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Telomere variability in the monocotyledonous plant order Asparagales

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A group of monocotyledonous plants within the order Asparagales, forming a distinct clade in phylogenetic analyses, was reported previously to lack the 'typical' *Arabidopsis*-type telomere $(TTTAGGG)_n$. This stimulated us to determine what has replaced these sequences. Using slot-blot and fluorescent *in situ* hybridization (FISH) to species within this clade, our results indicate the following. 1. The typical *Arabidopsis*-type telomeric sequence has been partly or fully replaced by the human-type telomeric sequence $(TTAGGG)_n$. Species in *Allium* lack the human-type variant. 2. In most cases the human variant occurs along with a lower abundance of two or more variants of the minisatellite sequences (of seven types evaluated), usually these being the consensus telomeric sequence of *Arabidopsis*, *Bombyx* $(TTAGG)_n$ and *Tetrahymena* $(TTGGGG)_n$. FISH shows that the variants can occur mixed together at the telomere. 3. Telomerases generate products with a 6 base pair periodicity and when sequenced they reveal predominantly a reiterated human-type motif. These motifs probably form the 'true telomere' but the error rate of motif synthesis is higher compared with 'typical' plant telomerases. The data indicate that the Asparagales clade is unified by a mutation resulting in a switch from synthesis of *Arabidopsis*-like telomeres to a low-fidelity synthesis of human-like telomeres.

Keywords: plant; human; *Tetrahymena*; telomere; evolution; TRAP assay

1. INTRODUCTION

Telomere sequences are highly conserved at the ends of chromosomes across eukaryotes. Indeed, only point mutations of the general, telomeric, minisatellite, oligonucleotide motif $(T_mA_nG_o)$ distinguish the telomeres of large groups of protozoa, algae, higher plants and animals. The conservation of telomere motifs results from their synthesis by telomerase, a nucleoprotein enzyme complex that adds oligonucleotide units to the telomere by reverse transcription. This system is considered to be evolutionarily ancient, as shown by homology between catalytic (protein) subunits of telomerases in different organisms, as well as by their similarity to viral reverse transcriptases.

The first exceptions noted to this 'telomerase totality' were *Drosophila* and other insects. *Drosophila* species use a strategy involving retrotransposition of HeT-A and TART sequences to chromosome ends (Biessmann *et al.* 1990; Casacuberta & Pardue 2003), whereas in *Chironomus* species and *Anopheles gambiae*, telomeric DNA is formed by blocks of satellite repeat sequences that are probably extended by gene conversion (see Biessmann *et al.* 2002 for review). These sequences are more typical of subtelomeric regions in organisms with telomeres maintained by telomerase. The second group of exceptions were found in yeasts, which possess irregular sequences at the telomeres. These yeasts have survived mutations in the telomerase-mediated chromosome elongation system and elongate their chromosomes using recombination. In these organisms, the structure of the chromosome ends and their dependence on recombination proteins point towards unequal recombination (gene conversion) occurring between telomeres (Lundblad & Blackburn 1993; McEachern & Blackburn 1996; Teng & Zakian 1999). Such alternative lengthening of telomere (ALT) mechanisms have also been shown to occur in rare cases of human telomerase-negative tumours and *in vitro* immortalized cell-lines (Bryan *et al.* 1997). In relation to ALT, telomeric loops observed in mammals (see Griffith *et al.* 1999), protozoa (see Murti & Prescott 1999; Munoz-Jordan *et al.* 2001) and recently in plants (J. D. Griffith, personal communication) could well serve, not only as the protective telomere capping structures, but also as substrates for rolling circle gene conversion.

In plants, the absence of *Arabidopsis*-type telomere $(TTTAGGG)_n$ was first observed in Alliaceae species (notably *Allium*) and it was suggested that the chromosomes may be terminated by satellite repeats, ribosomal DNA (rDNA) repeats or mobile elements (Fuchs *et al.* 1995; Pich *et al.* 1996; Pich & Schubert 1998). The list of plants 'lacking' typical plant telomeres was then extended to include *Aloe* species (family Asphodelaceae; Adams *et al.* 2000) in which the *Arabidopsis*-type telomeric sequence is apparently replaced by the human-type sequence $(TTAGGG)_n$ (Weiss & Scherthan 2002). The lack of *Arabidopsis*-type telomeric sequence has since been demonstrated in 16 other species from 12 families of Asparagales (Adams *et al.* 2001). In phylogenetic analyses

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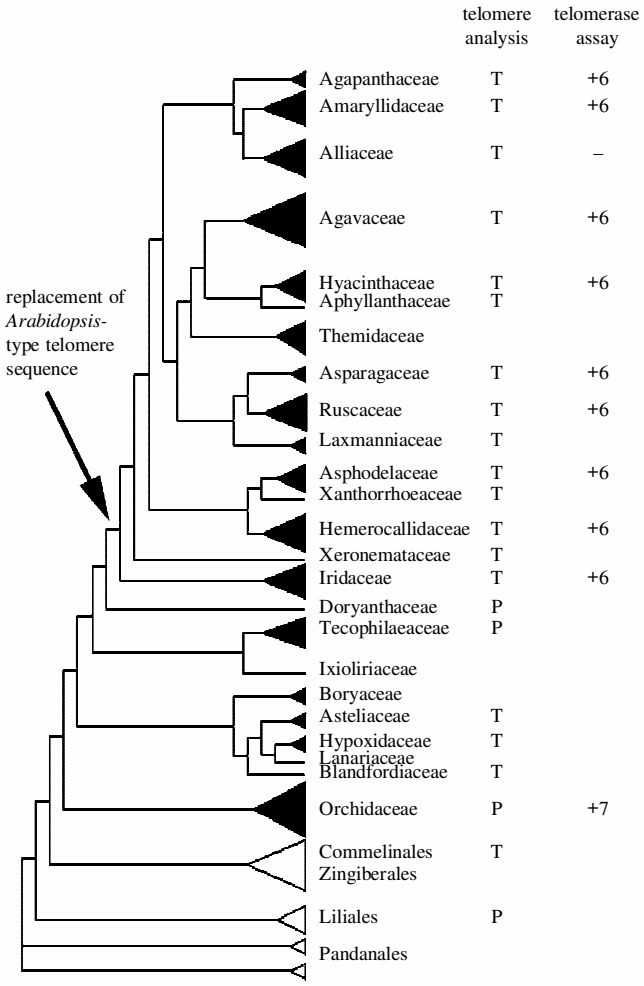

Figure 1. Phylogenetic tree of Asparagales (Fay *et al.* 2000). Arrow shows the node at which the *Arabidopsis*-type telomere sequences were substituted (considered as lost by Adams *et al.* 2001). T denotes that slot-blot analyses are presented here; P denotes additional families studied by Adams *et al.* (2001); + indicates that the TRAP assay generated a product; the subscript 7 or 6 denotes the periodicity in base pairs of the TRAP product; − indicates no TRAP products generated.

(Fay *et al.* 2000), these species cluster in a clade within Asparagales, thereby indicating that the *Arabidopsis*-type telomeric sequence was lost in a single evolutionary event *ca*. 80–90 Myr ago (figure 1). More recently, Sykorova *et al.* (2003) showed that the *Arabidopsis*-type telomere was also absent in some *Cestrum* species (family Solanaceae), although the minisatellite sequences were present at interstitial locations.

