J Mol Evol (1998) 47:544–550

JOURNAL OF MOLECULAR EVOLUTION

Actin Phylogeny Identifies *Mesostigma viride* as a Flagellate Ancestor of the Land Plants

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Received: 10 July 1997 / Accepted: 9 April 1998

Abstract. Green algae and land plants trace their evolutionary history to a unique common ancestor. This ''green lineage'' is phylogenetically subdivided into two distinct assemblages, the Chlorophyta and the Streptophyta. The Chlorophyta includes the Chlorophyceae, Trebouxiophyceae, Ulvophyceae, and Prasinopohyceae, whereas the Streptophyta includes the Charophyceae plus the bryophytes, ferns, and all other multicellular land plants (Embryophyta). The Prasinophyceae is believed to contain the earliest divergences within the green lineage. Phylogenetic analyses using rDNA sequences identify the prasinophytes as a paraphyletic taxon that diverges at the base of the Chlorophyta. rDNA analyses, however, provide ambiguous results regarding the identity of the flagellate ancestor of the Streptophyta. We have sequenced the actin-encoding cDNAs from *Scherffelia dubia* (Prasinophyceae), *Coleochaete scutata, Spirogyra* sp. (Charophyceae), and the single-copy actin gene from *Mesostigma viride* (Prasinophyceae). Phylogenetic analyses show *Mesostigma* to be the earliest divergence within the Streptophyta and provide direct evidence for a scaly, biflagellate, unicellular ancestor for this lineage. This result is supported by the existence of two conserved actin-coding region introns (positions 20-3, 152-1), and one intron in the 5′-untranslated region of the actin gene shared by *Mesostigma* and the embryophytes.

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Key words: Actin — Chlorophyta — Green algae — Land plants — *Mesostigma viride* — Phylogeny — Prasinophyceae — Streptophyta

Introduction

The monophyly of the green algal division, Chlorophyta [Classes Chlorophyceae, Prasinophyceae, Trebouxiophyceae, Ulvophyceae (*sensu* Sluiman 1985)], is supported by molecular sequence analyses (for exception see below) and a number of shared characters (e.g., stellate structure in the flagellar transition region, intraplastidial starch storage, two-membraned plastid containing chlorophylls-*a* and -*b*, and stacked thylakoids) that do not exist together in any other group of eukaryotes (Wilcox et al. 1993; Surek et al. 1994; Bhattacharya and Medlin 1995, 1998; Friedl 1995, 1997; Melkonian and Surek 1995). These cellular characters also demonstrate a common evolutionary history of the Chlorophyta with the Streptophyta (together, the ''green lineage''). The Streptophyta (*sensu* Bremer 1985) includes the Charophyceae plus all land plants (Devereux et al. 1990; Pickett-Heaps 1975; Mattox and Stewart 1984; Graham 1996). The separation of the Chlorophyta and Streptophyta into two distinct groups is supported by fundamental differences in the ultrastructure of the flagellated cells. The flagellates of the Chlorophyta have, for example, cruciate flagellar roots, whereas the flagellates of the Streptophyta have an unilateral flagellar root which is associated anteriorly with multilayered structures (MLS) (for details, see Friedl 1997).

Within the Streptophyta, the Charophyceae is basal to the embryophytes. The Charophyceae in turn trace their origin to flagellate, algal ancestors (Pascher 1918, 1931). The search for the extant relatives of these ancestors has interested biologists for many years because these cells will provide important clues to the morphological evolution of the Charophyceae and land plants. Extensive light and electron microscopic analyses suggest that the algal ancestors of the Streptophyta may be members of the Prasinophyceae (Christensen 1962; Pickett-Heaps 1975; Mattox and Stewart 1984; Moestrup and Throndson 1988; Melkonian 1989). Prasinophytes are a morphologically heterogeneous group of unicellular green algae whose bodies are covered with nonmineralized, square-shaped scales. Such scales are also found in the flagellate stages of the Charophyceae and the Ulvophyceae but not in other eukaryotes (Melkonian et al. 1995). The Prasinophyceae are divided into four orders (*sensu* Melkonian 1990): Mamiellales, Pseudoscourfieldales, Chlorodendrales, and Pyramimonadales.

