Dynamic Evolution of Telomeric Sequences in the Green Algal Order Chlamydomonadales

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Abstract

Telomeres, which form the protective ends of eukaryotic chromosomes, are a ubiquitous and conserved structure of eukaryotic genomes but the basic structural unit of most telomeres, a repeated minisatellite motif with the general consensus sequence TnAmGo, may vary between eukaryotic groups. Previous studies on several species of green algae revealed that this group exhibits at least two types of telomeric sequences, a presumably ancestral type shared with land plants (Arabidopsis type, TTTAGGG) and conserved in, for example, Ostreococcus and Chlorella species, and a novel type (Chlamydomonas type, TTTTAGGG) identified in Chlamydomonas reinhardtii. We have employed several methodical approaches to survey the diversity of telomeric sequences in a phylogenetically wide array of green algal species, focusing on the order Chlamydomonadales. Our results support the view that the Arabidopsis-type telomeric sequence is ancestral for green algae and has been conserved in most lineages, including Mamiellophyceae, Chlorodendrophyceae, Trebouxiophyceae, Sphaeropleales, and most Chlamydomonadales. However, within the Chlamydomonadales, at least two independent evolutionary changes to the Chlamydomonas type occurred, specifically in a subgroup of the Reinhardtinia clade (including C. reinhardtii and Volvox carteri) and in the Chloromonadinia clade. Furthermore, a complex structure of telomeric repeats, including a mix of the ancestral Arabidopsis-type motifs and derived motifs identical to the human-type telomeric repeats (TTAGGG), was found in the chlamydomonadal clades Dunaliellinia and Stephanosphaeria. Our results indicate that telomere evolution in green algae, particularly in the order Chlamydomonadales, is far more dynamic and complex than thought before. General implications of our findings for the mode of telomere evolution are discussed.

Key words: TRAP, dot-blot hybridization, terminal restriction fragments (TRFs), 18S rDNA phylogeny, telomere evolution, green algae.

Introduction

Telomeres are regarded as highly conserved features of eukaryotic genomes. These nucleoprotein structures protect the ends of linear chromosomes and distinguish them from double strand breaks (McClintock 1938). They are typically maintained by a special reverse transcriptase, telomerase, which adds telomeric repeats at chromosome ends to elongate telomeres. Telomerase consists of two subunits, a protein subunit (TERT) and an RNA subunit (TR). Telomeric DNA is formed by tandem repeats of very few variants of minisatellite sequence motifs TnAmGo that are conserved in individual groups of organisms, for example, TTAGGG in vertebrates and fungi (designed here as the human type), TTAGGG in plants (Arabidopsis type), or TTAGG in insects (Richards and Ausubel 1988; Meyne et al. 1989; Okazaki et al. 1993). The type of minisatellite motif produced by telomerase is directed by a short region inside the TR subunit that serves as a template for telomeric DNA synthesis. Besides telomerase-based telomere maintenance, alternative mechanisms are known, for example, retrotransposons in telomeres of Drosophila or satellite repeats in Chironomus (for review, see Biessmann and Mason 2003). There are also groups of
organisms where more types of telomere sequence were described and in some of these, the evolutionary switch points between the types of telomere sequences were identified in their phylogeny (fig. 1). In plants, two lineages were described with an evolutionary change or loss of the telomeric sequence. One is within the Solanaceae family, where the loss of the typical telomeric sequence was observed in Solanaceae and Alliaceae. In green algae, the switch to the telomere variant TTTAGGG has been described in Solanaceae and Alliaceae. In green algae, the switch to the telomere its replacement with an unknown sequence has been observed in (TTTAGGG–TTAGGG). The loss of minisatellite telomeric sequence and this sequence is seen in the monocotyledonous plant order Asparagales TTTAGGG is present in most green algae and land plants. Variation in green algae and plants (Chloroplastida). The ancestral telomeric motif Superscripts indicate references: 1) this work, 2) Derelle et al. (2006),3 ) Higashiyama et al. (1995),4 ) Petracek et al. (1990),5 ) Suzuki (2004),6 ) Fuchs et al. (1995),7 ) Fuchs and Schubert (1996),8 ) Richards and Ausubel (1988), 9) Sykorova et al. (2003a), 10) Sykorova et al. (2003b), 11) Sykorova et al. (2006).

**Materials and Methods**

**Algal Material, Control of Biological Contaminations, and DNA Extraction**

The algal material used in this study originated from culture collections as specified in supplementary table S1 (Supplementary Material online). Algae were grown in the recommended liquid media BBM (Bold's Basal Medium) or MASM (Modified Artificial Seawater Medium) on agar plates supplemented with suitable media. Only axenic algal cultures or cultures with only prokaryotic contaminants were accepted for this study. The absence/presence of contaminants was monitored microscopically and using algal cultures grown on LB and BBM agar plates. Genomic DNA for polymerase chain reaction (PCR) amplification of 18S rDNA sequences was isolated by the standard protocol of Della-porta et al. (1983) or by the CTAB method (Saghai-Maroo...
et al. 1984). For a wide range of algal samples, obtaining a higher yield of purified genomic DNA proved difficult using classical methods such as CTAB, the Dellaporta protocol, or commercially available DNA purification kits (not shown). Isolation of DNA from the majority of algal samples was thus performed according to a protocol similar to that for preparation of high-molecular-weight samples in agarose plugs (Sykorova et al. 2006). The algae were harvested from liquid cultures or agar plates, spun down, and the pellet was lyophilized. The samples were then incubated overnight at 55 °C in 2 ml tubes with slow rotation in lysis buffer (60 mM Tris–Cl, pH 8.0, 100 mM ethylenediaminetetraacetic acid, 0.5% sodium dodecyl sulfate [SDS]) supplemented with proteinase K (Sigma–Aldrich) to a final concentration of 500 μg/ml. DNA was purified using phenol:chloroform extraction and precipitated. Finally, the samples were gently dissolved in TE buffer and RNase A was added to a final concentration of 200 μg/ml. This protocol does not allow quantification of DNA concentration by spectrophotometry, so sample concentrations were estimated from agarose gels by a comparison to a series of standards of known concentration.