These data raise questions as to what sequences have come to replace the *Arabidopsis*-type consensus sequence and how do the new sequences function as telomeres. Here, we investigate these questions in Asparagales. Adams *et al.* (2001) noted by *in situ* hybridization that some species in the family Hyacinthaceae (Asparagales) appeared to have the *Arabidopsis*-type telomere when it might be expected from their position in the Asparagales phylogeny (Fay *et al.* 2000) that they would not. For this reason, extensive sampling across Hyacinthaceae was carried out in this work. We reasoned that the *Arabidopsis*-type consensus sequence may have been replaced by variants of the minisatellite repeat as found as the consensus sequence in vertebrates (including humans) $(TTAGGG)_n$, *Tetrahymena* $(TTGGGG)_n$, *Oxytricha* $(TTTTGGGG)_n$, *Bombyx* $(TTAGG)_n$, *Chlamydomonas* $(TTTTAGGG)_n$ and *Ascaris* $(TTAGGC)_n$. These sequence motifs were chosen for their sequence similarity to the *Arabidopsis* telomeric sequence, and we presumed that the simplest hypothetical event resulting in telomeric change to a different minisatellite repeat would be a mutation in the template region of telomerase.

We demonstrate that in most species investigated by slot-blot hybridization the telomere consensus motif of *Arabidopsis* is replaced by that typical of humans or by a mixture of the *Arabidopsis*-, human- and *Tetrahymena*-type motifs. To answer the question as to whether the human motif, detected *in situ* on chromosome ends of many Asparagales, is synthesized by telomerase and thus constitutes the 'true' telomere, the telomerase repeat amplification protocol (TRAP) was performed, and the products sequenced. The results were unambiguously positive in sampled Asparagales species, except for *Allium cepa*, in which no activity was detected. We also show that the lack of telomerase activity in *A. cepa* is not due to inhibition of the TRAP assay.

2. MATERIAL AND METHODS

(a) *Plant material*

The sources of plants used to extract total genomic DNA for slot-blot analyses are given in figures 2–4. Control genomic DNA for these experiments were *Silene latifolia* (Caryophilaceae; Institute of Biophysics, Czech Republic), *Nicotiana sylvestris* (Solanaceae, Queen Mary University of London, UK), *A. cepa* cv. Ailsa Craig (Alliaceae; Royal Botanic Gardens, Kew, UK; RBGK), *Homo sapiens* DNA (Cambio UK Ltd) and *Bombyx mori*, a gift of František Marec (Institute of Entomology, Czech Acad. Sci., České Budějovice, Czech Republic).

For FISH experiments, vigorously growing root tips were obtained from pot-grown plants of the following: *Ruscus aculeatus* (Ruscaceae), *Lycoris squamigera* (Amaryllidaceae), and *Ornithogalum umbellatum* (Hyacinthaceae; all grown at Royal Botanic Gardens, Kew); *Ornithogalum virens* (Hyacinthaceae; Chelsea Physic Gardens, London, UK); *Bowiea volubilis* (Hyacinthaceae) and *Iris versicolor* × *I. virginica* (Iridaceae; Queen Mary University of London).

For telomerase activity assays (TRAP assays), the following plants were used.

(i) Harvested seedlings of *A. cepa* cv. Stuttgarter Riesen (Alliaceae; Semo Smržice, Czech Republic), *Asparagus officinalis* (Asparagaceae; commercial cultivar), *Gennaria chaplinii* (RBGK 1986-5342; Amaryllidaceae), *Galtonia candicans* (RBGK 1969-19589; Hyacinthaceae), *Agapanthus umbellatus* (RBGK; Agapanthaceae), *Hosta rectiflora* (RBGK 1979-5048; Agavaceae), *Phormium cookianum* (RBGK 1996-434; Hemerocallidaceae), *Bulbine glauca* (RBGK 1998-4063; Asphodelaceae), *Iris pseudacorus* (RBGK; Iridaceae), and *Triticum aestivum* cv. Beaver (Poaceae Plant Breeding International, Cambridge, UK).

(ii) Excised terminal 3–5 mm of the roots of *Dracaena marginata* (Ruscaceae) and *Phalaenopsis* hybrid (Orchidaceae; Agro Tuřany, Brno, Czech Republic), *Muscari armeniacum* and *Scilla peruviana* (both Hyacinthaceae; Taylor Bulbs,

London, UK), and *O. virens* (Hyacinthaceae; Chelsea Physic Garden, London, UK).

(b) *Primers and probes*

For slot-blot hybridization, a range of probes was designed to investigate the telomeric consensus sequences reported from a diverse range of eukaryotes.

(i) *Arabidopsis*-type, ATSB: 5′ GGT TTA GGG TTT AGG GTT TAG GGT TTA G 3′
(ii) Human-type, HUSB: 5′ TTA GGG TTA GGG TTA GGG TTA GGG TTA G 3′
(iii) *Bombyx*-type, BOSB: 5′ TTA GGT TAG GTT AGG TTA GGT TAG GTT AG 3′
(iv) *Chlamydomonas*-type, CHSB: 5′ GTT TTA GGG TTT TAG GGT TTT AGG GTT TTA G 3′
(v) *Oxytricha*-type, OXSB: 5′ TTT TGG GGT TTT GGG GTT TTG GGG TTT T 3′
(vi) *Tetrahymena*-type, TTSB: 5′ TGG GGT TGG GGT TGG GGT TGG GGT TG 3′
(vii) *Ascaris*-type, ASSB: 5′ TTA GGC TTA GGC TTA GGC TTA GGC TTA G 3′
(viii) Control DNA was 18S nuclear ribosomal DNA (18S rDNA), a 1.7 kilobase *Eco* RI fragment of the 18S rRNA gene subunit from *Solanum lycopersicum* L. (tomato, accession number X51576) (Kiss *et al.* 1989).

The TRAP assays used the following primers.

(i) CAMV: 5′ CGT CTT CAA AGC AAG TGG ATT 3′ (Fajkus *et al.* 1998)
(ii) TELPR: 5′ CCG AAT TCA ACC CTA AAC CCT AAA CCC TAA ACC C 3′ (Fajkus *et al.* 1998)
(iii) HUTPR: 5′ CCG AAT TCA ACC CTA ACC CTA ACC CTA ACC C 3′ (designed here)
(iv) TS21: 5′ GAC AAT CCG TCG AGC AGA GTT 3′ (Fitzgerald *et al.* 1996)
(v) TS Primer (5′ AAT CCG TCG AGC AGA GTT 3′) and primer mix from TRAPeze telomerase detection kit (Intergen Company, USA).

For FISH experiments:

Concatemers of individual telomeric sequences were prepared by using the PCR protocol (Ijdo *et al.* 1991) with *Pfu* DNA polymerase (MBI Fermentas) and the G-rich forward primers ATSB, HUSB, TTSB (as above) with their complementary C-rich reverse primer to generate ATSB-concatemers, HUSB-concatemers and TTSB-concatemers, respectively. In some experiments the material was labelled for 45S nuclear ribosomal DNA (45S rDNA) with pTa71 which includes the 18-5.8-26S rDNA subunits and the intergenic spacer isolated from *Triticum aestivum* (Gerlach & Bedbrook 1979).

(c) *Slot-blot experiments*

To investigate the occurrence of variant minisatellite telomeric sequences in Asparagales we used slot-blot hybridization to screen a large range of species selected to best represent species diversity in Asparagales (figure 1). The probes were designed to make hybridization parameters as uniform as possible and to enhance probe specificity. As positive controls for individual repeat variants, either genomic DNAs (*S. latifolia*, *N. sylvestris*, *H. sapiens*, *B. mori*) or concatemers of the sequence motifs assayed (i.e. concatemers of ATSB, HUSB, BOSB, CHSB, OXSB, TTSB, ASSB using the methods in Ijdo *et al.* (1991)) were used to check for possible false negative results. *Silene latifolia* and *N. tabacum* were used as reference organisms because *N. tabacum* has an estimated 25-fold more telomeric sequence than *S. latifolia* despite having similar DNA C-values (Fajkus *et al.* 1995; Riha *et al.* 1998).

