verify that the profile represented a single clone type. Complete sequences were obtained for each unique restriction pattern. Before translation to the deduced amino-acid sequence, cloned *mxaF* sequences were screened for chimaeras by visual comparison with the *mxaF* sequences of extant strains. Sequence alignment and phylogenetic analyses were done with the ARB programme (http://www.mikro.biologie.tu-muenchen.de), omitting regions of sequence ambiguity and incomplete sequence from the analyses. Results were depicted as a consensus tree, combining the results of evolutionary distance (Jukes and Cantor model for DNA; Dayhoff PAM model for amino acids), maximum-parsimony and maximum-likelihood analyses of the datasets. Multifurcations indicate points where the branching order was not supported by all three methods.

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Ancestral chloroplast genome in *Mesostigma viride* reveals an early branch of green plant evolution

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Sequence comparisons suggest that all living green plants belong to one of two major phyla[1–3]: Streptophyta[4] (land plants and their closest green algal relatives, the charophytes); and Chlorophyta[5] (the rest of green algae). Because no green algae are known that pre-date the Streptophyta/Chlorophyta split, and also because the earliest diverging green algae show considerable morphological variation, the nature of the unicellular flagellate ancestor of the two green plant phyla is unknown[1,6,7]. Here we report that the flagellate *Mesostigma viride* belongs to the earliest diverging green plant lineage discovered to date. We have sequenced the entire chloroplast DNA (118,360 base pairs) of this green alga and have conducted phylogenetic analyses of sequences derived from this genome. *Mesostigma* represents a lineage that emerged before the divergence of the Streptophyta and Chlorophyta, a position that is supported by several features of its chloroplast DNA. The structure and gene organization of this genome indicate that chloroplast DNA architecture has been extremely well conserved in the line leading to land plants.

Mesostigma is a scaly, green biflagellate that belongs to the Prasinophyceae[8], a morphologically heterogeneous class that includes descendants of the earliest diverging green algae[2,7–9]. No unique character unites the Prasinophyceae to the exclusion of other green algal classes. In phylogenetic trees inferred from nuclear small subunit (SSU) ribosomal DNA[1–3,7,9] and actin-coding sequences[10], all prasinophytes examined so far, except *Mesostigma*, form independent lineages at the base of Chlorophyta. *Mesostigma* represents the earliest divergence within Streptophyta: in SSU rRNA trees[1], this position is supported by low bootstrap values (52%), whereas in actin-gene trees[10], it is more strongly supported (>81%). A specific affinity between *Mesostigma* and charophytes was previously predicted on the basis of the identical orientation of multilayered structures relative to flagellar roots[1,7,11].

The 135 genes in *Mesostigma* chloroplast DNA (cpDNA) (Fig. 1) represent the largest gene repertoire ever reported among green algal and land plant cpDNAs. We analysed the concatenated protein sequences (10,629 amino-acid positions) from 53 genes that are common to the cpDNAs of *Mesostigma*, three land plants (*Marchantia polymorpha*, *Pinus thunbergii* and *Nicotiana tabacum*) and three chlorophyte green algae (*Nephroselmis olivacea*, *Chlorella vulgaris* and *Pedinomonas minor*). The homologous proteins from the glaucocystophyte *Cyanophora paradoxa*, the red alga *Porphyra purpurea* and the cyanobacterium *Synechocystis* sp. PCC6803 were used to root the green algal phylogeny in these analyses. It is generally accepted that all chloroplasts were derived from a single primary endosymbiotic event involving the capture of a cyanobacterium[12]. The chloroplasts of glaucocystophytes, red algae and green algae are thought to be direct products of this primary endosymbiotic event[12]. Phylogenetic evidence indicates that glaucocystophyte chloroplasts evolved before those of red and green algae[13].