Dot-Blot Hybridization, Restriction Digestion, and Southern Hybridization

Genomic DNA samples (1–5 μg) were digested by restriction endonucleases Rsal, Alul, Mbol, or TaqI (NEB) and run on a 0.9% agarose gel in TAE buffer. DNA fragments were alkali blotted onto Hybond-XL nylon membrane (Amersham) using a standard protocol (Sambrook et al. 1989) and hybridized with radioactively end-labeled oligonucleotide probes (ATSB, T3AG2-SB, T3G3-SB, supplementary table S2, Supplementary Material online) as described in Sykorova et al. (2003b) with minor modifications according to Neplechova et al. (2005). Briefly, membranes were hybridized at 55 °C for 16 h and washed at 55 °C under low stringency conditions (2 × saline sodium citrate (SSC), 0.1% SDS); the final wash containing 0.01–1 SSC, 0.1% SDS); the final wash of 500 μg/ml. This protocol does not allow quantification of DNA concentration by spectrophotometry, so sample concentrations were estimated from agarose gels by a comparison to a series of standards of known concentration.

Pulsed Field Gel Electrophoresis

Agarose plugs with high-molecular-mass DNA samples were prepared from lyophilized algal samples and BAL-31 and restriction enzyme digestion was performed as described in Sykorova et al. (2006). Briefly, agarose plugs with high-molecular-weight DNA (TEL168 Tetracystis excentrica, TEL157 Chloromonas perforata, TEL180 Neospongiococcus gelatinosum) were digested with BAL-31 nuclease (all NEB) for 15 or 45 min, and then by the restriction endonuclease Scal-HF (TEL157, TEL180) or HindIII (TEL168) (all from NEB). The DNA was then analyzed by pulse-field gel electrophoresis using the CHEF Mapper (BioRad) under the following conditions: 1% agarose (Biorad) gel in 0.5 × TBE buffer, 6 V/cm, pulses 0.5–26 s for 20 h at 13 °C. Gels were alkali blotted and hybridized subsequently with the telomeric probes ATSB and HUSB.

Telomere Repeat Amplification Protocol

Telomerase activity was investigated using a protocol originally developed for plant telomerases (Fitzgerald et al. 1996; Sykorova et al. 2003b) and applied with modifications to dinoflagellates (Fojtova et al. 2010). Briefly, total proteins were extracted from 35 to 100 mg of lyophilized algal samples ground in liquid nitrogen and after centrifugation at 17,000 × g for 15 min, the telomerase-enriched fraction was purified from the supernatant by precipitation with 10% polyethylene glycol (PEG) 8,000 and the pellet was dissolved in telomerase extraction buffer. Alternatively, the samples of crude protein extracts (without PEG precipitation) were used as specified in Results. The amount of total protein in extracts was determined using the Bradford method (Bradford 1976). A control telomerase extract was prepared from 7 days-old Arabidopsis thaliana (Col-0) seedlings. The telomere repeat amplification protocol (TRAP) assay was performed in two phases (Sykorova et al. 2003b). In the extension step, 10 pmol of a substrate primer was elongated at 26 °C for 45 min in a reaction mix with the telomerase-enriched extract containing 0.01–1 μg of total protein. After the extension step, samples were heat inactivated and then a mixture containing 10 pmol of a reverse primer (TELPR30-3A) and 2 units of Dynazyme II Polymerase (Finnzymes) was added and PCR amplification of the TRAP product was performed (Sykorova et al. 2003b). Alternatively, the substrate primers 47F (Fojtova et al. 2002), CAMV (Fajkus et al. 1998), or GG(21) (Fitzgerald et al. 1996) and reverse primers representing different telomeric variants (TELPR, TELPR30-3A, CHTPR, HUTC, HUTPR, T3AG2-C, T3G3-C, supplementary table S2, Supplementary Material online) were used. Products were analyzed by polyacrylamide gel electrophoresis (PAGE), stained by GelStar(R) Nucleic Acid Gel Stain (Lonza) and visualized on a LAS3000 (Fujifilm). TRAP products from chosen algal species were cloned into the pCRITTOPO vector (Invitrogen) according to the manufacturer’s recommendations and sequenced (Macrogen).
PCR and Sequencing of the 18S Ribosomal RNA Gene

To build a phylogeny for the strains investigated, we sequenced the 18S rDNA region of those strains (46 in total) for which it was not available from previous studies or for which the reported sequence seemed questionable (sub-standard). In a few cases, we relied on 18S rDNA sequences previously determined from presumably identical strains from a culture collection different from that used here for telomere investigations. To confirm that the previously sequenced strains were the same as our cultures, we checked the respective cultures by careful microscopic observation and in some cases, by sequencing the internal transcribed spacer (ITS) regions (data not shown). Genomic DNA isolated via the modified IRRI protocol (Collard et al. 2007) was used as a template for amplification of the 18S rDNA region using DyNazyme II Polymerase (Finnzymes) under PCR conditions described in Katana et al. (2001). For difficult DNA templates, we alternatively used Robust KAPA Polymerase (KAPA). The PCR products were gel purified using a Gel Extraction Kit (Qiagen) and subjected to direct sequencing using specific primers (Katana et al. 2001). PCR products that revealed unclear results from direct sequencing were cloned and sequenced (Macrogen). Newly determined sequences were deposited in GenBank with accession numbers JN903973–JN904007, JN968580–JN968586, JN968588, JN968589, and JN982286 (supplementary table S1, Supplementary Material online).