To address the issue of a prasinophyte ancestry of the Streptophyta, we have isolated and sequenced actin-encoding cDNAs from *Mesostigma viride*, *Scherffelia dubia* (Prasinophyceae) and from *Coleochaete scutata*, *Spirogyra* sp. (Charophyceae). These sequences were included in phylogenetic analyses with actin-coding regions from other members of the green lineage. Actin sequences have proven to be an important addition to phylogenetic studies because they provide a relatively conserved molecular marker, independent of rDNA, for reconstructing eukaryotic relationships (Baldauf and Palmer 1993; Drouin et al. 1995; Bhattacharya and Weber 1997). Our present understanding of actin phylogeny within the green lineage suggests that the common ancestor of this group contained a single copy of the actin gene (Bhattacharya and Ehlting 1995). This coding region subsequently underwent multiple gene duplications within the land plants and in the Ulvophyceae [e.g., *Acetabularia cliftonii* (GenBank No. Z28698), *Cladophora rupestris* (T. Friedl, personal communication)].

Materials and Methods

Isolation of Actin Sequences. Total RNA was isolated from an actively growing culture of *Mesostigma viride* Lauterborn (SAG 50-1) using the Trizol reagent according to the manufacturer's intructions (BRL). A cDNA bank was made from the mRNA using the λgt11 phage vector. A total of 83,000 recombinant primary clones was amplified and screened with a 1100-nucleotide (nt) partial *Scherffelia* actin cDNA fragment under standard conditions (Stratagene). The *Scherffelia* actin probe was isolated by the reverse transcription/polymerase chain reaction (RT/PCR) method and PCR primers that recognize conserved regions near the 5′ terminus (Ac1) and at the 3′ terminus (Ac3) of actin sequences [shown in boldface letters in Fig. 1A (see Bhattacharya et al. 1993a)] and labeled with [^{32}P]dCTP (Pharmacia) with the random primed method according to the manufacturer's instructions. A phage that hybridized with the actin probe was purified and the insert was subcloned into the pBluescript vector for sequencing by the dideoxy termination method using T3, T7, and actin-specific oligonucleotides (Bhattacharya et al. 1991) with radioactively labeled [^{35}S]dATP and the Sequenase 2.0 kit (USB). PCR primers (sequences underlined in Fig. 1A) were used to amplify the genomic actin sequence from *Mesostigma.* This PCR fragment was subcloned and sequenced using a fluorescent-labeled dideoxy terminator sequencing method (Medigene). Procedures similar to those described above were used to isolate and sequence full-length actin cDNAs from *Coleochaete scutata* de Brébisson (SAG B 50–90; bank kindly provided by R. Kaemmerer) and *Scherffelia dubia* [SAG B 17.89 (Bhattacharya et al. 1993b)] cDNA libraries, but a *Pythium irregulare* [Oomycota (Bhattacharya et al. 1991)] actin probe was used to isolate this sequence from the *Scherffelia* cDNA bank. Partial actin cDNAs were also isolated from *Spirogyra* sp. (strain 253; SVCK, Hamburg) using the RT/PCR method and the Ac1 and Ac3 PCR primers (Bhattacharya et al. 1993a). Single-stranded template DNA from *Spirogyra* was prepared for sequencing with biotinylated PCR primers and the dynabead M-280 system (Dynal). PCR products were sequenced directly using the dideoxy sequencing method. The actin sequences from *Mesostigma*, *Coleochaete*, *Scherffelia*, and *Spirogyra* will appear in the GenBank/EBI/DDBJ databases under the accession numbers AF061020, AF061019, AF061018, and AF061021, respectively.

Actin Gene Copy-Number Analyses. Total *Mesostigma* genomic DNA was extracted with the plant DNeasy kit (Qiagen) and treated with the *Bam*HI and *Pvu*II endonucleases under standard conditions (NEB). The digested DNA was transferred onto a nylon membrane (Southern blot) and probed with the PCR fragment encoding the *Mesostigma* genomic actin fragment described above using a nonradioactive method according to the manufacturer's instructions (Gene Images, Amersham). The hybridization was done in 5× standard saline citrate (SSC), 0.1% (w/v) sodium dodecyl sulfate (SDS), and 5% (w/v) dextran sulfate at 60°C overnight. Filters were initially washed for 15 min at 60°C in 1× SSC and 0.1% SDS and then for 15 min at the same temperature in 0.1× SSC and 0.1% SDS prior to detection. DNA extraction and Southern analysis of *Scherffelia* were done as by Bhattacharya et al. (1993b) using the homologous partial actin fragment isolated by the RT/PCR method as described above. Hybridization was done in 5× SSC, 100 m*M* K_2PO_4 (pH 7.0), and 1× BFP at 65°C overnight. Filters were initially washed for 1 h at 60°C in 1× SSC and 0.1% SDS and then for 30 min at the same temperature in 0.1× SSC and 0.1% SDS prior to autoradiography.