Trees constructed with distance, maximum parsimony and maximum likelihood inference methods using *Cyanophora* proteins as an outgroup were found to be congruent in showing a strongly supported topology, 'T1' (Fig. 2a), in which *Mesostigma* emerges before the divergence of Streptophyta and Chlorophyta. Only T1
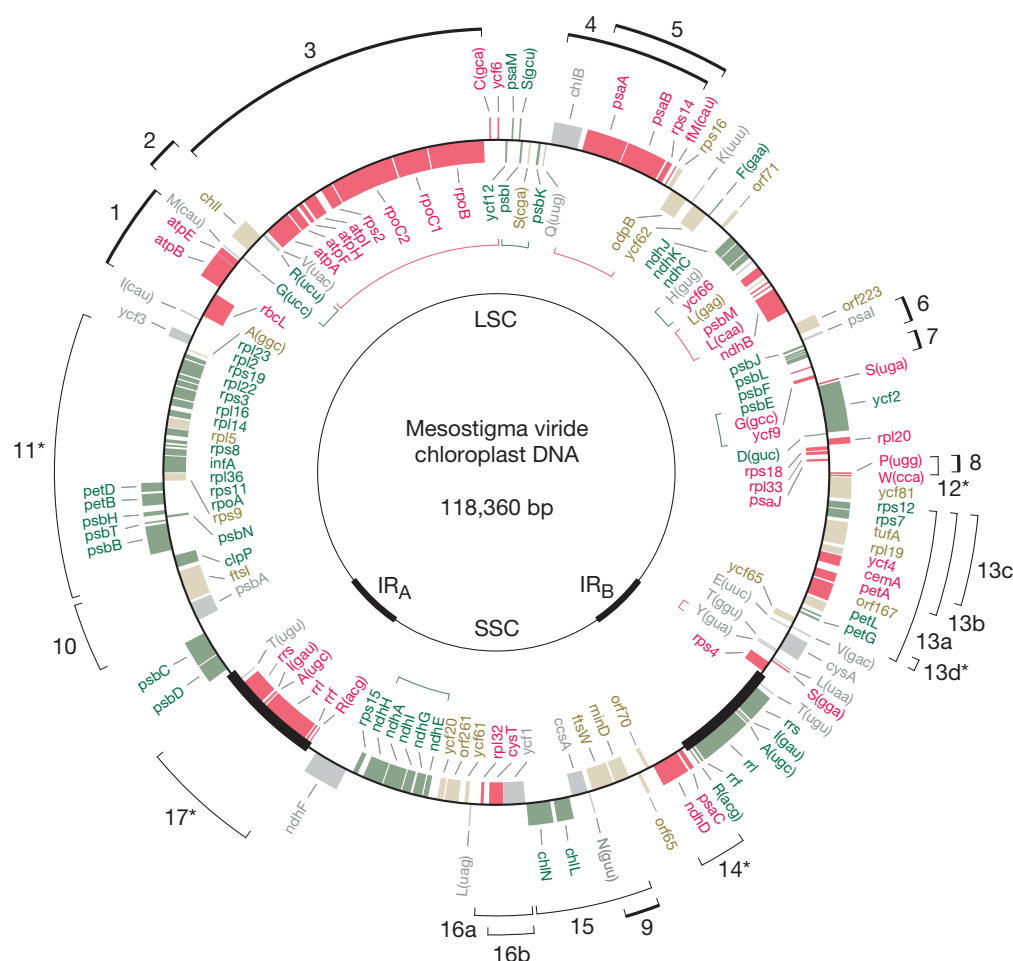
Figure 1 *Mesostigma* cpDNA. Inverted repeat sequences (IR$_A$, IR$_B$) separate small (SSC) and large (LSC) single-copy regions. Genes outside the map are transcribed clockwise. Genes absent from *Marchantia* cpDNA are represented in beige. Gene clusters shared with *Marchantia* are shown as series of green and red boxes; those corresponding to brackets inside the map are shared exclusively with land plants. Genes present in *Marchantia* but located outside conserved clusters are shown in grey. Thick brackets indicate clusters that are shared with some non-green algae but are absent from *Synechocystis*. Thin brackets denote clusters that are shared specifically with both chlorophytes and streptophytes (asterisks) or only chlorophytes.

was detected in quartet-puzzling and distance-based analyses under the amino-acid substitution models of Jones *et al.*[14] (JTT-F) and Dayhoff *et al.*[15], with either a uniform or a gamma-distributed rate of substitution across sites. In maximum-parsimony analysis, bootstrap support for T1 was 97%, and two alternative topologies, 'T2' and 'T3' (Fig. 2b), differing with respect to the position of *Mesostigma*, were each recovered in 1.5% of the bootstrap samples. T1, T2, and T3 were also the only topologies recovered in maximum-likelihood analysis under the JTT-F model with a uniform rate of substitution; T1 was found in 98.9% of bootstrap samples, and both T2 and T3 were significantly worse than T1 (T2, $P < 0.05$; T3, $P < 0.01$) in the Kishino-Hasegawa test[16]. Removal of constant sites from the data set had negligible effect on bootstrap support for T1 in distance and maximum-likelihood analyses, and on the confidence limit of tree topologies under the Kishino-Hasegawa test (see Supplementary Information). Considering that most constant sites (5,585 out of 5,628) were estimated to be invariable using SPLITSTREE, these observations eliminate the possibility that misleading effects of invariable sites[17] contributed to the recovery of T1. Even when the *Cyanophora* sequences were excluded from the data set, or when other outgroup sequences (*Porphyra* proteins, or a combination of *Cyanophora*, *Porphyra* and *Synechocystis* proteins) were used, trees compatible with T1 remained the best topologies (see Supplementary Information).