Phylogenetic Analyses

Initial analyses of newly obtained sequences and alignment construction were performed as described (Nemcova et al. 2011; Neustupa et al. 2011). A set of sequences for the final phylogenetic analysis was constructed to comprise representatives of all primary clades of Chlamydomonadales delineated by Nakada et al. (2008), other lineages of Chlorophyceae (Sphaeropleales, Oedogoniales, Chaetophorales, and Chaetopeltidales), and the remaining groups of the “core” chlorophytes (Trebouxiophyceae, Ulvophyceae, and Chlororodendrophyceae). 18S rDNA sequences from the trebouxiophytes C. variabilis NC64A and Coccomyxa sp. C-169 were extracted from the genomic scaffolds retrieved from the respective databases at the Joint Genome Institute (http://genome.jgi.doe.gov/ChlNC64A_1/ChlNC64A_1.home.html, scaffold_3, contig 410, 9,283–12,032 bp; and http://genome.jgi.doe.gov/Coc_C169_1/Coc_C169_1.home.html, scaffold_4, 2,976,163–2,978,999 bp). After removing unreliably aligned regions, the final alignment comprised 150 taxa and 1,647 positions (the alignment is available upon request). A maximum-likelihood (ML) tree was inferred using RAxML 7.2.8 available on the CIPRES Portal (Miller et al. 2010; http://www.phylo.org/sub_sections/portal/), employing a rapid bootstrapping algorithm followed by a thorough ML search on the original data set with the GTR + I substitution model (Stamatakis et al. 2008). A Bayesian inference was performed using the program MrBayes 3.1 (Huelsenbeck and Ronquist 2001). Two parallel Markov chain Monte Carlo runs were carried out for 3 million generations, each with one cold and three heated chains employing the GTR + I + COV evolutionary model. Trees were sampled every 100 generations. The initial 5,001 trees from each run were discarded as “burn-in” based on plotting log likelihood values. Posterior probabilities of tree bipartitions were calculated on the basis of the consensus of the remaining 50,000 trees.

Results

Sample Collection and Analyses

For this study, we tested a large range of algal strains from culture collections (see Materials and Methods) to sample broadly the phylogenetic diversity of the Chlorophyta and particularly the order Chlamydomonadales (class Chlorophyceae, fig. 1). All strains were examined for non-algal contaminants and 66 axenic algal strains or strains with bacterial contamination only (i.e., contamiantes lacking telomeres and telomerase) were used for telomere/telomerase analyses. In attempt to determine what forms the ends of chromosomes (i.e., what is synthesized by telomerase), we investigated 62 algal strains by the TRAP assay for telomerase activity and cloned the TRAP products from 39 strains. In a subset of these strains (34 in total), the occurrence of variant minisatellite telomeric repeats was investigated by Southern hybridization (terminal restriction fragment [TRF] analysis and/or dot-blot hybridization) using telomeric oligonucleotide probes (table 1).

A Phylogenetic Framework for the Telomere Sequence Evolution in Chlorophyceae

We used a standard and widely used marker for green algal phylogeny, the 18S ribosomal RNA gene, to infer the phylogenetic position of the algal strains investigated in this study for the type of telomeric sequences. The overall topology of the resulting tree (fig. 2; supplementary fig. S1, Supplementary Material online) is in accord with previous analyses and shows the basal split of the “core” chlorophytes into the four major lineages (Leliaert et al. 2012), Chlororodendrophyceae (represented by the genus Tetraselmis), Ulvophyceae, Trebouxiophyceae, and Chlorophyceae. The Chlorophyceae is further divided into two previously defined major clades (Turmel et al. 2008), OCC (Oedogoniales, Chaetophorales, Chaetopeltidiales), and CS (Chlamydomonadales and Sphaeropleales). As usual in 18S rDNA-based phylogenies, most of these major groups are poorly supported by bootstrap or posterior probability values. Within Chlamydomonadales (=Volvocales), all clades defined by Nakada et al. (2008) were reconstructed, generally with
Table 1
Hybridization Probes and Primers Used in This Study for Investigating Green Algal Minisatellite Telomeric Repeats

| Reference Organism Telomere Type | Minisatellite Repeat Unit | Hybridization Southern Blot | TRAP C-Rich Reverse Primer |
|----------------------------------|---------------------------|----------------------------|-----------------------------|
| Arabidopsis                      | TTAGGG                    | ATSB                       | TELPR                       |
| Chlamydomonas                    | TTTAGGG                   | CHSB                       | CHTPR                       |
| Human                            | TTAGGG                    | HUSB                       | HUTPR, HUTC                 |
| Chlorarachniophyte Nucleomorph   | TCTAGGG                   | CASB                       | n.a.                        |
| Arabidopsis-derived              |                           |                            |                             |
| Exchange TTAGGG                  |                           |                            |                             |
| TTCAGGG                          |                           | TTAGGG-SB                  | n.a.                        |
| TTTAGGC                          |                           | TTAGGG-SB                  | n.a.                        |
| Deletion TTAGGG                  |                           |                            |                             |
| T3AG2                            |                           | T3AG2-SB                   | T3AG2-C                     |
| T3G3                             |                           | T3G3-SB                    | T3G3-C                      |

**NOTE.**—SB, Southern Blot, n.a., not analyzed.

high statistical support. Strains for which the telomeric sequences have been determined previously, or in this study, represent four major groups—Chlororodendrophyceae, Trebouxiiophyceae, Sphaeropleales, and Chlamydomonadales. Their phylogenetic position fits the expectation based on previous taxonomic and phylogenetic studies, with the exception of several cocoid or capsal strains (attributed to the genera Chlorococcum, Tetracystis, and Neospungiococcum) that have not been previously studied by molecular means and that are very difficult to classify based on morphological features only; all these strains diverge within clades previously defined for the order Chlamydomonadales. As previously shown, some traditional genera of chloromonadalean algae (Chlamydomonas, Chlorococcum, and Tetracystis) are polyphyletic in the 18S rDNA tree. *Borodinellopsis texensis* SAG 17.95 belongs to the Chlamydomonadales, but does not fall into any of the major chloromonadalean clades. It may be sister to the Moewusinia clade, but the statistical support for this position is inconclusive, hence it potentially represents a new hitherto unrecognized chlamydomonadalean lineage.

