phasmida), whereas it was absent only in the order Dermaptera (Table 2 and references therein; Fig. 6). In addition, the pattern of Southern hybridization signals in most TTAGG-positive "lower" neopterans (this study; also see *Locusta migratoria* in Sahara et al. 1999) indicated (*i*) considerable lengths of the telomeric DNA with a large fraction of restriction fragments longer than 21 kb and (*ii*) homogeneity in the telomere lengths among different chromosomes and among different tissues of the organism. Taken together with the telomerase activity demonstrated by Sasaki and Fujiwara (2000) in the American cockroach *P. americana* and the cricket *Teleogryllus taiwanemma*, we conclude that the telomerase-dependent mechanism of elongation of the $(TTAGG)_n$ telomeric sequence is conserved in the "lower" Neoptera with the exception of earwigs (Dermaptera).

Like the "lower" Neoptera, the Paraneoptera appeared relatively homogeneous with respect to the presence of TTAGG repeats (Table 2; Fig. 6). Although FISH data confirmed the telomeric location of TTAGG repeats only in a bark lice (Psocoptera) (this study) and two species of aphids (Sternorrhyncha) (Bizzaro et al. 2000; Spence et al. 1998), our results of single-primer PCR and Southern hybridization furnished evidence of the sequence presence in the genome of a leafhopper (Auchenorrhyncha), the greenhouse whitefly (Sternorrhyncha), and a thrip (Thysanoptera). Thus, the TTAGG repeats were lost only in the true bugs (Heteroptera), as found by Okazaki et al. (1993) and Sahara et al. (1999). However, the Southern hybridization pattern in the TTAGG-positive paraneopterans was remarkably different from that in the "lower" Neoptera. A large number of discernible bands at a wide molecular mass range indicated that some TTAGG restriction fragments might represent nontelomeric sequences (cf. Frydrychová and Marec 2002).

In contrast with the "lower" Neoptera and Paraneoptera, the Endopterygota exhibited apparent heterogeneity in TTAGG presence (Table 2; Fig. 6). In earlier studies, the $(TTAGG)_n$ motif was shown to be well conserved in the telomeres of Hymenoptera and Lepidoptera (Okazaki et al. 1993; Meyne et al. 1995; Sahara et al. 1999; Lorite et al. 2002). Our FISH data confirmed that the motif is also conserved in the sister order of Lepidoptera, the Trichoptera. On the other hand, a number of studies showed that the genomes of Diptera lack this telomere motif, and instead, telomerase-independent mechanisms of telomere maintenance had evolved (Biessmann et al. 2002). In addition, our study revealed the absence of TTAGG repeats in two orders closely related to Diptera, the Siphonaptera and Mecoptera. Since these three orders form a common monophyletic clade, the so-called Antliophora (Kristensen 1991), the above data indicate that the $(TTAGG)_n$ motif could already have been lost in a common ancestor of the Antliophora some 250 million years ago (cf. paleontological data in Carpenter 1992*a*, 1992*b*).

Heterogeneous data were also obtained in three other closely related orders of Endopterygota, forming a common clade Neuropterida (i.e., Megaloptera, Raphidioptera, and Neuroptera; Kristensen 1991) (Table 2; Fig. 6). The $(TTAGG)_n$ motif was present in the telomeres of an alderfly (Megaloptera) but absent in a snakefly (Raphidioptera) and the green lacewing (Neuroptera) (this study). Okazaki et al. (1993) reported positive Southern hybridization signals in another neuropteran species, the ant lion *Protidricerus japonicus*. However, they erroneously classified this species to the Mecoptera (mistaken also in Sahara et al. 1999 (see table 1 therein)). The highest heterogeneity was found in beetles, the Coleoptera, where the TTAGG repeats were present in seven of 13 tested families, absent in five families, and both present and absent in one family (Frydrychová and Marec 2002). The scattered distribution of TTAGG indicated that the telomeric motif was lost repeatedly, at least five to six times, during the evolution of beetles. Providing that the main lineages of the Endopterygota phylogenetic tree are set properly, we suppose that a common ancestor of Coleoptera and Neuropterida possessed the $(TTAGG)_n$ motif that was then independently lost in or even within some orders.