Systematic biases in amino-acid composition can give rise to incorrect relationships in reconstructed phylogenetic trees[18]. Chi-squared analysis of amino-acid composition at variable sites reveals that the 53 concatenated proteins of *Mesostigma* do not significantly differ ($P < 0.05$) from those of *Cyanophora*, *Marchantia*, *Pedinomonas* and *Pinus*. Our failure to detect a systematic bias in amino-acid composition thus suggests that the basal position of *Mesostigma* is the result of a genuine phylogenetic signal. In support of this conclusion, neighbour-joining analysis of LogDet distances calculated after removal of all constant sites retrieved the T1 topology in 93% of bootstrap samples, with T2 being the only alternative topology detected. LogDet distances allow the recovery of the correct tree when sequences differ markedly in amino-acid frequencies for cases when substitution processes are otherwise uniform across the underlying tree[19].

We also carried out maximum-likelihood analyses of concatenated chloroplast SSU and large subunit (LSU) rDNA sequences (4,016 positions, of which 1,026 are variable) from the taxa shown in Fig. 2, using the HKY model of nucleotide substitution[16] (for analyses with other models, see Supplementary Information). The reconstructed trees were found to be congruent with those inferred from chloroplast proteins in providing unequivocal support for T1. T1 and T3 accounted for 99.0% and 1.0% of bootstrap samples, respectively (T2 was not recovered), and T3 was significantly worse
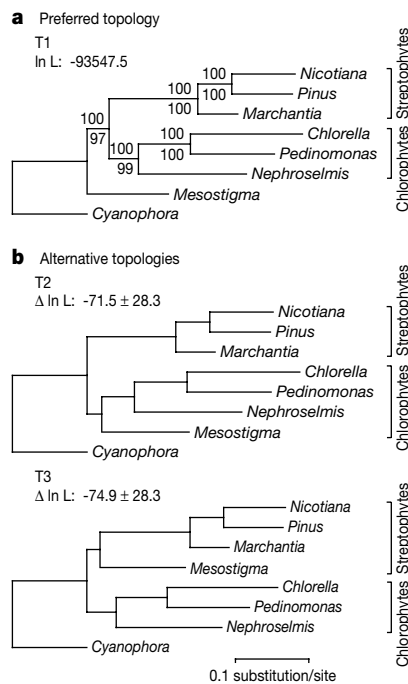
Figure 2 Phylogenetic position of *Mesostigma* as inferred from 53 chloroplast proteins. **a**, Preferred tree topology found in all analyses. The log likelihood (ln L) value was calculated using PROTML and the JTT-F model. The numbers above the nodes indicate the bootstrap percentages and reliability percentages obtained in distance and puartet-puzzling analyses, respectively. The numbers below the nodes indicate the bootstrap percentages in maximum-parsimony analysis. **b**, Alternative tree topologies detected in maximum-likelihood, maximum-parsimony and LogDet analyses. The ΔlnL value indicates the difference in log likelihood relative to T1 and the standard error of that difference.

missing from Streptophyta; (4) the presence of nine gene clusters that are found in some non-green algal cpDNAs but not in the *Synechocystis* genome (Fig. 1)—only subsets of these clusters have been retained in other green plants (Table 1); and (5) the absence of introns—a feature that is also shared with the cpDNAs of *Nephroselmis*[20] and most non-green algae[12].

Many genes in *Mesostigma* cpDNA form clusters that are shared exclusively with chlorophytes and/or streptophytes (Table 1), thus strengthening the notion that *Mesostigma* represents the most basal green plant lineage. These clusters provide clues into how the green plant chloroplast genome diverged from the ancestral pattern of gene organization during the evolution of Chlorophyta and Streptophyta.