We also studied the number of actin genes in *Mesostigma* using PCR methods. In this analysis, forward primers that recognize different conserved sequences close to the 5′ terminus of actin-coding regions were used in combination with the highly conserved Ac3 reverse primer and *Mesostigma* genomic DNA. We postulated that the two introns in the *Mesostigma* actin gene would vary in length (or be lost) from other members of its actin gene family. The actin genes from this taxon would therefore be resolved as PCR fragments of different sizes in agarose gels. The forward primers used for this analysis were a green plant-specific primer (Fern5, 5′-CTTGTYTGYGACAATGGATCWGGAATGGT-3′) and two general actin primers [142F, 5′-ATGGACCAGAAGGACGC-3′; 244F, 5′-GAYATGGAAAAGATCTGC-3′ (see Bhattacharya et al. 1993a)]. The Fern5 primer recognizes a sequence just upstream of the first conserved plant intron (20-3), whereas the 142F and 244F primers recognize conserved sequences between the conserved 20-3 and 152-1 introns (i.e., after the first nucleotide of the 152nd codon). The PCR conditions were a pretreatment at 95°C for 10 min followed by 35 cycles of a denaturing step at 95°C for 2 min, an annealing step at 60°C for 2 min, and an extension step at 72°C for 4 min. A final extension step at 72°C for 10 min was included after the 35 cycles.

Phylogenetic Analyses of Actin-Coding Regions. A tree was inferred with the maximum-likelihood method [fastDNAml (Olsen et al.

A

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1     ATGGAGTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCAATTCGCGGCCGCTCGCACGGCAAGAAGCCTCCTAGGACCACTCTTACTTATACGTCTCTAGgtaagaggagatggaaactctctttattgatccatcgttgt

151   ttccgcttttaatgagactgtgaaagcattatccgcgttggcccatttagggctgtcgagatttcttgttgcagtcatgcgttttgtgaaacgcctttaaaatggcgtcacttatcaatggaagagtggtgcacatcaaccggtgctata

301   ctttctaagagtacagccttttctatagatccttctgtggcgttgaatgccgcaccttggaaatgaaggtcacttcgccggacgcgtgaagcccgcctgcagacatctcatcacttgtggcattttctctcgatgcatgatctggcgcat

451   tttgcagGAACAATGGCTGACGAAGGAGAGGTTAGCGCCCTTATGTGCGACAATGGATCCGGAATGGTCAAGgtaaggcttttcccgcagagcatgcgtcgaggtcaagtgtgctgtctgcttggctgatctgcgaatgaaagttgaatg
451                 M--A--D--E--G--E--V--S--A--L--V--C--D--N--G--S--G--M--V--K----------

601   catcttttccccacgaatctacctggctcccagcctcgccattgaacgtctgtgcatattgctctctcgcaggcCGGCTTCGCCGGTGAtGATGCTCCCCGTGCCGTGTTTCCCAGTATAGTCGGTCGTCCCCGCCACCAGGGTGTTATG
601   ----------------------------------------------------------------------A--G--F--A--G--D--D--A--P--R--A--V--F--P--S--I--V--G--R--P--R--H--Q--G--V--M-

751   GTCGGCATGGGTCAGAAGGACGCCTACGTCGGTGATGAGGCTCAGTCCAAGCGTGGTATTCTTACCCTGAAGTATCCCATCGAGCACGGTATTGTGACCAACTGGGATGATATGGAGAAGATCTGGCACCACACTTTCTACAATGAGCTC
751   -V--G--M--G--Q--K--D--A--Y--V--G--D--E--A--Q--S--K--R--G--I--L--T--L--K--Y--P--I--E--H--G--I--V--T--N--W--D--D--M--E--K--I--W--H--H--T--F--Y--N--E--L-

901   CGTGTTGCTCCCGAGGAGCACCCCGTGCTGCTGACTGAGGCTCCCCTGAACCCCAAGGCCAACCGTGAGAAGATGACCCAGATCATGTTTGAGACGTTCAACGTCCCAGCCATGTATGTCGCCATCCAGGCCGTCCTGTCCCTGTACGCT
901   -R--V--A--P--E--E--H--P--V--L--L--T--E--A--P--L--N--P--K--A--N--R--E--K--M--T--Q--I--M--F--E--T--F--N--V--P--A--M--Y--V--A--I--Q--A--V--L--S--L--Y--A-

1051  TCCGGGCGTACCACAGgtatgcatagcacatctttttaaagaaactgagcttgctttttccgttggggctttttctgattgggactttcgctttttggtttgctgtctgaatgcaatgtgctggttatgcagtggtgggtatatttgcga
1051  -S--G--R--T--T----------.----------------------------------------------------------------------------------------------------------------------