The experiments were conducted using radioactively labelled probes that have higher sensitivity in slot-blot hybridization than was reported by Adams *et al.* (2001). Species used here and in Adams *et al.* (2001) enabled direct comparisons. In carrying out slot-blot hybridizations, attention was paid to ensure that the DNA concentrations were accurately measured for blotting 1 μg of genomic DNA of each species. However, this does not enable calculations of relative copy numbers of minisatellite repeats between species because DNA C-values vary substantially between angiosperm species (Bennett & Leitch 2003). Furthermore, attention was paid to the control DNAs on each membrane to enable the evaluation of overall signal strength, uniformity of signal between blots and specificity of probe hybridization.

Genomic DNA (1 μg) or telomeric concatemers (500, 100 and 20 pg) was loaded onto Hybond-N+ membrane (Amersham Biosciences) using a Bio-Dot SF apparatus (Bio-Rad, USA) according to manufacturer's instructions; to this was hybridized a [^{32}P]end-labelled oligonucleotide probe (Sambrook *et al.* 1989) at 55 °C, in hybridization buffer (0.5 M sodium phosphate, pH 7.5; 7% SDS) overnight. Membranes were washed at 55 °C in 2× SSC with 0.1% SDS. These were exposed to X-ray films and a Phosphoimager Typhoon 9410 (Amersham Biosciences) screen. The control genomic DNA samples enabled comparison of the strength of hybridization signals between membranes.

(d) *Fluorescent* in situ *hybridization (FISH)*

FISH was carried out as described in Leitch *et al.* (2001) with modifications as in Adams *et al.* (2001). The probes were labelled with either digoxigenin-11-dUTP or biotin-16-dUTP. Slides were denatured in 70% formamide in 2 × SSC at 70 °C for 2 min. The hybridization mix contained 2 μg ml^{-1} of labelled ATSB- or TTSB-concatemers and 2 μg ml^{-1} of labelled HUSB-concatemers in 50% (v/v) formamide, 10% (w/v) dextran sulphate and 0.1% (w/v) sodium dodecyl sulphate in 2 × SSC (0.3 M sodium chloride, 0.03 M sodium citrate). After overnight hybridization at 37 °C, the slides were washed in 20% (v/v) formamide in 0.1× SSC at 42 °C at an estimated hybridization stringency of 80–85%. Sites of probe hybridization were detected using 20 μg ml^{-1} of fluorescein-conjugated anti-digoxigenin IgG (Roche Biochemicals, giving green fluorescence) and 5 μg ml^{-1} of Cy3-conjugated avidin (Amersham Biosciences, giving red fluorescence). Chromosomes were counterstained with 2 μg ml^{-1} DAPI (4′,6-diamidino-2-phenylindole, giving blue fluorescence) in 4 × SSC, mounted in Vectashield (Vector Laboratories) medium, examined using a Leica DMRA2 epifluorescent microscope, photographed with an Orca ER camera and analysed by using Improvision Openlab software. Images were processed for colour balance, contrast and brightness uniformly.

(e) *Telomerase activity (TRAP) assays*

In an attempt to determine what forms the ends of chromosomes (i.e. is synthesized by telomerase), a modified version of the TRAP assay was used. In this assay, different substrate primers (used because of the unknown selectivity of telomerase in the analysed species) and reverse primers complementary both

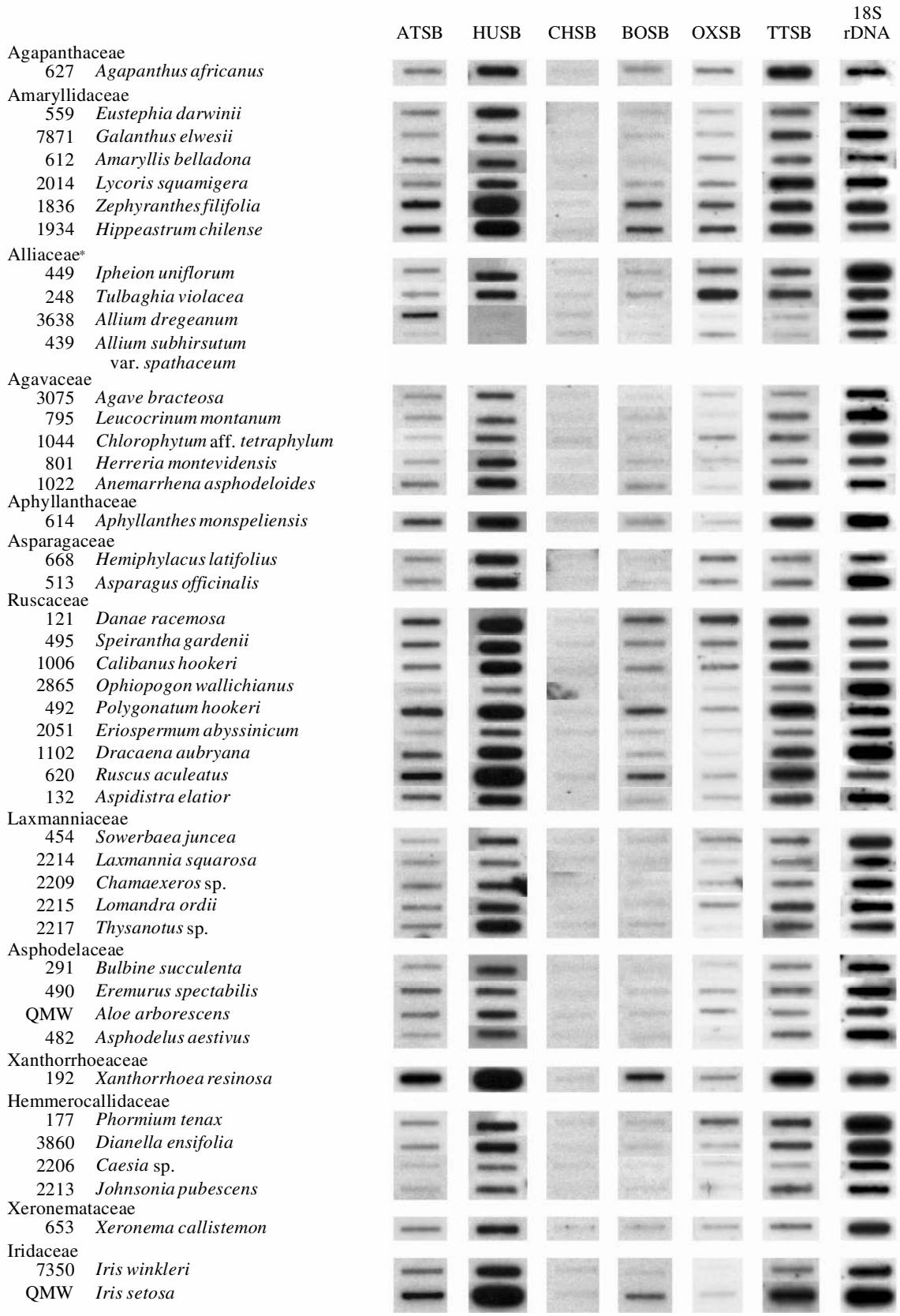

Figure 2. Slot-blot hybridization of species within the clade marked by the arrow in figure 1 (excluding Hyacinthaceae and Themidaceae) using the probes indicated. The left-hand column gives the source of material for DNA samples—numbers (Royal Botanic Gardens, Kew, accession numbers), QMW (Queen Mary University of London). **Allium cepa*, another member of Alliaceae is used as a control (figure 4).