**Dot-Blot Hybridization Screening of Chlamydomonadales and Sphaeropleales**

We investigated 29 strains from Chlamydomonadales and two species from Sphaeropleales for the occurrence of variant minisatellite telomeric repeats by dot-blot hybridization (fig. 3 and table 1). Sonicated human genomic DNA and genomic DNA of the model plants *Nicotiana tabacum* (Solanaceae), *Arabidopsis*-type telomere and *Ipheion uniflorum* (Asparagales, human-type telomere) were used as controls (fig. 1). Dot-blot hybridization results showed the presence of several types of telomeric minisatellite repeats in genomes of Chlamydomonadales and Sphaeropleales and an abundance of the *Arabidopsis*-type variant of telomeric sequence (ATSB), except in species from the clade Dunaliellina (fig. 3). The *Chlamydomonas*-type variant (CHSB) revealed strong signals comparable to the *Arabidopsis*-type variant in all samples from the clade Chloromonadinia suggesting similar telomeric features in the species of this group. In several species, the abundance of the *Arabidopsis*-type variant was reflected in the abundance of the repeats that are most closely related to it; these variants occur in the following order of abundance: *Arabidopsis* type > *Chlamydomonas* type > T3AG2 type > human type. A human-type variant probe hybridized strongly to genomic DNA of the samples *C. perforata* (TEL157) and *Chlorococcum sphacosum* (TEL106) (*Stephanosphaeria*) and the sample *Planktosphaeria gelatinosa* (TEL83) (Sphaeropleales).

**TRF Analysis**

The typical telomere lacks recognition sites for restriction enzymes due to its minisatellite repeat sequence organized in tandem. Thus, most restriction enzymes will cut DNA at the closest site in the subtelomere, splitting a TRF from the rest of the chromosome. Digestion of genomic DNA, separation of fragments on agarose gel, and Southern hybridization with a telomeric probe enable estimation of the length of algal telomeres as TRF. The dot-blot results suggested a large abundance of the *Arabidopsis*- and the *Chlamydomonas*-type variants in species from the Chloromonadinia clade and of the human-type variant in the genomes of *C. sphacosum* (TEL106) and *C. perforata* (TEL157). To characterize the distribution and occurrence of potential telomere-like minisatellites, we investigated species from Chloromonadina, Reinhardtinia, Stephanosphaeria, and Dunaliellina clades for the presence of several types of minisatellite repeat sequences using an analysis of the TRF length (fig. 4; supplementary fig. S2, Supplementary Material online). Southern hybridization results showed a smear of TRF fragments between 0.3 and 2 kb in *Chloromonas actinochloris* (TEL151) and *Chloromonas reticulata* (TEL159) (Chloromonadinia) and revealed colocalization of the *Arabidopsis*-type and the *Chlamydomonas*-type variants in TRF of both genomes (fig. 4; supplementary fig. S2, Supplementary Material online). The TRFs from *C. sphacosum* (TEL106)
include technical problems in group (3) samples, we also used and/or by an inadequate reverse primer sequence. To exclude relatively long telomere-associated sequences. A crude telomerase extract in the TRAP assay (supplementary fig. S3A, Supplementary Material online) and alternative substrate primers (47F, GG(21), CAMV) were used to meet possible different substrate sequence requirements for the telomerase action. However, omitting the PEG purification step or alteration of the substrate primer did not increase the telomerase activity in samples (not shown). When we used reverse primers with variant minisatellite telomeric sequences—CHTPR (Chlamydomonas type, not shown), HUTPR, HUTC (human type), T3G2-C (TTTAGG type), or T3G3-C type (TTTGGG type), ladders of TRAP products with a six-nucleotide periodicity were observed for reactions containing the reverse primers HUTC, HUTPR (not shown), and T3G2-C in samples TEL106, 175, 173, 170 (fig. 5E–G).

Fig. 2.—A maximum-likelihood phylogenetic tree based on 18S ribosomal RNA sequences from “core” chlorophytes with a denser sampling of the order Chlamydomonadales. Only the part of the tree corresponding to Chlamydomonadales is shown here; the full version of the tree is available as supplementary fig. S1 (Supplementary Material online). ML bootstrap percentage values/Bayesian posterior probabilities values higher than 80%/0.95 are shown above branches. Accession numbers of sequences from GenBank are given before the species name, and newly determined sequences are highlighted in bold. The “true” telomeric types are indicated in color (Arabidopsis type, green; Chlamydomonas type, magenta; human type, blue; not
Cloning the TRAP Products Reveals the “True” Telomere-Type Sequence Synthesized by Telomerase

Telomerase synthesizes telomeric repeats using a short template region of its RNA subunit (TR). The sequence type of the telomeric repeat then can be estimated from cloned and sequenced TRAP products. To confirm the observed TRAP pattern and to determine the sequence of the telomerase product, we sequenced TRAP products of 36 algal strains from the chlamydomonadalean clades Golenkinia, Radicar-teria, Oogamochlamydinia, Reinhardtinia, Chloromonadi-nia, Monadinia, and Moewusinia, and of three species from the sister group Sphaeropleales (supplementary fig. S1, Supplementary Material online). For cloning, we chose reactions that expressed high enzyme processivity starting with the combination TS21/C2/TELPR30-3A (i.e., the reverse primer has the Arabidopsis type of sequence). To avoid artifacts, the TRAP products from another combination of a substrate primer (CAMV, 47F, GG(21)) and an alternative Arabidopsis-type reverse primer TELPR (differs from TELPR30-3A at the 3’-end) were cloned. Sequencing results confirmed the Arabidopsis-type (22 strains, table 2) and the Chlamydomonas-type variant (12 strains, table 2) synthesized by telomerase as deduced from the periodicity of TRAP products resolved on polyacrylamide gels (see above). The sequence analysis of TRAP products revealed different fidelity of telomerases independently of their phylogeny position, with T-slippage errors most common (table 2, examples of a detailed analysis are shown in supplementary fig. S4, Supplementary Material online). These errors are probably caused by inaccurate annealing of the telomere DNA strand to the anchor site of the TR template region that results in synthesis of a telomeric repeat shorter or longer by one “T” residue; however, the “errors” are incorporated randomly. Unusually high error rates were observed in products synthesized by telomerases of H. inaequalis (TEL80) and Chloromonas rubrifilum (TEL161), resulting in a mix of Arabidopsis- and Chlamydomonas-type telomeric repeats (table 2). The TRAP products of H. inaequalis contained predominantly the Arabidopsis-type variant and the products from C. rubrifilum contained a similar number of the Arabidopsis-type and the Chlamydomonas-type repeats in sequences of all clones. Considering the phylogenetic relationship between algal species, we also cloned TRAP rehybridized with the control 18S rDNA probe. Control samples were from the green alga Chlorella vulgaris (Trebouxiophyceae), sonicated human DNA, and from plants with the typical Arabidopsis-type telomere sequence (Nicotiana tabacum, Solanaceae), and the human-type telomere sequence (Iphion uniflorum, Asparagales). Accession numbers for all algal strains (identified by the TEL number) are listed in supplementary table S1 (Supplementary Material online). It should be noted that DNA concentration had to be estimated from agarose gels due to the purification method used, and the loaded amount of genomic DNAs might vary among samples.