The gene organization of *Mesostigma* cpDNA is highly similar to that of land plant cpDNAs, with 81% of its genes being found in clusters that are shared with land plant cpDNAs (Fig. 1). This observation indicates that the chloroplast genome from the common ancestor of chlorophytes and streptophytes has been highly preserved in structure and gene order during the long evolutionary period (~800 Myr (ref. 6)) separating this ancestor from land plants. Such an exceptional conservation of gene order was unexpected, as *Nephroselmis* cpDNA, the green algal cpDNA previously known to display the most ancestral characters, shares a limited number of gene clusters with its land plant counterparts[20]. Our results predict that cpDNA rearrangements occurred relatively infrequently in Streptophyta and that analysis of these events will be useful not only to study how the chloroplast genome evolved during the transition from the most ancestral green flagellate to land plants, but also to clarify phylogenetic relationships among streptophytes.

The morphological characteristics[8] of *Mesostigma* support the view that the most ancestral green flagellate was a biflagellated and asymmetric cell that had an underlayer of square scales (a character unique to green algae), an eye spot and a cruciate flagellar root system with multilayered structures[1,7]. The number of flagella is highly variable among prasinophytes and other green algae. Square-shaped scales are found in members of both Chlorophyta and Streptophyta; however, the presence of an eye spot has been noted

than T1 ($P < 0.05$) in the Kishino-Hasegawa test. Removal of all constant sites from the data had little effect on support for T1.

In favouring the placement of *Mesostigma* within Streptophyta (a position corresponding to T3), actin-gene trees[10] contrast with those that we inferred from chloroplast sequence data. We used the Kishino-Hasegawa test under the HKY model to assess the confidence limit of actin-tree topologies (see Supplementary Information) and found that the phylogenetic information in the actin data set (198 variable sites) is not sufficient to eliminate the hypothesis that *Mesostigma* branches at the base of Chlorophyta and Streptophyta (T1). The actin trees that support this hypothesis represented a meaningful proportion (12.7%) of bootstrap samples in maximum-likelihood analysis and were not significantly worse ($P \geqslant 0.30$) than those compatible with T3 (best maximum-likelihood trees). From this result, we conclude that the discrepancy between the chloroplast and the actin tree is only apparent.

The positioning of *Mesostigma* as the earliest divergence in the phylogeny of green plants is supported by our finding that this prasinophyte has retained more ancestral cpDNA features than previously examined green plants. The most relevant of these ancestral features are (1) a quadripartite structure in which common genes reside in corresponding genomic regions—a feature that is also shared with the cpDNAs of some non-green algae, most land plants and *Nephroselmis*[20]; (2) the presence of five genes (*trnA*(ggc), *odpB*, *ycf20*, *ycf61* and *ycf65*) that were previously identified only in *Porphyra* cpDNA; (3) the presence of all genes that were lost specifically in Chlorophyta or Streptophyta[20], with the exception of one (*rpl21*) of the eight genes specifically missing from Chlorophyta and three (*rne*, *rnpB* and *rpl12*) of the sixteen genes

Table 1 Distribution of the *Mesostigma* chloroplast gene clusters that are shown in Figure 1

Cluster	Non-green algae				Streptophyte	Chlorophytes		
	Cyan	Porp	Guil	Odon	Marc	Neph	Pedi	Chlo
1	●				●			
2		●	●					
3	●	●	●	●	●		●	
4	●							
5			●		●			
6	●	●	●					
7	●	●	●	●	●	●		
8	●	●	●		●	●	●	●
9	●					●	●	
10					●	●		
11					●	●	●	
12					●	●	●	
13a					●	●	●	
13b						●	●	
13c						●	●	
13d					●	●	●	
14					●	●		
15						●		
16a						●		●
16b						●	●	●
17					●	●		

Filled circle denotes the presence of a gene cluster: 1, *rbcL-atpB-E*; 2, *chlI-R*(ucu)-*V*(uac); 3, *rpoB-C1-C2-rps2-atpI-H-F-A*; 4, *chlB-psaA-B*; 5, *psaA-B-rps14*; 6, *psaI-psbJ-L-F-E*; 7, *ycf9-G*(gcc); 8, *psaJ-P*(ugg); 9, *ftsW-N*(guu); 10, *ftsI-psbA*; 11, *clpP-psbB-T-N-H-petB-D-rps9-rpoA-rps11-rpl36-infA-rps8-rpl5-14-16-rps3-rpl22-rps19-rpl2-23*; 12, *psaJ-P*(ugg)-*W*(cca); 13a, *rps12-7-tufA-rpl19-ycf4-cemA-petA-L-G*; 13b, *rps12-7-tufA-rpl19-ycf4-cemA*; 13c, *rps12-7-tufA-rpl19-ycf4*; 13d, *petL-G*; 14, *psaC-ndhD*; 15, *ftsW-N*(guu)-*ccsA-chlL-N*; 16a, *rpl32-cysT-ycf1*; 16b, *cysT-ycf1*; 17, *rrs-I*(gau)-*A*(ugc)-*rrl-rrf-R*(acg). Abbreviations of genus names and references for corresponding cpDNAs: Cyan, *Cyanophora*[25]; Porp, *Porphyra*[26]; Guil, *Guillardia*[27]; Odon, *Odontella*[28]; Meso, *Mesostigma* (this study); Marc, *Marchantia*[29]; Neph, *Nephroselmis*[20]; Pedi, *Pedinomonas* (our unpublished data); Chlo, *Chlorella*[30].