to the human (HUTPR) and the plant (TELPR) sequence were used in separate experiments. Under the conditions used in the PCR step of the assay, the specificity of reverse primer annealing is low, and TELPR can anneal to the human sequence. Extracts for telomerase assays were prepared from seedlings or root tips as previously described (Fitzgerald *et al.* 1996) with minor modi-

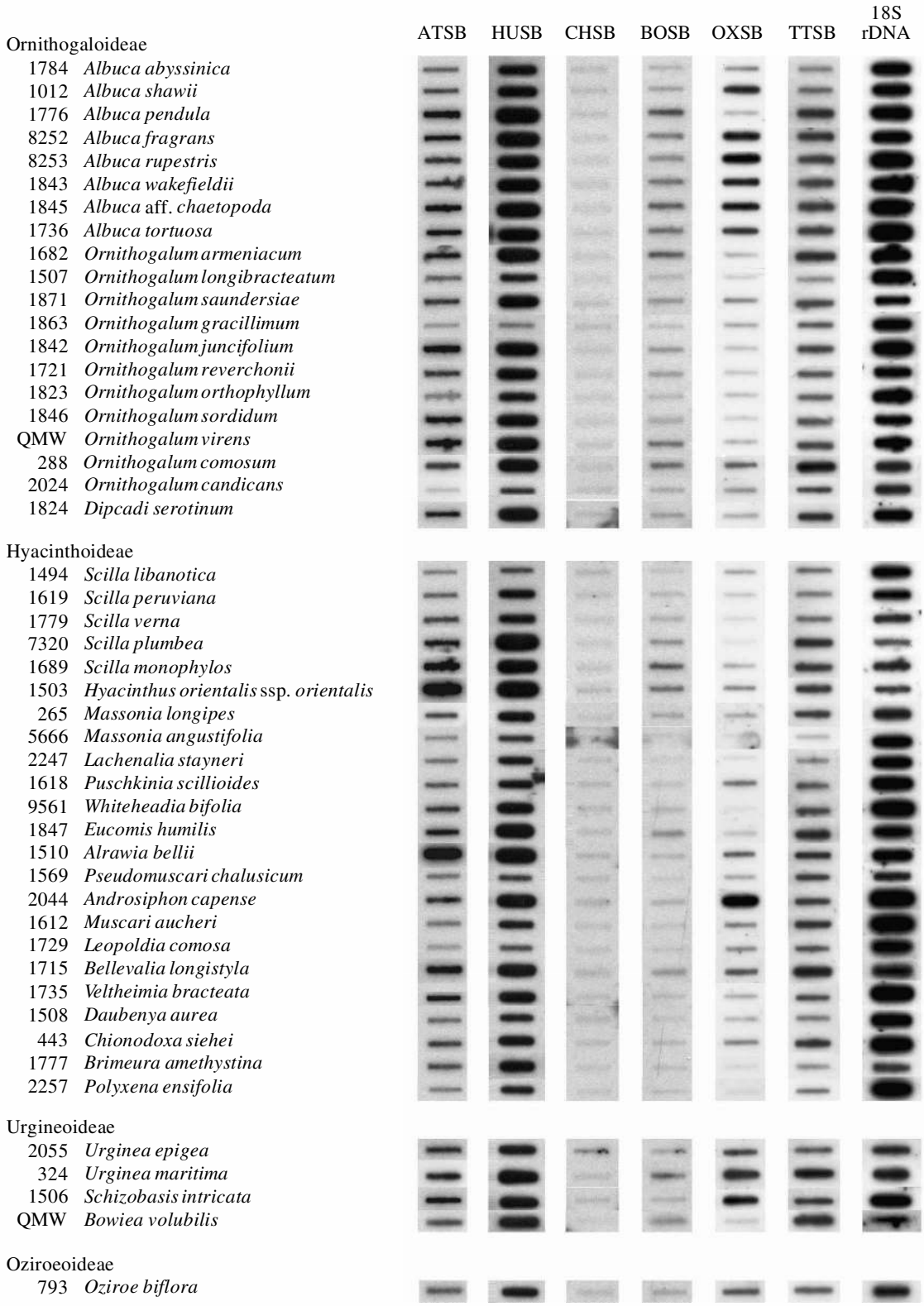

Figure 3. Slot-blot hybridization of species in Hyacinthaceae using the probes indicated. The left-hand column gives the source of material for DNA samples—numbers (Royal Botanic Gardens, Kew, accession numbers), QMW (Queen Mary University of London).

fications (Fajkus *et al.* 1998). The amount of protein in extracts was determined as previously described (Bradford 1976).

TRAP assay was performed in two phases. First, an extension step with substrate primer was run for 45 min at 26 °C in reaction mix containing 46 μl of TRAP buffer (Fitzgerald *et al.* 1996), 1 μl of telomerase extract containing 50–500 ng of total protein and 1 μl of 10 μM substrate oligonucleotide (CAMV, TS21 or TS). After an extension step, reactions were heated to 95 °C for 10 min. After cooling to 80 °C, a mixture containing two units of Dynazyme II DNA polymerase (Finnzymes) and 1 μl of 10 μM reverse primer (TELPR, HUTPR or TRAPeze Primer mix, Intergene) was added to each reaction, after which

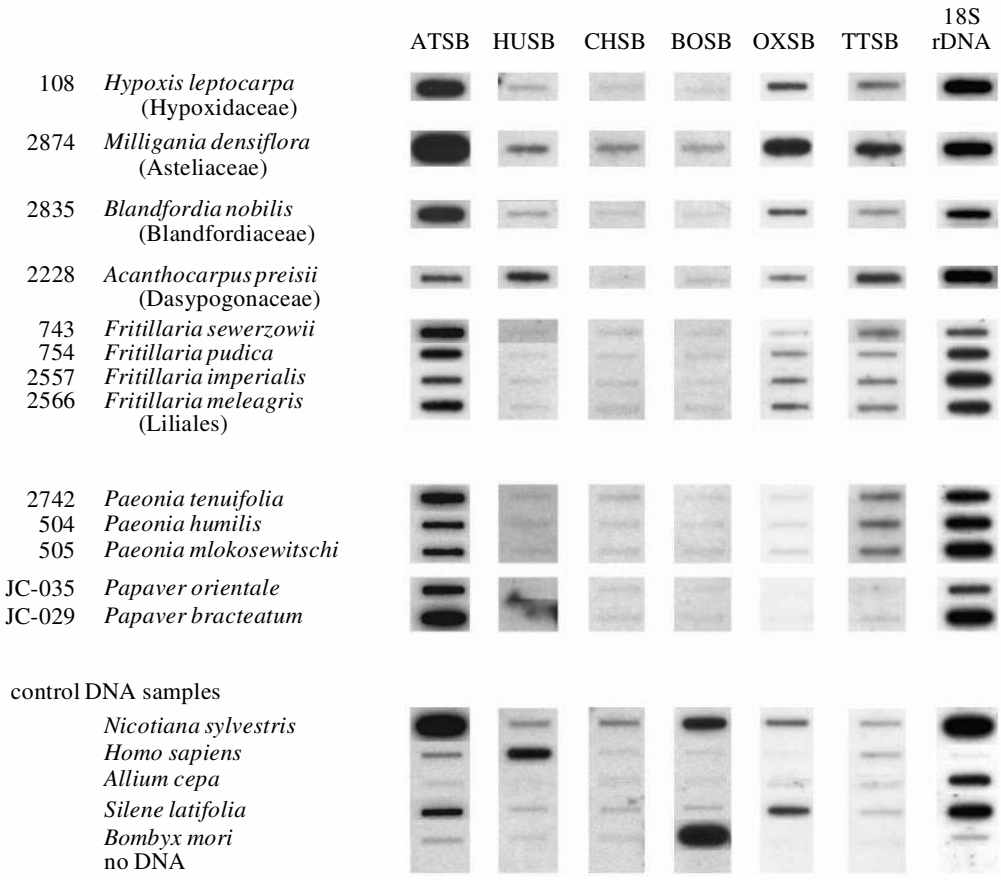

Figure 4. Slot-blot hybridization to species outside the clade defined by the arrow in figure 1 using the probes indicated. The left-hand column gives the source of material for DNA samples—numbers (Royal Botanic Gardens, Kew, accession numbers), JC (J. Carolan, Trinity College, Dublin). The families in brackets are to be found in figure 1 except for *Acanthocarpus preisii* (Dasypogonaceae) which is in Commelinales/Zingiberales. The remaining taxa are outside the phylogeny presented.