**Fig. 3.**—Genomic DNA dot-blot hybridization data and their correlation with the green algal clades in the 18S rDNA-based phylogeny of the group (fig. 2; supplementary fig. S1, Supplementary Material online). Genomic DNA was hybridized with different types of minisatellite sequences representing typical telomeric types—the Arabidopsis type (ATSB), the Chlamydomonas type (CHSB), the human type (HUSB), and their variants T3A2-SB, T3G3-SB, (see table 1, supplementary table S2, Supplementary Material online). All membranes were

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**Table 1.** —Genomic DNA dot-blot hybridization data and their correlation with the green algal clades in the 18S rDNA-based phylogeny of the group (fig. 2; supplementary fig. S1, Supplementary Material online). Genomic DNA was hybridized with different types of minisatellite sequences representing typical telomeric types—the Arabidopsis type (ATSB), the Chlamydomonas type (CHSB), the human type (HUSB), and their variants T3A2-SB, T3G3-SB, (see table 1, supplementary table S2, Supplementary Material online). All membranes were

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**Table 2.** —Genomic DNA dot-blot hybridization data and their correlation with the green algal clades in the 18S rDNA-based phylogeny of the group (fig. 2; supplementary fig. S1, Supplementary Material online). Genomic DNA was hybridized with different types of minisatellite sequences representing typical telomeric types—the Arabidopsis type (ATSB), the Chlamydomonas type (CHSB), the human type (HUSB), and their variants T3A2-SB, T3G3-SB, (see table 1, supplementary table S2, Supplementary Material online). All membranes were
products from *Heterochlamydomonas rugosa* (TEL229), which revealed results similar to *H. inaequalis* (TEL80) (table 2). In conclusion, the major *Chlamydomonas*-type variant was identified in all species investigated from the clade Chloromonadinia, making this clade uniform with respect to telomeres. Surprisingly, only two *C. reinhardtii* accessions (TEL224, 225) from the Reinhardtinia clade revealed synthesis of the *Chlamydomonas*-type telomeric sequence, whereas the telomerase of the remaining accession of *C. reinhardtii* (TEL223) synthesized predominantly *Arabidopsis*-type repeats (table 2; supplementary fig. S3B, Supplementary Material online).

Since *Tetracystis intermedia* (TEL170), *T. pulchra* (TEL173) and *Tetracystis texensis* (TEL175) from the Dunaliellinia clade did not show a clear TRAP pattern using the primer combinations mentioned above, TRAP products were cloned from reactions utilizing the primer set CAMV/C2T3AG2-C (fig. 5E). The TRAP products of *T. excentrica* (TEL168) were cloned using primer set GG(21)/C2TELPR30-3A (supplementary fig. S4, Supplementary Material online). All four species investigated from the Dunaliellinia clade (TEL168, 170, 173, 175) revealed telomerases producing human-type repeats with an error rate similar to telomerases from algal groups with a “noncanonical” telomeric sequence (table 2). The...
### Table 2
Results of TRAP Assays