1201  gagaatggctagagttggacaccatgctctgctagccaatgacattgactgatccctgtgatattctgtttctccccttgctacagGTATTGTGCTGGACTCCGGTGATGGTGTCACCCATACCGTCCCCATCTACGAGGGTTACGCCCT
1201  ----------------------------------------------------------------------------------G--I--V--L--D--S--G--D--G--V--T--H--T--V--P--I--Y--E--G--Y--A--L

1351  GCCGCACGCCATCCTGCGTCTCGACCTGGCTGGCCGCGACTTGACAGACTACCTGATGAAGATCCTGACGGAGCGTGGCTACTCCTTCACCACGACTGCCGAGCGTGAAATCGTGCGTGACATCAAGGAGAAGCTGGCGTACGTCGCCAT
1351  --P--H--A--I--L--R--L--D--L--A--G--R--D--L--T--D--Y--L--M--K--I--L--T--E--R--G--Y--S--F--T--T--T--A--E--R--E--I--V--R--D--I--K--E--K--L--A--Y--V--A--I

1501  TGACTATGAGGCTGAGCTCGCCACTGCCAACACCAGCTCTTCCATCGAAAAGAGCTACGAGCTGCCCGATGGCCAGGTCATCACCATTGGCAATGAACGCTTCCGTTGCCCAGAGGTGCTGTTCAACCCAGGCTACGTCGGCATGGAATC
1501  --D--Y--E--A--E--L--A--T--A--N--T--S--S--S--I--E--K--S--Y--E--L--P--D--G--Q--V--I--T--I--G--N--E--R--F--R--C--P--E--V--L--F--N--P--G--Y--V--G--M--E--S

1651  CGCGGGTATCCACGAGACCACCTACAACTCGATCATGCGGTGCGATGTCGATATTCGGAAGGACCTGTACGGCAACATTGTGCTGTCGGGTGGTTCCACCATGTNTCCTGGCATTGCTGACCGCATGAGCAAGGAGATCACTGCCTTGGC
1651  --A--G--I--H--E--T--T--Y--N--S--I--M--R--C--D--V--D--I--R--K--D--L--Y--G--N--I--V--L--S--G--G--S--T--M--?--P--G--I--A--D--R--M--S--K--E--I--T--A--L--A

1801  CCCCAGCAGCATGAAGATCAAGGTCGTGGCCCCCCCCGAGCGGAAGTACTCCGTCTGGATCGGTGGCTCAATCCTGGCCTCCCTGAGCACATTCCAGGCTATGTGGATCGCCAAGAGCGAGTACGACGAGTCTGGGCCATCTATTGTGCA
1801  --P--S--S--M--K--I--K--V--V--A--P--P--E--R--K--Y--S--V--W--I--G--G--S--I--L--A--S--L--S--T--F--Q--A--M--W--I--A--K--S--E--Y--D--E--S--G--P--S--I--V--H

1951  CAGGAAGTGCTTCTAAATCACGGCATCATTGTTTGCAGCACGAACCATGTTATGTGGTCAGGTGCTCATAATAGTCTCCCTCGACCGTGCATAGCTGCGATAGAGATGTGAGATAGCAGGTGGAGGCGGATAATGTGCTGGGTCTATTAT
1951  --R--K--C--F--*
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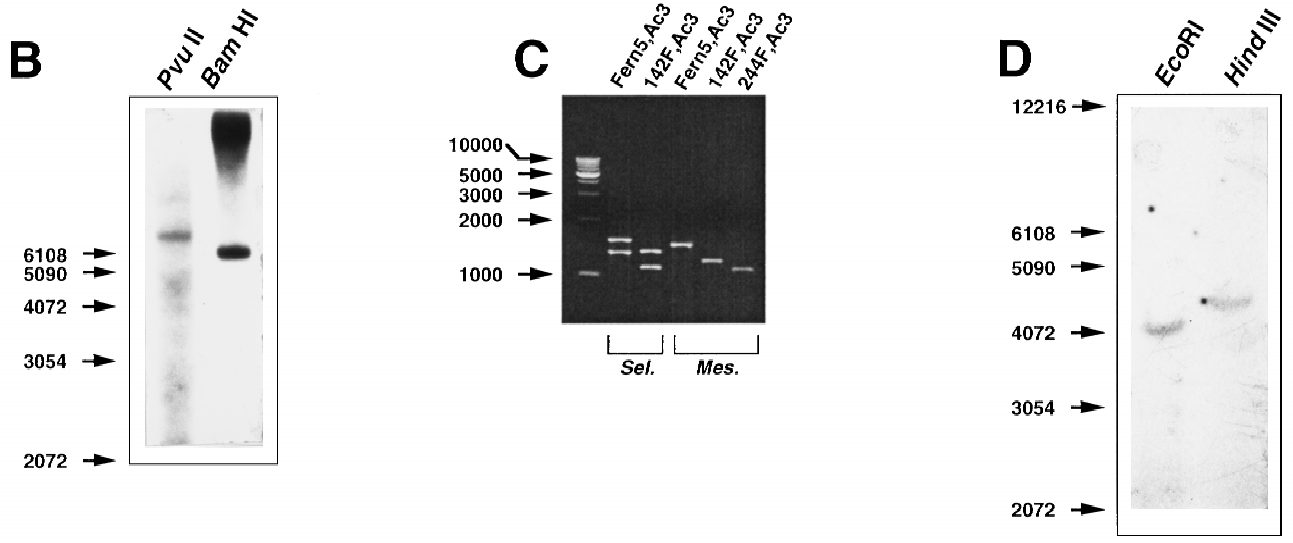