it was subjected to 35 cycles of 95 °C/30 s, 65 °C/30 s, 72 °C/30 s, followed by a final extension at 72 °C for 10 min. Alternatively, a TRAPeze telomerase detection kit (Intergen) was used following the manufacturer's protocols. Products were analysed by polyacrylamide gel electrophoresis (PAGE) as described previously (Fajkus *et al.* 1998).

(f) *Sequence analysis of TRAP products*

Aliquots of the TRAP reactions coming from CAMV × HUTPR and CAMV × TELPR primer combinations were precipitated and used for cloning into the *Eco* RV site of the pZErO plasmid vector (Invitrogen). Cloned DNA was sequenced using a Big Dye Terminator kit and ABI PRISM 310 sequencing apparatus (Applied Biosystems) following the manufacturer's protocols (Applied Biosystems).

3. RESULTS

(a) *Slot-blot hybridization screening of the clade identified in figure 1 (excluding Hyacinthaceae and Themidaceae)*

Slot-blot hybridization using the probes ATSB, HUSB, CHSB, BOSB, OXSB, TTSB and ASSB revealed the following results (figure 2).

(i) There was an abundance of the human-type variant of the telomere sequence $(TTAGGG)_n$ that exceeds the *Arabidopsis*-type variant.

(ii) All species tested had some, or considerable, amounts of the *Tetrahymena*-type $(TTGGGG)_n$ although in less abundance than the human-type.

(iii) The *Arabidopsis*-type of the sequence was present in many species in these experiments, but the signal for this type is also reduced compared with that observed for the human-type.

(iv) The *Bombyx*-type $(TTAGG)_n$ was found in some species at an abundance less than the human variant and usually equally or less abundant than the *Arabidopsis*-type variant.

(v) The abundance of the human-type was frequently reflected in the abundance of the repeats that are most closely related to it; these variants occur in the following order of abundance: human-type > *Tetrahymena*-type ⩾ *Arabidopsis*-type > *Bombyx*-type.

(vi) The *Oxytricha* $(TTTTGGGG)_n$ variant had a variable but generally low abundance. *Tulbaghia violacea* (Alliaceae) was an exception in having a considerably higher proportion of *Oxytricha*-type than *Arabidopsis*-type repeat.

(vii) Little or no signal was observed by using probes for the variants typical of *Chlamydomonas* $(TTTTAGGG)_n$ or *Ascaris* $(TTAGGC)_n$ (the latter is not shown).

(viii) The genus *Allium* differs from the rest of Alliaceae in not having a high abundance of the human mini-

satellite repeat. *Allium dregeanum* was unusual in possessing the *Arabidopsis*-type repeat as the most abundant sequence. This is in contrast to all other Alliaceae.

(b) *Slot-blot hybridization to Hyacinthaceae*

Slot-blot hybridization using the probes ATSB, HUSB, CHSB, BOSB, OXSB, TTSB and ASSB revealed the following results (figure 3).

(i) The pattern of hybridization generally followed that described in the section above, i.e. there was no indication that Hyacinthaceae as a whole was different from related families within their clade.
(ii) Most *Albuca* species had particularly large amounts of *Oxytricha*-type repeat. These plants are unusual for the clade as a whole in that the abundance of these repeats exceeds the abundance of that of the *Arabidopsis*-type.
(iii) The abundance of the *Arabidopsis*-type in *Hyacinthus orientalis* and *Alrawia bellii* followed the trend for the clade as a whole in that the *Arabidopsis*-type occurred at lower abundance than the human-type, but the difference in their relative abundance was small.
(iv) There was an unusual abundance of the *Oxytricha*-type sequence variant in *Androsiphon capense* that greatly exceeded that of the *Arabidopsis*-type and was only slightly less than that of the human-type. *Scilla peruviana* uniquely also possessed the *Ascaris*-type (not shown).

(c) *Slot-blot hybridization to species outside the clade identified in figure 1*

Slot-blot hybridization using the probes ATSB, HUSB, CHSB, BOSB, OXSB, TTSB and ASSB revealed the following results (figure 4).

(i) All species had abundant *Arabidopsis*-type repeats, the abundance of which exceeds that of all species described above, with the exception of *Acanthocarpus preisii* (Dasypogonaceae).
(ii) There were varying distributions of the other variants in the four species analysed, but, except for *A. preisii*, they were in lower abundance than the *Arabidopsis*-type telomeric repeat.
(iii) *Milligania densiflora* (Asteliaceae) had high numbers of the *Oxytricha*-type repeat $(TTTTGGGG)_n$ exceeding other species with a predominance of the plant-type repeat.

(d) *Fluorescent* in situ *hybridization*

FISH analyses were conducted on species inside the clade identified in figure 1. In *Lycoris squamigera* (figure 5*a*), *Ruscus aculeatus* and *Iris versicolor* × *virginica* (not shown, outside Hyacinthaceae) and *Bowiea volubilis* (Hyacinthaceae, not shown), no FISH signals were obtained using the ATSB-concatemer probe. The metaphase of *L. squamigera* is green (figure 5*a*) because high grain and contrast has been applied in an attempt to reveal any faint specific ATSB-concatemer probe signal. None was found but the image processing has made the chromosomes green since the method also amplifies low levels of background fluorescence. However, all these species were labelled at their telomere with HUSB-concatemers as shown for *L. squamigera* (figure 5*b*), *I. versicolor* × *I. virginica* (figure 5*g*) and *R. aculeatus* (figure 5*h*). *Ornithogalum virens* (Hyacinthaceae) labelled with both ATSB- and HUSB-concatemers at their telomere (figure 5*c–e*). The ATSB-concatemer probe was faint but labelled the end of all chromosome arms (figure 5*c*) whereas the HUSB-concatemer labelled the telomere strongly (figure 5*d*,*e*). At interphase, the chromatin at some telomeric regions was more decondensed, enabling the spatial resolution of ATSB- and HUSB-concatemer signals (figure 5*f*). The ATSB repeat was always linked to HUSB signal and in some cases occurred within it. Similar results were obtained by using the ATSB-concatemer on metaphases of *Ornithogalum umbellatum* (not shown) although only a subset of arms showed a signal, perhaps because the abundance of these repeats is closer to the signal threshold for FISH as shown in this species by Adams *et al.* (2001). Probing *O. umbellatum* metaphases with TTSB-concatemer also showed a terminal signal to some chromosome arms (figure 5*i*) and the signals coincided with stronger label from the HUSB-concatemer probe, the latter labelled strongly the ends of all chromosome arms (figure 5*j*).