only in chlorophytes. Multilayered structures are found in chlorophytes and streptophytes, and appear to be an ancestral character of algae, as similar structures have been described in other algal groups[6].

Methods

DNA sequencing

Chloroplast DNA from *Mesostigma viride* (NIES-296) was isolated from total cellular DNA as an AT-rich fraction by CsCl-bisbenzimide isopycnic centrifugation[20]. This DNA preparation was sheared by nebulization, and 1,500–3,000-bp fragments were recovered by electroelution after agarose gel electrophoresis. These fragments were treated with *Escherichia coli* Klenow fragment and T7 DNA polymerase, and cloned into the *Sma*I site of Bluescript II KS+. After hybridization of the clones with the original DNA used for cloning, DNA templates from positive clones were prepared with the QIAprep 8 Miniprep kit (Quiagen). Nucleotide sequences were determined with the PRISM dye terminator cycle sequencing kit (Applied Biosystems) on a DNA sequencer (model 373; Applied Biosystems) using T3 and T7 primers. Sequences were assembled and analysed as described[20]. Short genomic regions not represented in the clones analysed were sequenced from PCR-amplified fragments.

Phylogenetic analysis

Genome sequences were retrieved from GenBank. *Pedinomonas* cpDNA sequences are from our unpublished data. Individual protein and rRNA gene sequences were aligned with CLUSTALW 1.74 (ref. 21), alignments were concatenated, and ambiguously aligned regions containing gaps were excluded. The alignments and data sets are available in Supplementary Information. The program packages MOLPHY 2.3b3 (ref. 16), PHYLIP 3.573c2[22], PUZZLE 4.0.2[23] and SPLITSTREE 2.4[24] were used for phylogenetic analyses. Symmetric distance matrices were computed with PUZZLE and PROTDIST[22], whereas Logdet distances were calculated with SPLITSTREE. Distance trees were constructed with NEIGHBOR[22] and/or FITCH[22], maximum-parsimony trees were obtained with PROTPARS[22], and quartet-puzzling trees were generated with PUZZLE. The robustness of distance and maximum-parsimony trees was assessed by bootstrap percentages after 100 replications. In the case of quartet-puzzling trees, reliability percentages of the occurrence of the nodes were estimated after 10,000 puzzling steps. Maximum-likelihood analyses of protein and DNA sequences were carried out with PROTML[16] and NUCML[16], respectively, and local bootstrap probability was estimated by resampling of the estimated log likelihood[16].

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Pattern recognition and active vision in chickens

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Recognition of objects or environmental landmarks is problematic because appearance can vary widely depending on illumination, viewing distance, angle of view and so on[1]. Storing a separate image or 'template' for every possible view requires vast numbers to be stored and scanned, has a high probability of recognition error and appears not to be the solution adopted by primates[2,3]. However, some invertebrate template matching systems can achieve recognition by 'active vision' in which the animal's own behaviour is used to achieve a fit between template and object[4], for example by repeatedly following a set path[5–7]. Recognition is thus limited to views from the set path but achieved with a minimal number of templates. Here we report the first evidence of similar active vision in a bird, in the form of locomotion and individually distinct head movements that give the eyes a similar series of views on different occasions. The hens' ability to recognize objects is also found to decrease when their normal paths are altered.

When leaving a nest or newly discovered food source, bees and wasps use special orientation flights and then attempt to recapitulate the same set of views on subsequent occasions by following similar flight paths[5–7]. The functional significance of such active vision[4] is that by learning only a limited set of views of an object, the task of recognizing it is greatly simplified, although at the cost of recognition failure if the object is viewed from an unfamiliar