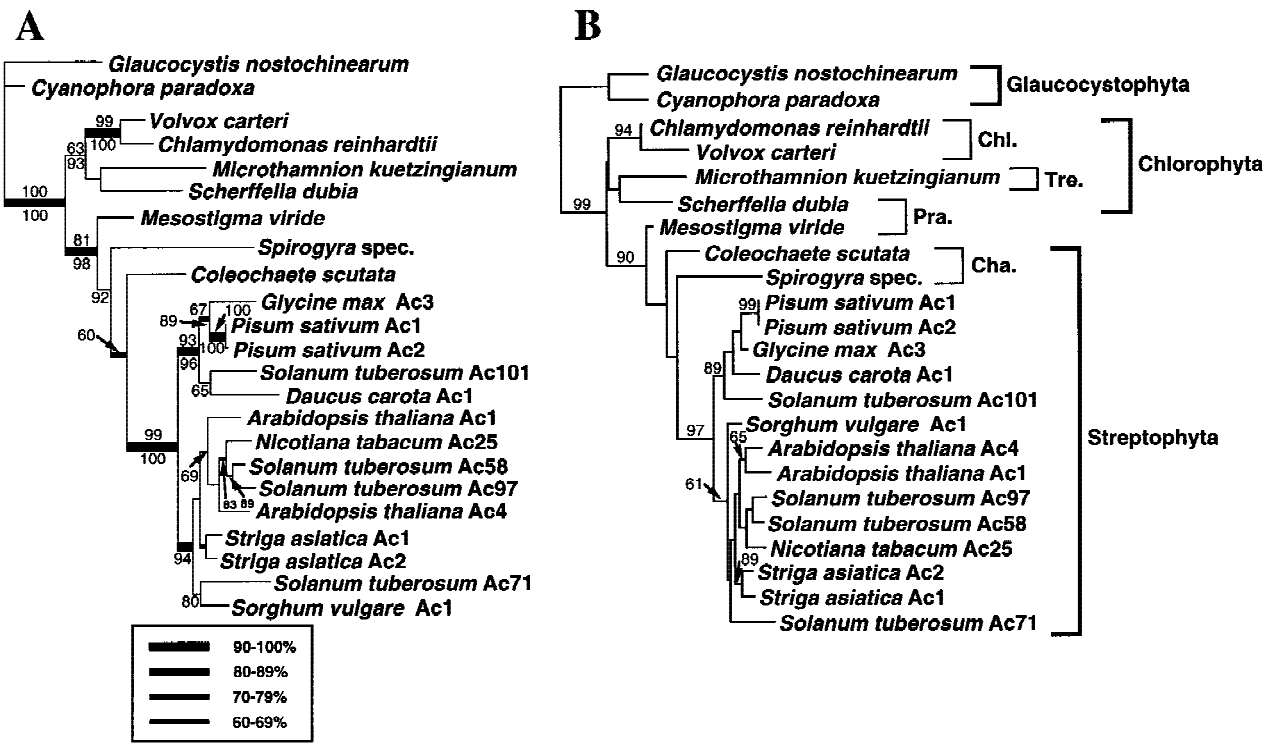

Fig. 2. **A** Maximum-likelihood phylogeny of actin-coding regions within the green lineage. Only first and second codon positions were used in the analysis (730 sites). Results of a bootstrap analysis (200 replications) are shown *above the internal nodes.* The bootstrap values (500 replications) shown *below the internal nodes* were inferred from a weighted maximum-parsimony analysis. The results of bootstrapped distance analyses (200 replications) are shown as branch lengths of differing thicknesses (see legend) *below the branch lengths.* Only bootstrap values ≥60% are shown. **B** LogDet phylogeny of actin-coding regions. A total of 126 parsimony sites was used in the analysis; gaps were excluded. The LogDet distance matrix was used as input for a neighbor-joining tree reconstruction. The values shown *at the branches* are the results of a Quartet Puzzling analysis. Only values ≥60% are shown. Chl., Chlorophyceae; Tre., Trebouxiophyceae; Pra., Prasinophyceae; Cha., Charophyceae. The trees shown in A and B have been rooted within the branch leading to the glaucocystophyte actin sequences. All actin sequences except those from *Glaucocystis nostochinearum* and *Microthamnion kuetzingianum* (T. Friedl, unpublished data) are available from the GenBank/EBI/DDBJ databases.

1994)] using the global search option and a transition/transversion ratio of 2 using only first and second positions of actin codons (730 nucleotides). Two hundred bootstrap replications were done to test the stability of internal nodes in this phylogeny. The maximum-likelihood tree was rooted on the branch length leading to the glaucocystophyte actin sequences. A bootstrap analysis (500 replications) was also done with the actin data set using a weighted (RC index over the interval 1–1000) maximum-parsimony analysis [PAUP Version 3.1.1 (Swofford 1993)] with a heuristic search procedure and a branch-swapping algorithm. The tree resulting from the maximum-parsimony analysis was loaded into the MacClade program (Version 3.04) (Maddison and Maddison 1992) and the divergence points of *Mesostigma* and the other green algae were rearranged to determine the costs of these changes in overall tree length. In addition, the actin data were subjected to a distance analysis with a Kimura model and a neighbor-joining tree reconstruction [PHYLIP Version 3.5c (Felsenstein 1997)]. Five hundred bootstrap replications were done with the distance method.