(e) *Telomerase assays*

Using both the human (HUTPR) and the plant (TELPR) sequence of reverse primers, products were obtained with Asparagales species including Hyacinthaceae (figure 6), as well as with *G. chaplinii*, *P. cookianum* and *D. marginata* (all Asparagales; not shown). Two positive controls for each assay were used, extracts from human control cells from the TRAPeze telomerase detection kit with the HUTPR primer and extracts from *T. aestivum* (Poaceae, Poales) (expected to have the plant-type repeat) with the TELPR primer. PAGE analysis showed that all species within the clade identified in figure 1 (except *A. cepa*) had TRAP products containing a ladder of fragments with a six-nucleotide periodicity (identical to human control cells) and in contrast to the plant controls (*T. aestivum*, *Phalaenopsis* hybrid), which had a seven-nucleotide periodicity. The *Phalaenopsis* hybrid (Orchidaceae) lying outside the clade identified in figure 1, also had a seven-nucleotide periodicity. Similar results were also obtained when using the TRAPeze telomerase detection kit (not shown).

Special attention was paid to *A. cepa*, which was the first plant species to be reported to lack the *Arabidopsis*-type telomere (Fuchs *et al.* 1995). The results from the TRAP assay, using primers suitable for the detection of either plant- or human-type repeat were all negative under various extract concentrations (figure 7). Negative results were also obtained in experiments with several alternative substrate primers (not shown). Inhibition of the PCR step of TRAP by some component of the *Allium* extract was excluded by the internal control sequence included in the TRAPeze reaction buffer (figure 7*a*). To find out whether the lack of telomerase activity in *Allium* was due to the presence of inhibitors of telomerase activity in the *Allium* extracts, admixture experiments were performed by using

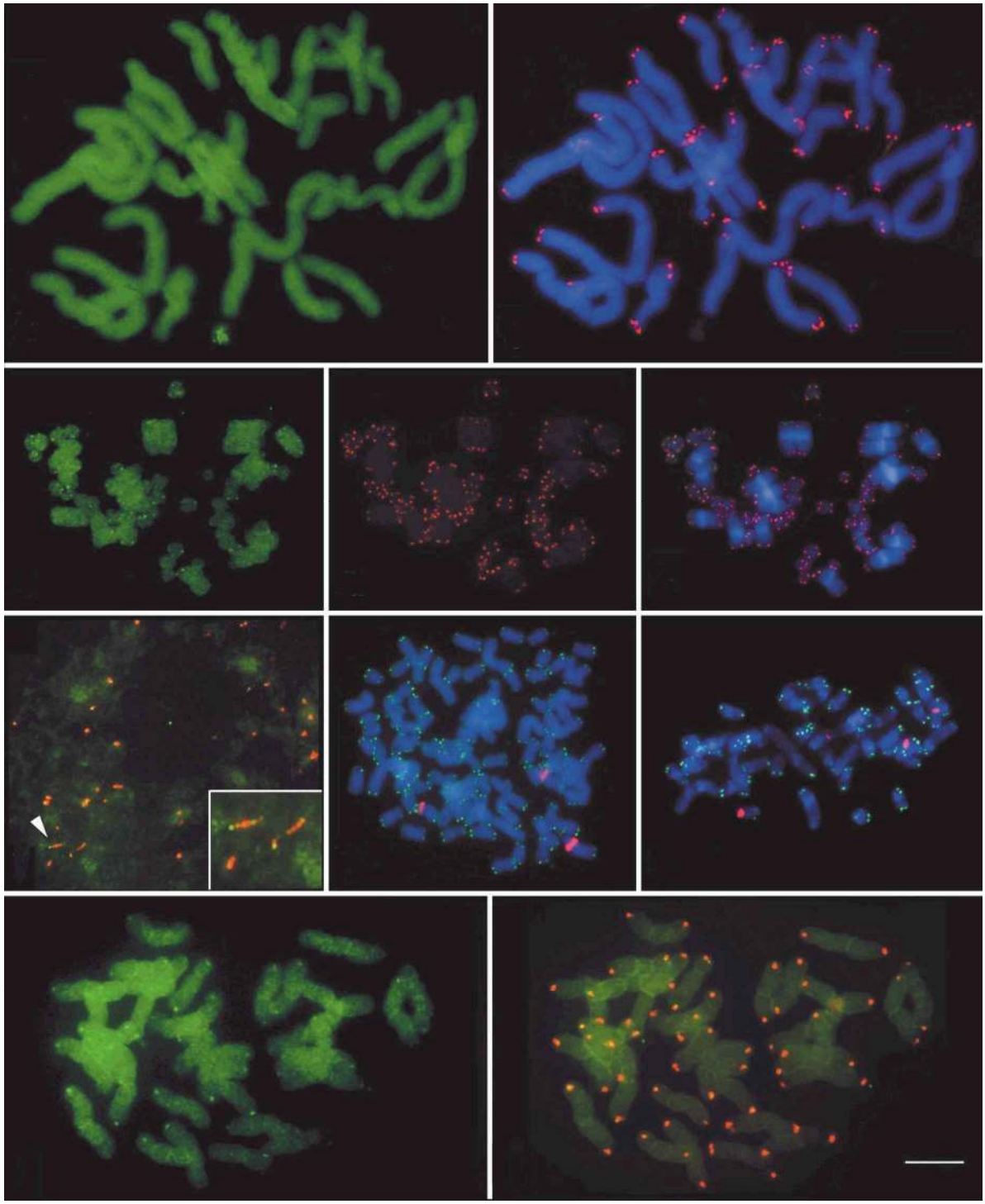

Figure 5. Metaphase of *Lycoris squamigera* (Amaryllidaceae) probed with (*a*) ATSB-concatemers (green) and (*b*) HUSB-concatemers (red) and counterstained with DAPI for DNA (blue). Note that here is no specific ATSB-concatemer signal (the chromosomes are green because the image has very high contrast and grain; without this the image would be black). Metaphase (*c–e*) and interphase (*f*) of chromosomes of *Ornithogalum virens* probed with (*c*) ATSB-concatemers (green fluorescence), (*d*) HUSB-concatemers (red fluorescence), (*e*) merged image of (*c*) and (*d*) and superimposed onto DAPI-stained chromosomes (blue); (*f*) interphase labelled with HUSB-concatemers (red) and ATSB-concatemers (yellow). The inset shows an enlarged region of the nucleus (arrowhead) with interspersed ATSB within arrays of HUSB. (*g*) Metaphase of an *Iris* hybrid (*I. versicolor* × *virginica*) showing FITC-labelled HUSB-concatemers and Cy3-labelled pTa71 for 45S rDNA with DAPI counterstain. (*h*) Metaphase of *Ruscus aculeatus* (Ruscaceae) probed HUSB-concatemers (green) and pTa71 (red) counterstained with DAPI. (*i,j*). Metaphase of *Ornithogalum umbellatum* labelled with TTSB-concatemers (yellow) and image merged with label from HUSB-concatemers (red). Scale bar, 5 μm.

combinations of *Allium* and *S. latifolia* extracts (figure 7*b*). The results clearly showed that the presence of *Allium* extracts did not decrease activity of *Silene* telomerase. Indeed the presence of *Allium* extract had a slightly beneficial effect on activity and processivity of *Silene* telomerase.