| Class/Order Clade          | TEL       | Species              | TRAP Products (type) / CLONED | Number of Clones | Number of Repeats (T2) hu | T-Slippage (T(n)AGGG) (%) of all | Variant Repeats (G-Slippage, Mis-Incorporation) |
|----------------------------|-----------|----------------------|------------------------------|-----------------|--------------------------|----------------------------------|---------------------------------|
| Chlorodendrophyceae        | 211       | Tetraselmis chui     | 7-nt (ttttaggg) n.a.         | 3               | 55 1 53 0               | 1.8 2                             | 0 0.0 0 0 0 0 0 0 0               |
| Trebouxiophyceae           | 101       | Chlorella vulgaris   | TTTAGGG 10 35 0             | 30 3 1          | 11.4 0 3                |
| Chlorophyceae/Sphaeropleales | 83       | Planktosphaera gelatinosa | TTTAGGG 5 27 0 77 0 0 0 0.0 0 |
|                           | 87        | Coelastrella vacuolata | TTTAGGG 2 17 0 17 0 0 0 0 0 0 0 |
|                           | 91        | Mychonastes homosphaera | TTTAGGG 3 33 0 33 0 0 0 0 0 0 0 |
|                           | 138       | Pseudomuniella aurantiana | 7-nt (ttttaggg) n.a.        |
|                           | 188       | Bacteoacoccus cohaerens | 7-nt (ttttaggg) n.a.        |
| Chlorophyceae/Chlamydomonadaceae | 178     | Golenkinia longispicula | TTTAGGG 7 55 1 53 0 0 1.8 2 0 |
|                           | 177       | Cartenia lunensis    | TTTAGGG 3 80 0 74 6 0      | 7.5 2 0          |
| Tatrensinia               | 150       | Chlamydomonas sp     | 7-nt (ttttaggg) n.a.        | 3               | 55 1 53 0               | 1.8 2                             | 0 0.0 0 0 0 0 0 0 0               |
| Oogamochlamydiina         | 115       | Lobochlamys segnis   | TTTAGGG 5 65 5 53 5 1       | 16.9 3 2        |
|                           | 223       | Chlamydomonas ‘reinhardtii’ | TTTAGGG 4 86 1 57 25 3 33.7 1 0 |
| Reinhardtinia             | 80        | Heterochlamydomonas inaequalis | TTTAGGG 8 230 0 153 63 6 30.0 8 10 |
|                           | 166       | Tetracystis diplobionticoidea | TTTAGGG 2 27 1 25 0 1      | 7.4 0 0          |
|                           | 229       | Heterochlamydomonas rugosa | TTTAGGG 4 90 0 62 26 0      | 32.2 4 2        |
|                           | 114       | Chlamydomonas asymmetrica | TTTAGGG 2 39 3 35 1 0      | 10.2 0 0        |
|                           | 179       | Heterotetracystis akinetos | TTTAGGG 4 86 1 77 7 0       | 9.3 1 1        |
|                           | 155       | Chlamoromonas oogama | TTTAGGG 3 66 1 56 8 0       | 13.6 1 1        |
|                           | 219       | Chlamydomonas debaryana | 7-nt (ttttaggg) n.a.        | 5               | 71 1 64 6 0             | 9.8 4 1                            |
|                           | 220       | Chlamydomonas debaryana | TTTAGGG 8-nt (ttttaggg) n.a. | 3               | 55 1 53 0               | 1.8 2                             | 0 0.0 0 0 0 0 0 0 0               |
|                           | 224       | Chlamydomonas reinhardtii | 8-nt (ttttaggg) n.a.        | 4               | 56 0 0 56 0 0 0.0 0 0 0 |
|                           | 225       | Chlamydomonas ‘reinhardtii’ | +/− TTTAGGG 4 56 0 0 56 0 0.0 0 0 0 |
| Chloromonadina            | 4         | Chlamoromona sp.     | TTTAGGG 7 99 0 7 89 2 9.0 0 1 |
|                           | 104       | Chlamoromona hydra   | TTTAGGG 4 63 0 10 47 5 23.8 0 0 |
|                           | 105       | Chlamydomonas meslini | TTTAGGG 4 77 0 10 64 0 12.9 1 2 |
|                           | 151       | Chlamoromonas acutiores | TTTAGGG 5 104 1 15 88 0 14.4 1 2 |
|                           | 152       | Chlamoromonas acutiores | TTTAGGG 5 47 0 12 35 0 25.5 0 1 |
|                           | 153       | Chlamoromona asteroides | +/− 8-nt (ttttaggg) n.a. 4 56 0 0 56 0 0.0 0 0 0 |
|                           | 154       | Chlamoromona carnoenses | TTTAGGG 5 72 1 10 57 3 18.0 1 1 |
|                           | 156       | Chlamoromona palmeloides | TTTAGGG 3 58 2 8 48 0 13.8 5 0 |
|                           | 158       | Chlamoromona radiata  | TTTAGGG 3 48 0 6 40 2 16.7 1 0 |
|                           | 159       | Chlamoromona reductula | 8-nt (ttttaggg) n.a.        | 5               | 100 0 3 85 10 13.9 1 2 |
|                           | 160       | Chlamoromona roseae  | TTTAGGG 5 171 0 82 85 2 49.1 5 2 |
|                           | 161       | Chlamoromona rubrifilum | TTTAGGG 5 66 1 56 8 0       | 13.6 1 1        |
|                           | 217       | Chlamydomonas macrostellata | Neg. n.a.                  |
| Monadina                  | 162       | Chlamoromona subdivisa | 7-nt (ttttaggg) n.a.        |
|                           | 172       | Tetracystis pampae   | 7-nt (ttttaggg) n.a.        |
|                           | 176       | Tetracystis vinateri | 7-nt (ttttaggg) n.a.        |
|                           | 226       | Chlamydomonas monadina | TTTAGGG 3 65 1 60 4 0       | 7.7 0 0        |

**Note:** G-Slippage and Mis-Incorporation are not shown in the table.
| Class/Order Clade | Species               | TEL Ladder* (type) / CLONED | TRAP Products | Number of Clones | Number of Repeats | T-Splippageb (T(n)AGGG) Variant Repeats (% of all) | G-Slippage | Mis-Incorporation |
|------------------|-----------------------|-----------------------------|---------------|------------------|------------------|-------------------------------------------------|------------|------------------|
| Borodinelopsis   | *Borodinelopsis texensis* | TTIAGGG 4 | 76 | 27 | 40 | 7 | 1 | 46.0 | 0 | 1 |
| Moewusinia       | Tetracystis aeria | TTIAGGG 3 | 50 | 0 | 50 | 0 | 0 | 0.0 | 0 | 0 |
|                  | Tetracystis aggregata | TTIAGGG 7 | 109 | 0 | 106 | 3 | 0 | 2.7 | 1 | 0 |
|                  | *Tetracystis aplanospora* | 7-nt (tttaggg) | n.a. | | | | | | |
|                  | *Tetracystis dissociata* | TTIAGGG 6 | 101 | 0 | 99 | 0 | 0 | 0.0 | 3 | 0 |
|                  | Tetracystis isobilateralis | 7-nt (tttaggg) | n.a. | | | | | | |
|                  | Tetracystis illinoisensis | 7-nt (tttaggg) | n.a. | | | | | | |
|                  | Chlamydomonas moewusii | TTIAGGG 4 | 35 | 0 | 35 | 0 | 0 | 0.0 | 0 | 0 |
|                  | Chlamydomonas moewusii | 7-nt (tttaggg) | n.a. | | | | | | |
|                  | Chlamydomonas raudensis | TTIAGGG 5 | 69 | 0 | 66 | 3 | 0 | 4.3 | 0 | 0 |
|                  | Chlamydomonas noctigama | TTIAGGG 4 | 32 | 0 | 32 | 0 | 0 | 0.0 | 0 | 0 |
|                  | Chlamydomonas noctigama | TTIAGGG 4 | 54 | 2 | 50 | 2 | 0 | 7.4 | 0 | 0 |
|                  | Chlorococcum hypnoxuporum | 7-nt (tttaggg) | n.a. | | | | | | |
| Dunaliellinia    | *Tetracystis excentrica* | TTIAGGG 4 | 114 | 74 | 30 | 4 | 1 | 36.8 | 4 | 2 |
|                  | Tetracystis intermedia | +/- TTAGGG 4 | 38 | 21 | 11 | 5 | 0 | 42.1 | 0 | 1 |
|                  | Tetracystis pulchra | +/- TTAGGG 1 | 18 | 16 | 0 | 2 | 0 | 11.1 | 3 | 2 |
|                  | Tetracystis texensis | TTAGGG 3 | 75 | 59 | 14 | 0 | 0 | 17.5 | 4 | 0 |
| Stephanosphaeria | *Chlorococcum sphacosum* | +/- | n.a. | | | | | | |
|                  | *Chlorococcum minutum* | +/- | n.a. | | | | | | |
|                  | *Chloromonas perforata* | +/- TTAGGG 4 | 52 | 4 | 45 | 2 | 0 | 11.5 | 1 | 1 |
|                  | Neospongiococcum gelatinosum | +/- | n.a. | | | | | | |
|                  | Chlorococcum ellipsoideum | +/- | n.a. | | | | | | |