The results of these phylogenetic analyses were further tested with a LogDet analysis (Lockhart et al. 1994). The LogDet is a transformation method for converting aligned sequences into pairwise distances. This method is applicable even when sequences vary in nucleotide frequencies. And finally, the Quartet Puzzling method (Strimmer and von Haeseler 1996) was used to test the topologies of the trees inferred from the other phylogenetic methods. The Quartet Puzzling method used the HKY model of the substitution process (Hasegawa et al. 1985) with the transition/transversion ratio calculated from the data set with a gamma distribution model of rate heterogeneity (four rate categories). One thousand puzzling steps were used for the analysis.

Results and Discussion

Actin Gene Copy Number. The single bands resolved in the Southern analyses of *Mesostigma* and *Scherffelia* genomic DNA are consistent with there being single-copy actin genes in these taxa (Figs. 1B and D). These results are not proof, however, of single actin genes in *Meso-*

Fig. 1. **A** Actin gene of *Mesostigma viride* inferred from cDNA and genomic sequences. The PCR primers used to isolate the genomic actin sequence from *Mesostigma* are *underlined.* Introns within the *Mesostigma* actin gene are shown in *lowercase letters.* The intron located at the 5′ terminus of the gene is positioned outside the protein-coding sequence. The actin-specific PCR primer sequences used to isolate partial actin cDNA sequences from *Spirogyra* are shown in *boldface letters.* **B** Southern analysis of *Mesostigma* genomic DNA. DNAs were restricted with *Bam*HI and *Pvu*II. **C** Photograph of a 1% agarose gel containing the PCR products from an analysis of actin gene-copy number in genomic DNA from *Selaginella* (Sel.) and *Mesostigma* (Mes.). **D** Southern analysis of *Scherffelia* genomic DNA using the restriction enzymes *Eco*RI and *Hind*III. These Southern and PCR analyses are consistent with there being single-copy actin genes in *Mesostigma* and *Scherffelia.*

stigma and *Scherffelia* because the actin-annealing fragments may be loci which contain multiple genes. Alternatively, the probes may have hybridized exclusively, or with a significantly higher efficiency, with their homologues than with paralogous actin gene family members. To address these issues, we tested for the presence of multiple actin genes in *Mesostigma* with PCR analyses using different combinations of conserved amplification primers. The results of these analyses with genomic DNA from *Mesostigma* and from the spike-moss *Selaginella apoda* (for comparison) are shown in Fig. 1C.