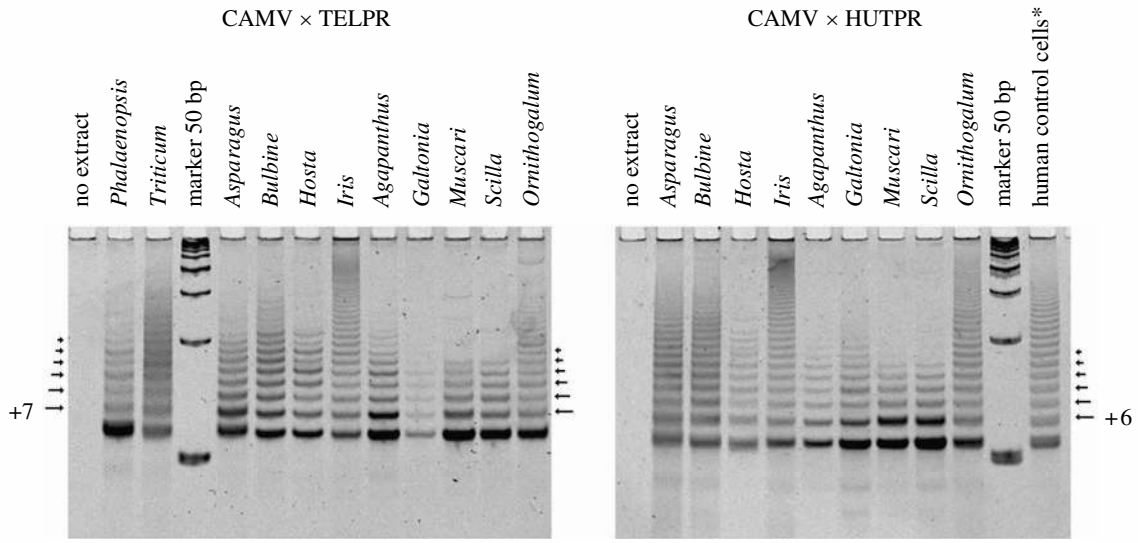

Figure 6. TRAP assay of Asparagales species using two different sets of primers—CAMV × HUTPR (human-type repeat) or CAMV × TELPR (*Arabidopsis*-type repeat). The same amount of total protein (50 ng) was used for all species shown: *Bulbine glauca* (Asphodelaceae), *Iris pseudacorus* (Iridaceae), *Agapanthus umbellatus* (Agapanthaceae), *Galtonia candicans* (Hyacinthaceae), *Muscari armeniacum* (Hyacinthaceae), *Scilla peruviana* (Hyacinthaceae), *Ornithogalum virens* (Hyacinthaceae), *Triticum aestivum* (Poaceae), *Phalaenopsis* sp. (Orchidaceae), *Asparagus officinalis* (Asparagaceae) and *Hosta rectiflora* (Agavaceae). *A control TRAP assay on human cells was performed with primers TS21 × HUTPR. Marker—50 bp ladder. Arrows with numbers denote the length increments of telomerase products (6 or 7 bp periodicity).

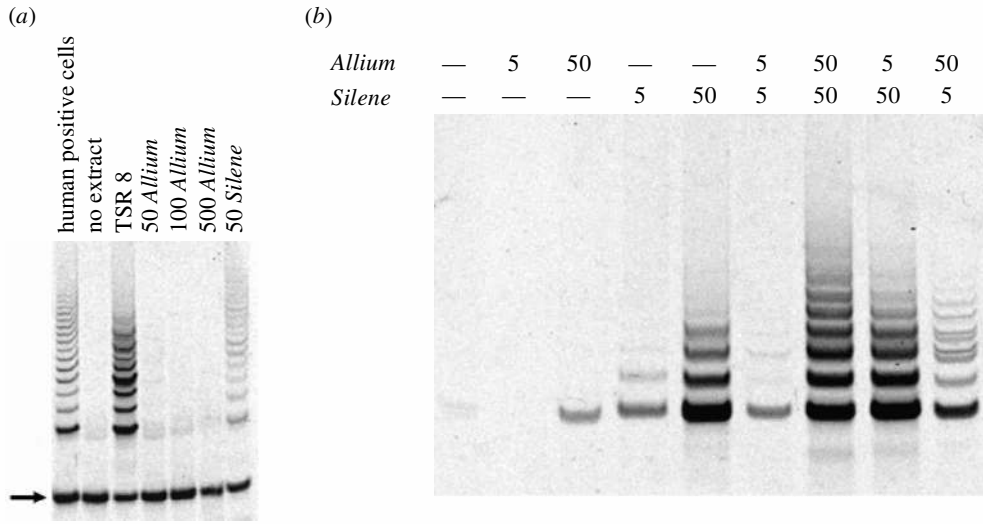

Figure 7. TRAP assay with *Allium cepa* (Alliaceae). (*a*) TRAP assay performed by TRAPeze detection kit—a different amount of total proteins of *A. cepa* was used, but the result remained negative; control lanes of TSR8 and human control cells indicate a typical pattern of TRAP products. The *Silene latifolia* lane shows a weak 7 bp ladder resulting from mismatches in the telomerase product (plant-type repeat) to reverse primer sequence (human-type repeat). The internal control product (arrow) reveals that there is no inhibition in the PCR step. (*b*) Admixture experiments (CAMV × TELPR)—*Allium* extracts do not decrease the activity of *Silene* telomerase. Combinations of both extracts were used as is indicated.

(f) *Sequence analysis of cloned telomerase products*

To determine the sequence of the telomerase product, the products of TRAP assays were cloned in reactions using CAMV × TELPR (i.e. the reverse primer is the *Arabidopsis*-type of sequence) or CAMV × HUTPR (the reverse primer is the human-type of sequence) in extracts of *B. glauca* (Asphodelaceae) and *O. virens* (Hyacinthaceae). Both species, in both types of TRAP assay, revealed the same kinds of products (see table 1). The most common unit was the human-type minisatellite $(TTAGGG)_n$, but other variants were also present (*Arabidopsis* > *Tetrahymena*). As with 'typical' plant telomerases (Fitzgerald *et al.* 2001), T- or G-slippage is the most common type of error, but in contrast with these telomerases, errors were not restricted to the first two synthesized repeats (table 1*b*).

4. DISCUSSION

(a) *Telomeres of Asparagales species*

The loss of *Arabidopsis*-type telomeric repeats occurred as a unique event during Asparagales evolution, and most plants examined in the modified clade appeared to lack these sequences (Adams *et al.* 2001). Such changes may

Table 1. Sequence analysis of TRAP products.
((*a*) Insert sequences cloned; the ATT at the beginning of the sequence denotes the 3′ end of the substrate primer; the reverse primer sequence was removed. Errors classified as T- or G-slippage (bold underlined letters) are additional T or G nucleotides incorporated into the reiterated unit. Other errors, mostly A/G substitution, are considered nucleotide misincorporations (letters in bold). (*b*) Summary statistics. Note the high number of errors reflecting the low accuracy of synthesis by telomerases. (*c*) Telomere motifs detected among telomerase products.)

(*a*) *Bulbine glauca*	*Ornithogalum virens*
CAMV × HUTPR (human-type reverse primer)	CAMV × HUTPR (human-type reverse primer)
ATTagggttag	ATT(agggttagggtt**T**)$_2$agggtta
ATT(agggtt)$_5$**T**a	ATTagggtt**T**(agggtt)$_7$a
ATT(agggtt**T**)$_2$agggtt (agggttagggtt**T**)$_3$(agggtt)$_3$a	ATTagggtt**T**a
ATT(agggtt)$_5$**T**a	ATT(agggtt)$_2$a
ATT(agggtt)$_6$**T**a	ATTagggTGttGgggttagggtta
ATTtGgggttagggtt**T**(agggtt)$_6$a	ATTagggtta
ATT(agggtt)$_5$a	ATT(agggtt)$_7$aAggtt(agggtt)$_{10}$a
ATTagggGtt(agggtt)$_6$a	ATT(agggtt)$_5$agggtt**T**a
ATT(agggtt**T**)$_2$(agggtt)$_4$agggGttagggtta	
ATTGgggttagggGtt**TT**agggtt**TT**(agggtt)$_5$a	
CAMV × TELPR (*Arabidopsis*-type reverse primer)	CAMV × TELPR (*Arabidopsis*-type reverse primer)
ATT(agggtt)$_6$a	ATT(agggtt)$_5$Ggggtt(agggtt)$_2$**T**(agggtt)$_6$a
ATTagggtt(agggtt**T**)$_2$(agggtt)$_3$a	ATTagggTGttagggtt**T**agggttaA
ATT(agggtt)$_2$**T**(agggtt)$_6$a	ATTagggtta
ATTagggttagggGtt(agggtt)$_2$a	ATT(agggtt**T**)$_3$a
ATTtGgggtta	ATT(agggtt)$_3$a
	ATT(agggGttagggtt)$_2$a