**Note:**—nt, nucleotide, n.a., not analyzed. Major telomere type is underlined.  
*+/− low telomerase activity in TRAP, neg. no ladder, negative.  
b Various number of T residues categorizes repeats to human type (hu), Arabidopsis type (at), and Chlamydomonas type (ch).  
c TRAP products were cloned using the primer sets CAMV × TELPR (5 clones) and CAMV × HUTPR (5 clones), variant repeats were identified only in the TRAP products resulting from CAMV × HUTPR primer combination.
cloning of TRAP products from the Stephanosphaeria species showed synthesis of the Arabidopsis-type variant in C. sphacosum (TEL157), but the sequenced products from other species showed non-telomeric sequences resulting probably from PCR artifacts (not shown).

**Discussion**

For the purpose of interpreting the data reported in this study, it is important to distinguish between the telomeric sequences synthesized by telomerase, which form only the ends of chromosomes and are designated as “true” telomeric type in figures 1 and 7, and the telomere DNA, which consist of these distal sequences and of telomeric sequence variants present in more proximal telomeric regions constituting the telomere structure. Our data suggest that a change of the “true” telomere type happened independently in at least three green algal groups when the ancestral Arabidopsis type of telomere sequence (TTTAGGG) was repeatedly replaced by the Chlamydomonas type.
(TTTAGGG) or by the human-type (TTAGGG) variant (fig. 7). Mapping the distribution of the telomere types onto a green algal phylogeny inferred from 18S rDNA sequences showed that the Arabidopsis type of telomeric sequence is ancestral not only for the whole Chlorophyceae group (figs. 1 and 2; supplementary fig. S1, Supplementary Material online) but also for the order Chlamydomonadales, whereas the Chlamydomonas type of telomeric sequence is present in only some chlamydomonadalean clades (Chloromonadinia and Reinhardtinia; fig. 3 and table 2). The human-type variant occurs in terminal positions in genomes of several species from the clades Dunaliellinia and Stephanosphaeria (fig. 6). These derived telomeric types are discussed separately in more detail below.

At Least Two Separate Origins of the Chlamydomonas Type of Telomeres in the Chlamydomonadales

Initial investigations of the telomeric sequences in the standard strain NO+ and a field isolate of C. reinhardtii (Petracek et al. 1990) revealed a novel telomeric sequence type, which has been confirmed by sequencing the whole genome of another C. reinhardtii accession (CC-503 cw92 mt+, GenBank accession number ABCN01000000; Merchant et al. 2007). To reevaluate the telomeric data for C. reinhardtii, we investigated three more accessions of this species from the culture collection. Furthermore, we tested several additional strains from the Reinhardtinia clade to determine the distribution of the Chlamydomonas-type telomeric sequences. Surprisingly, this derived telomeric type could be identified in only two C. reinhardtii accessions (table 2; supplementary table S1, Supplementary Material online), and based on the genome sequence reported by Prochnik et al. (2010), it is also present in a related alga Volvox carteri (GenBank accession number ACJH00000000). Two of the C. reinhardtii strains studied (TEL 224 and TEL 225) belong to standard strains of this species (Proschold et al. 2005). However, the C. reinhardtii accession (TEL223) that possesses the Arabidopsis-type variant in telomeres and a telomerase producing a high number of errors in the repeats synthesized in the in vitro testing system (table 2) has been apparently misidentified, as it occupies a phylogenetically remote position in the Oogamochlamydinia clade. This strain, a minus (−) mating type originally labeled as Chlamydomonas smithii, was found, unlike the plus (+) mating type strain of C. smithii, not to be able to interbreed (to mate) with standard C. reinhardtii strains (Harris 1989). It differs from standard C. reinhardtii also in morphology. A very different ITS sequence rather similar to C. culleus and homothallic zygote formation in clonal cultures, were also reported (Coleman and Mai 1997) and support our findings.
Complex Telomere Evolution in the Dunaliellinia and the Stephanosphaeria

Special attention was paid to telomeres of two chlamydomonadalean clades that showed unclear dot-blot and TRAP results, the Dunaliellinia and the Stephanosphaeria. We investigated the possibility that the telomeric sequence in this clade has changed to a minisatellite type derived from the ancestral TTAGGG by a single nucleotide change, for example, to the TCTAGGG variant found in the chlorarachniophyte nucleomorph (Gilson and McFadden 1995), or minisatellite types TTCAGGG and TTTAGGC. However, none of these variants seem to form telomeres in the Dunaliellinia or Stephanosphaeria. Finally, cloning of the TRAP products suggested telomerase synthesizes the human-type telomeric repeat in four species representing a distinct subclade within the Dunaliellinia (figs. 2 and 7). The number of telomerase errors is very high with the majority of the Arabidopsis-type “errors” synthesized (table 2). Interestingly, the telomerase inaccuracy of Dunaliellinia species is similar to those of other groups with a noncanonical telomere type (see above). A Blast search of whole genome shotgun data from an ongoing Dunaliella salina genome sequencing project available in Trace Archive (http://www.ncbi.nlm.nih.gov/Traces/) revealed the presence of short stretches of telomere-like sequences and also of long tracts consisting of the Arabidopsis-type variant or of a mixture of the human-type and the Arabidopsis-type variants (not shown). The terminal position of these sequences cannot be assessed without genome/scaffold assembly, but they leave open the possibility that the human-type variant contributes to telomeres also in other species from this clade.