The Fern5 and 142F forward primers, in combination with the Ac3 reverse primer, result in two distinct bands in the PCR reactions with *Selaginella* genomic DNA. Sequence analyses show that the *Selaginella* PCR fragments encode two closely related actin genes with intron lengths accounting for their size difference. Southern analyses with genomic DNA from this taxon show two cross-reacting fragments when probed with the larger actin-encoding fragment (Bhattacharya and S.S. An, unpublished data). These same primer combinations result in single fragments in the *Mesostigma* PCR amplifications. The 244F and Ac3 primer combination also results in a single, clear band when *Mesostigma* genomic DNA is used in the PCR reaction. The lengths of the *Mesostigma* genomic actin PCR fragments are consistent with those predicted from the complete genomic sequence shown in Fig. 1A (e.g., 1466 nt with primers Fern5 and Ac3). These results provide additional support for a single actin gene in *Mesostigma* but do not rule out the possibility that the actin primers selectively anneal to a particular actin gene which then forms the ''founder'' sequence for subsequent amplifications. This would result in the absence of other, more divergent, gene family members in the PCR-products. Though we do not directly address this point, we have successfully used the Fern5, 142F, and 244F PCR primers, in combination with the Ac3 reverse primer, to identify members of divergent actin gene families from a wide variety of green plants including the ferns, *Psilotum triquetrum, Anemia phyllitidis, Adiantum* sp., and *Cycas* sp. (Bhattacharya and An, unpublished data). We therefore do not think that these primers recognize sequences in only one subset of actin genes. A more thorough analysis using a known single-copy gene probe from *Mesostigma* as a control in Southern analyses is required, however, to prove the presence of a single actin gene in this taxon.

Phylogeny of Actins. The maximum-likelihood, maximum-parsimony and neighbor-joining phylogenetic analyses of actin sequences show strong bootstrap support for the subdivision of the green lineage into two groups defined by the Chlorophyta (excepting *Mesostigma*) on the one side and the Streptophyta on the other (Fig. 2A). The LogDet and Quartet Puzzling trees agree with this result (Fig. 2B). These trees are consistent with previous molecular phylogenies (Manhart and Palmer 1990; Steinkötter et al. 1994; Malek et al. 1996; Friedl 1997; Huss and Kranz 1997). That the Prasinophyceae (i.e., *Scherffelia* and *Mesostigma*) appear as paraphyletic lines within the green lineage in Fig. 2 is also not surprising. Analyses of SSU rDNA sequences have demonstrated that other members of the Prasinophyceae arise as multiple independent lineages at the base of the radiation of the chlorophytes, ulvophytes, and trebouxiophytes (Steinkötter et al. 1994; Melkonian and Surek 1995; Melkonian et al. 1995). *Scherffelia,* for example, is positioned here within the near-simultaneous radiation of the ''advanced'' prasinophytes (Chlorodendrales) and the Trebouxiophyceae and Chlorophyceae as in the rDNA trees (Steinkötter et al. 1994). Members of the remaining orders of prasinophytes that have been positioned in rDNA trees [*Mantoniella squamata* (Mamiellales), *Nephroselmis olivacea, Pseudoscourfieldia marina* (Pseudoscourfieldiales), *Pterosperma cristatum* (Pyramimonadales)] also fall on the Chlorophyta side of the green lineage radiation (Melkonian et al. 1995). Importantly, *Mesostigma* (Pyramimonadales) is positioned here, with significant bootstrap support, at the base of the charophytes and land plants in all the phylogenetic analyses. This result not only suggests a paraphyletic origin of the Pyramimonadales but identifies the single-celled *Mesostigma* as the earliest divergence within the Streptophyta. Our phylogenies provide the first unambiguous evidence for the basal position of a prasinophyte within the land plant lineage and is supported by previous SSU rDNA sequence comparisons that tentatively placed *Mesostigma* in this position, however, without bootstrap support [52% (Melkonian et al. 1995)]. The actin-coding region appears to have greater resolving power in this portion of the green lineage phylogeny.

We also used the MacClade program (Maddison and Maddison 1992) to alter the topology of the consensus tree inferred from the weighted maximum-parsimony bootstrap analysis. This phylogeny had a length of 499 steps when included in an unweighted parsimony analysis. In these analyses, *Mesostigma* was repositioned (1) at the base of the Chlorophyta, (2) as a monophyletic sister taxon of *Scherffelia,* and (3) as a monophyletic sister taxon of *Spirogyra* or *Coleochaete.* These analyses show that five characters change unambiguously on the branch uniting *Mesostigma* with the Streptophyta and that an additional five steps are required to move *Mesostigma* to the base of the Chlorophyta. An additional 16 steps are required to force monophyly of *Mesostigma* and *Scherffelia* (i.e., a monophyletic Prasinophyceae), whereas an additional 10 and 8 steps are required for the monophyly of *Mesostigma* and either *Spirogyra* or *Coleochaete,* respectively. Taken together, these results provide additional support for the paraphyly of the Prasinophyceae shown by all the phylogenetic analyses and are consistent with the positioning of *Mesostigma* as an early divergence within the Streptophyta with its branch

point preceding those of the Charophyceae (i.e., *Spirogyra, Coleochaete*).