In summary, the distribution of TTAGG telomeric repeats in insects (Fig. 6) favors the conservation of the $(TTAGG)_n$ telomere motif (in "primarily apterous insects", "lower" Neoptera, and Paraneoptera) and occasional loss (in Palaeoptera, Dermaptera, and Heteroptera) during the evolution of insects until the Endopterygota had originated. In the Endopterygota, repeated losses of the telomeric motif had occurred. The TTAGG losses are particularly frequent in beetles. According to Frydrychová and Marec (2002), these relatively frequent losses suggest a predisposition or a backup mechanism of telomere maintenance in the genome of beetles that enabled them evolutionary frequent changes in telomere composition. This hypothesis is applicable to the entire clade Endopterygota. In other words, it assumes the

existence of an alternative mechanism that would serve as a deposit function for cases when the primary mechanism is inactivated. Such a mechanism would not be a novelty. Biessmann and Mason (1997) suggested that eukaryotes may possess the dual capacity of telomere elongation by telomerase and recombination. In the yeast *Sacharomyces cerevisiae*, and possibly in humans (Mefford and Trask 2002), both of which normally use telomerase, recombination can be used for telomere length compensation when the telomerase is inactive or inactivated. Furthermore, at least two alternative pathways of telomere elongation already exist in insects, as found in the Diptera. These are the recombination-based elongation of long terminal satellite repeats and targeted transposition of non-long-terminal-satellite retrotransposons to existing chromosome ends (review: Biessmann et al. 2002).

Insects are not the only group of eukaryotes in which losses of the ancestral telomere structure have occurred. There is an increasing number of examples of a similar phenomenon in flowering plants. The telomeres of plant species are predominantly composed of the telomerase-dependent *Arabidopsis*-type sequence, $(TTTAGGG)_n$ (reviewed in Fajkus and Zentgraf 2002). However, Adams et al. (2000, 2001) reported the loss of TTTAGGG repeats in 12 related families of Asparagales. The results of Weiss and Scherthan (2002) suggested that in *Aloe*, the *Arabidopsis*-type telomeric repeats might be replaced with the human-type telomeric repeats, TTAGGG. The recent data of Sýkorová et al. (2003*a*) indicate that this switch from the plant repeats to human repeats is caused by a mutation. Finally, in spite of the early reported presence of TTTAGGG repeats in several Solanaceae species (*Nicotiana* and *Solanum*), Sýkorová et al. (2003*b*) found no evidence for the presence of conventional telomerase-dependent telomeric repeats in other representatives of Solanaceae (*Cestrum*, *Vestia*, and *Sessea*). Thus, the loss of telomeric repeats in very distant organisms, such as insects and plants, suggests that the alternative mechanisms of chromosome capping may be much more widespread.

Acknowledgements

We wish to thank Marie Korchová for her technical assistance. We also thank Karel Novák for collecting caddisflies, Aleš Bezděk for collecting scorpion flies, Marie Červenská and Zdeněk Růžička for supplying us with green lacewings from their laboratory cultures, Tomáš Soldán for determination of water insects and collecting alderflies, Jiří Zelený for determination of snakeflies and damselflies, Jan Havelka for bark lice collection and Paraneoptera determination, Barbora Konopová for gifting us walking sticks, Jan Šobotník (Prague) for supplying us with termites, David Doležel for laboratory culture of the firebrat, Hana Seňadová for laboratory culture of cockroaches, and František Šťáhlavský (Prague) for his help with collecting bristletails. Finally, our thanks are owed to Petr Švácha for his comments on insect phylogeny, Jan Zrzavý for critical reading of the manuscript, and Jaroslav Marek and Jiří Král (both Prague) for their help with interpretation of paleontological data. This work was supported by grant No. 206/00/0750 from the Grant Agency of the Czech Republic (Prague) and by Entomology Institute projects Z5007907 and K5052113.

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