The Stephanosphaeria clade displayed divergent results showing the Arabidopsis type of telomeric repeats synthesized by telomerase of C. perforata (TEL157, table 2) and an abundance of the human-type minisatellite in the genome of TEL157 and in the other two species C. sphacosum (TEL106, fig. 3) and N. gelatinosum (TEL180, fig. 6). The terminal position of the Arabidopsis- and the human-type minisatellites in chromosomes of TEL157 and TEL180 was also confirmed by BAL-31 digestion, suggesting the co-occurrence of both telomeric variants in mixed arrays (fig. 6). These genomic features are shared by all Stephanosphaeria species investigated as well as by TEL168 from the Dunaliellinia clade (see above). Due to a low telomerase activity/processivity resulting in an unclear TRAP pattern (table 2 and fig. 5E–G) and a failure in cloning of the TRAP products (except TEL157), the question about the steps in telomere evolution of the Stephanosphaeria remains open.

Nevertheless, according to the 18S rDNA phylogeny, Dunaliellinia and Stephanosphaeria, together with several
other chlamydomonal clades, constitute a higher order clade dubbed Caudivolvoxa (Nakada et al. 2008; fig. 2). It is, therefore, possible that the telomere synthesis started to depart from the ancestral state early in the Caudivolvoxa evolution, before the split of the Dunaliellinia and Stephanosphaeria lineages. Obviously, a much broader survey of telomere sequences of Caudivolvoxa coupled with a more precise phylogenetic scheme for this group are required to shed more light on this issue.

Implications for the General Mode of Telomere Evolution

The data about telomeric types currently available have come mostly from common model organisms. A majority of eukaryotic groups have telomeres formed by minisatellite repeats synthesized by telomerase. By now, several groups with more than one minisatellite telomeric type have been described, for example, ciliates (TTGGGG in Tetrahymena and TTTTGGGG in Oxytricha), plants (TTAGGG as the major type, TTAGGG in several families of Asparagales), or fungi (TTAGGG as a major type, different repeats in yeasts; for details, see the Telomerase database telomerase.asu.edu/sequences.html; Podlevsky et al. 2008). A specific group of organisms with two telomeric types is represented by chlorarachniophytes and cryptomonads with both telomeres of the nuclear and the nucleomorph chromosomes (Gilson and McFadden 1995; Zauner et al. 2000). In this study, we have substantially expanded the diversity of telomeric sequences known for green algae (fig. 7), showing evidence for two evolutionarily independent transitions from the ancestral TTTAGGG (Arabidopsis type) motif to the derived TTTTGGGG (Chlamydomonas type) motif in a Reinhardtinia subclade and in the Chloromonadina clade, and for the emergence of the TTAGGG (human type) motif in Dunaliellinia and Stephanosphaeria clades. It should be mentioned that there is also an increasing number of reports about species or groups of organisms with an “unknown” telomeric type or at least with the lack of typical telomeric types. Some of these cases have later been reevaluated, for example, in insects, where the originally described multiple loss of a typical telomeric sequence (TTAGG) in some Coleoptera (Frydrychova and Marec 2002; Frydrychova et al. 2004) was explained for the Tenebrionoidea superfamily by a switch to the novel telomere type TCAGG first identified in Tribolium (Mravinac et al. 2011).

The sequence of the telomeric repeat synthesized by telomerase is determined by the sequence in the template region of its RNA subunit (TR). The simplest hypothetical event resulting in a change to a different telomeric minisatellite repeat would be a mutation in this template region. Such a hypothesis is supported by our observation of recently diverged telomere variants in green algae (TTTTAGGG, Chlamydomonas type; TTAGGG, human type), which differ very little from the ancestral telomere-type variant (TTTAGGG).

This divergence likely arises from a single nucleotide change (insertion or deletion) in the TR template region. This mode of TR evolution is the most common process in the evolution of telomere motifs, as has been suggested for the evolution of land plant and insect telomerases. Another possible cause of variant repeat synthesis could be the change in telomerase template usage caused by mutation in the catalytic telomerase subunit TERT (Sykorova, Leitch, et al. 2006). The change of the minisatellite type forming the telomeres probably influences other aspects of telomere function, for example, the DNA-binding activity of telomere-associated proteins. However, a high flexibility of plant telomeric proteins has been described in land plants, where 1) the Arabidopsis-type variant of telomeric motif was changed to the human type in several families of Asparagales and proteins binding both telomeric motifs were found (Rotkova et al. 2004, 2007); 2) the human type was lost in the genus Allium of the Alliaceae family and similar proteins binding both telomere variants were detected in vitro (Fajkus et al. 2005); 3) the typical Arabidopsis-type telomeres were lost in three plant genera of the Solanaceae family and the DNA-binding activity of Cestrum proteins to the ancestral Arabidopsis-type variant was reported (Peska et al. 2008). The telomere-binding properties may also be a subject of rapid adaptive coevolution of function as occurs with another essential structure, centromeric repeats and specific centromeric histone types (Malik and Henikoff 2001). How a mutation in a telomere sequence synthesized by telomerase becomes fixed is unknown, but we would presume that the inner parts of the telomere formed by ancestral sequences (still binding telomere-associated proteins) could form a buffer zone that may help to deal with the new situation. Also the observed high number of errors in telomere synthesis, especially in groups with “recently” changed telomere types, could participate and make the passage to new telomere type easier.

Supplementary Material

Supplementary tables S1 and S2 and figure S1–S4 are available at Genome Biology and Evolution online (http://www.gbe.oxfordjournals.org/).

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