Actin Intron Positions. To test our findings further we isolated and sequenced the single-copy (see Fig. 1A) actin gene from *Mesostigma* to determine the distribution of spliceosomal introns. The *Mesostigma* actin gene is interrupted by only two introns within the protein-coding region, which occur at positions, 20-3 and 152-1, whereas the vast majority of the embryophyte actin genes [over 50 genes have been characterized (see Moniz de Sá and Drouin 1996)] contain these same two introns and have a third intron at position 356-3. Some embryophytes have lost secondarily one or more of these conserved introns [e.g., the *Striga asiatica SAAc*-2 gene lacks the 152-1 intron (Florea and Timko 1997)]. Thus, the actin genes of *Mesostigma* and the embryophytes show a remarkably similar intron pattern. In contrast, actin genes of the Chlorophyceae have a much more complex gene organization. *Volvox* and *Chlamydomonas* have eight introns (20-3, 52-3, 101-3, 123-3, 139-3, 197-1, 248-3, 310-1), of which only one (20-3) is shared with *Mesostigma* and the embryophytes. Given the ongoing discussion on intron loss versus intron gain or possibly both events during eukaryotic evolution (Kwiatowski et al. 1995; de Souza et al. 1996), we cannot discriminate between a massive intron loss in the *Mesostigma*/embryophyte lineage versus the Chlorophyceae and a massive gain of introns in the latter lineage. This situation may change when actin gene sequences from charophytes and additional prasinophytes become available. Currently, the phylogenetic relationship between *Mesostigma* and the embryophytes is supported by the structure of the actin genes in these taxa and is consistent with an ancestral position of *Mesostigma.* Interestingly, the 5′-untranslated region of the *Mesostigma* actin gene also contains an intron (see Fig. 1A). Introns in a similar position have been found in the actin genes of embryophytes (McDowell et al. 1996) and metazoans (Ortega et al. 1996). Although homology of the *Mesostigma* and plant introns cannot be determined unequivocally due to the high divergence of the untranslated region sequences, this result provides further support for an evolutionary relationship between these taxa.

Are there morphological data that support the positioning of *Mesostigma* as the unicellular ancestor of the charophytes/embryophytes? This result has been predicted for a number of years based on the unique cell ultrastructure of *Mesostigma* [e.g., the only prasinophyte to lack flagellar hair scales, an extremely compressed cell body along the anterior–posterior axis (Melkonian 1989)], though, until now, no clear positioning was possible with the morphological data due to their equivocal nature (for review, see Melkonian and Surek 1995). Most important of the morphological characters that unite *Mesostigma* with the charophytes/embryophytes is the presence of two MLS in this alga that are located in the identical orientation to the flagellar roots as are the MLS in the charophytes (Melkonian 1989). The existence of MLS alone does not, however, provide clues to the ancestry of charophytes/embryophytes since these structures are also found in other prasinophytes [e.g., *Pterosperma cristatum* (Inouye et al. 1990)] and in other algal groups [e.g., Trentepohliales, Ulvophyceae (Graham and McBride 1975), Glaucocystophyta (see Bhattacharya et al. 1995)] and are likely of a primitive origin (i.e., found in the common ancestor of the crown group radiation).

In conclusion, we provide phylogenetic evidence that the charophytes/embryophytes had a flagellate ancestry and that *Mesostigma* is an extant descendant of this common ancestor. Since *Mesostigma* has an eyespot, it is likely that this character is primitive and has been secondarily lost within the charophytes. Interestingly, the genus *Mesostigma* is found only in freshwater habitats, in contrast to most other prasinophytes, which mainly live in marine habitats or have both marine and freshwater members (Melkonian 1990). This easily cultivable green alga, which is available from culture collections, therefore provides a unique opportunity to study the development of genetic complexity within a evolutionarily homogeneous group of organisms that spans the gradient of evolution from single cells to morphologically complex land plants.

Acknowledgments. This work was supported by a grant from the Deutsche Forschungsgemeinschaft to D.B. We thank J. Steinkötter, J. Ehlting (University of Cologne), and A. Besendahl (Göttingen) for technical assistance, C. Burgeff (Göttigen) for construction of the *Mesostigma* cDNA library, R. Kaemmerer (Berlin) for provision of the *Coleochaete* cDNA library, T. Friedl (University of Bayreuth) for providing access to actin sequences prior to publication and H.A. Schmidt (Heidelberg) for doing the maximum-likelihood analyses.

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