(*b*)	no. of sequenced base pairs	no. of repeats	no. of error repeats (% of all)	errors in first two repeats	T-slippage	G-slippage	mis-incorporation
Bulbine glauca	586	91	23 (25%)	7	16	4	3
Ornithogalum virens	463	72	17 (24%)	10	10	2	5

(*c*) telomere motif	*Bulbine glauca*	*Ornithogalum virens*
TTGGGG	6	4
TTAGGG	69	55
TTTAGGG	13	10
TTTTAGGG	2	0
TTTTGGGG	1	0

be rare and irreversible, or the loss and subsequent gain of typical telomeres may occur repeatedly as has been indicated in insects (Frydrychova & Marec 2002). The present work aimed to distinguish between these possibilities by examining additional Asparagales species using more sensitive methods. Recently, Weiss & Scherthan (2002) described the presence of the human-type repeat in *Aloe*, a genus shown to lack the *Arabidopsis*-type (Adams *et al.* 2000). We have extended the search for this and other variants of the minisatellite telomeric repeat in a range of selected Asparagales.

It is usual to consider that the $(TTTAGGG)_n$ repeat is typical of plant telomeric sequences because it has been detected in the model plants used commonly in molecular genetics (*Arabidopsis thaliana*, *Nicotiana tabacum*, *Oryza sativa* and *Zea mays*). However, even the telomeres of these plants do have variants of the consensus sequence (Ganal *et al.* 1991; Richards *et al.* 1993), and it is also known that plant telomerases, as well as telomerases from other organisms, are not 100% precise in substrate/template annealing and frequently generate repeats with variable numbers of T and G nucleotides (Tsujimoto *et al.* 1997, 1999; Fitzgerald *et al.* 2001). Consequently, the occurrence of variants of the minisatellite found typically at the telomeres of human, *Chlamydomonas* or *Bombyx* in plant telomere arrays is not surprising. However, our slot-blot analyses revealed a diversity of minisatellite repeats in Asparagales species that we did not expect. The clade identified by Adams *et al.* (2001), lacking *Arabidopsis*-type telomeric sequences that were distinct in that their predominant signal, was the human-type repeat $(TTAGGG)_n$, whereas those outside the group had the *Arabidopsis*-type $(TTTAGGG)_n$ as the most abundant minisatellite (with the exception of *A. preisii*). The slot-blot data also demonstrate that the Asparagales species with the predominant human-type repeat have other variants, notably the *Arabidopsis*, *Tetrahymena* and *Bombyx*-types. The abundance of these variants reflected the abundance of the human-type of repeat. In contrast, plant species with predominantly the *Arabidopsis*-type

repeat have highly inconsistent patterns of abundance of other repeat variants (especially the *Chlamydomonas* and human variants).

TRAP assays to all species within the clade identified in figure 1 (except *A. cepa*) gave a product with a 6 bp ladder pattern, and subsequent sequencing showed a common human-type, TTAGGG-iterated unit. In the *Phalaenopsis* hybrid (Orchidaceae) that is outside this clade, and in *T. aestivum* and other plants outside Asparagales examined previously, a 7 bp ladder pattern is generated. These data suggest that telomerase is functional across most Asparagales but preferentially synthesizes the human-variant of repeat in the clade identified in figure 1. The selected species from this clade examined by FISH using probes against the human- and *Arabidopsis*-type sequence variant were all heavily labelled with the human variant at the telomeres. The *Arabidopsis* variant of the minisatellite sequence was detected by FISH to *O. umbellatum*. The sequences occurred with the larger blocks of the human-variant and were sometimes interspersed within the human-type arrays. Likewise, in *O. virens* the *Tetrahymena* variant was localized to the telomere where it formed very faint dots at the same location as stronger signals derived from the human variant. Possibly, when telomere elongation changed to generating the human variant of the telomeric minisatellite, the new version of telomerase had lower fidelity, resulting in greater numbers of synthesis errors. This would give a higher proportion of repeat variants and explain the abundance of the variants in slot-blot assays. Perhaps the relatively high ratio of errors in the cloned TRAP products is further evidence of reduced telomerase fidelity. A mixed array of repeats might also help to maintain the functions of other telomere-associated proteins adapted to the *Arabidopsis*-sequence variant.

Hyacinthaceae share features of other Asparagales in the clade identified in figure 1, both in slot-blots and TRAP assays, and appear not to be distinct except in having a higher relative abundance of the *Arabidopsis*-type. This is also the case for *A. bellii* and *H. orientalis* in which our slot-blot data indicate substantial proportions of the *Arabidopsis*-type repeat, even though it remains in a slightly lower abundance than the human variant. All data show that the previously defined evolutionary point of the 'loss' of typical telomeres in Asparagales is more probably a point of substitution of the *Arabidopsis*-type telomeric sequence by another.

(b) *Telomerase of Asparagales species*

The TRAP assay involves an *in vitro* elongation of non-telomeric substrate primers, and the assay models *de novo* synthesis of telomeres. The most common errors of this type of reaction are the addition or omission of dT (or dG) residues, called T-slippage (or G-slippage) (Tsujimoto *et al.* 1997, 1999; Fitzgerald *et al.* 2001). This might generate the few *Arabidopsis*-type repeats by Asparagales telomerases in the TRAP assay (table 1). The positions of the *Arabidopsis*-type repeats differed between clones, including positions near to the substrate primer where mis-incorporations are often generated by telomerases (Fitzgerald *et al.* 2001), especially in *in vitro* assay using non-telomeric substrate primers. The minisatellite variant typical of *Tetrahymena* $(TTGGGG)_n$ and observed *in situ* and by slot-blot represents only a single nucleotide change from the human type of repeat (A to G).

Plant and protozoan telomerases studied previously favour longer substrate primers (21 nt) (Wang & Blackburn 1997; Fitzgerald *et al.* 2001) rather than shorter primers (18 nt, TS primer) commonly used in human TRAP assays. Telomerases from Asparagales species examined here could use the TS primer efficiently, revealing similarities to human telomerase (not shown). No RNA telomerase subunit has been characterized in plants so we can only speculate on the length of its template region. If the template region is short enough, the sequence generated by telomerase could be explained by a single point mutation (deletion). Another possibility is that the template motif is not read through completely in most reaction cycles, perhaps owing to structural changes in the catalytic subunit or the RNA subunit or loss of some factor ensuring precise positioning of substrate and template.

It remains unknown as to how the mutation became fixed and whether the new variant had any effect on all other aspects of telomere function. Possibly, plant proteins associated with telomeres are flexible in their activity; for example, the putative rice telomere-binding protein, RTBP1, is able to bind the vertebrate telomeric repeat sequence *in vitro* (Yu *et al.* 2000). Alternatively, there may be rapid adaptive coevolution of function as occurs with centromeric repeats and specific centromeric histone types (Malik & Henikoff 2001). These possibilities are not mutually exclusive. Perhaps plant telomeres are less vulnerable to changes in telomeric DNA or proteins because plant telomeres are lost more slowly after DNA replication than in animals (Riha *et al.* 1998). This would provide more time for the accumulation of adaptive changes.

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