

Charophyte algae and land plant origins

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The charophyte algae are six distinct groups of mostly freshwater green algae that are related to modern land plants. Charophyte algae exhibit diverse morphologies and reproductive strategies, from unicells to branching erect forms, and from swimming asexual spores to sex involving eggs and sperm, respectively. The green algae known as stoneworts (Charales) are suggested to be the extant sister group to all land plants, although the phylogeny is not conclusive. Here we review recent molecular phylogenetic work on the charophyte algae and its implications for our understanding of the origins of land plants and of characters in their aquatic ancestors that might have played a role in the explosive diversification of plants on land.

The emergence of green algae from an aquatic habitat and the colonization of the land was a pivotal event in evolution [1,2]. Evolutionary transitions into the terrestrial realm have occurred many times among the green algae, which thrive on and under rocks, soil, and upon leaves and stems of so-called higher plants. But the diversity of these land algae pales in comparison to that of embryo-producing land plants, or embryophytes. **Plants with protected embryos and a diploid phase in the life cycle radiated into new niches in numbers that exceeded by several orders of magnitude the species diversity of their immediate sibling groups among the green algae, which generally exhibit only one diploid cell in the life cycle [1].** The explosion of land-plant species not only transformed the landscape and atmosphere, but also set the stage for the subsequent emergence of animals onto land. However, similar to other epochal evolutionary events, the initial movement of green plants onto land must have been a subtle event that involved green algae of relatively simple morphology that have left little, if any, known fossil record [1]. Nevertheless, **the origin of the land flora has been fertile ground for research for more than 100 years [1,3,4].** Now, at the beginning of the 21st century, gene-sequence analysis, including sequencing of several key organellar genomes, has enabled hypotheses to be tested about relationships among living green algae and embryophytes.

Chlorophyte and charophyte algae

The close evolutionary relationship of green plants to green algae (*Chlorophyta sensu lato*) has been long accepted, but the diversity of form (unicells to multicellular) and life cycles (with one or two free-living stages in the life cycle) among green algae led to diverse hypotheses about which taxa gave rise to land-dwelling descendants. Frederick O. Bower's [3] early 20th-century book about the evolution of land plants and their life cycles framed the debate about the origin of a land flora for >50 years. Ultrastructural research on cell morphology and division re-framed the debate during the second half of the 20th century, with the result that **Chlorophyta were split into two lineages [1,5–7]. One lineage comprises most of the traditional Division Chlorophyta [7],** which we term 'chlorophytes' or 'chlorophyte green algae', and includes most extant green algae (hundreds of genera and upwards of 10 000 species), such as *Chlamydomonas*, *Volvox*, *Cladophora*, *Ulva* and other organisms that are important as model organisms and primary producers in freshwater and marine ecosystems. **A second lineage, Charophyta [8,9], contains relatively few extant green algae (some 65 genera and a few thousand species),** which we term 'charophyte green algae', along with approximately half a million species of land plants. Biochemical characters of glycolate metabolism [5] and ultrastructural work on cellulose synthesizing terminal complexes, or rosettes [10–12], has revealed other characters that are unique to charophyte algae and land plants, but that are absent from chlorophyte algae. In other words, charophyte algae shared a more recent common ancestor with land plants than with chlorophyte green algae. Thus, Charophyta represents a monophyletic lineage only if embryophytes are included with charophyte green algae.

Genes and genomes unravel charophyte algal phylogeny

Recent molecular phylogenetic studies support the monophyly of individual charophyte algal groups and of most of the charophyte algal groups plus land plants (the exception being *Mesostigma*, which is discussed in detail below). However, the branching order of groups within the phylogeny has been uncertain [13–21]. Hypotheses of relationships among charophyte algae have developed in a piecemeal fashion, as subsets of taxa from several groups were sampled one gene at a time from either the nucleus

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Box 1. Major groups in the Charophyta

Mesostigmatales (mesostigmatophytes) (*Mesostigma viride*)

Mesostigma is a freshwater, scaly, asymmetrical unicell with a pair of flagella. The monotypic taxon was considered one of many primitive unicells in a group called prasinophytes until ultrastructural features revealed it to be a member of the land-plant lineage [49]. Other studies [53,54] suggest that this species occupies a unique position sister to all green algae and plants (see text). The species has never been observed to reproduce sexually. (Figure 1a. Scale bar = 32 µm.)

Chlorokybales (chlorokybophytes) (*Chlorokybus atmophyticus*)

Another monotypic taxon, *Chlorokybus* exists as packets of a few cells that grow in moist terrestrial habitats and produce asymmetrical flagellate motile cells (zoospores). Sexual reproduction is unknown in this species. (Figure 1b. Scale bar = 32 µm.)

Klebsormidiales (klebsormidiophytes) (*Klebsormidium* sp.)

Thirty species in two genera are all unbranched filaments or short chains of cells that produce zoospores through pores in the cell wall. Zoospore flagella are lateral, as in all motile cells of charophyte algae known. Sexual reproduction is unknown in the group. (Figure 1c. Scale bar = 32 µm.)

Zygnematales (conjugating green algae) (*Spirogyra* sp.)

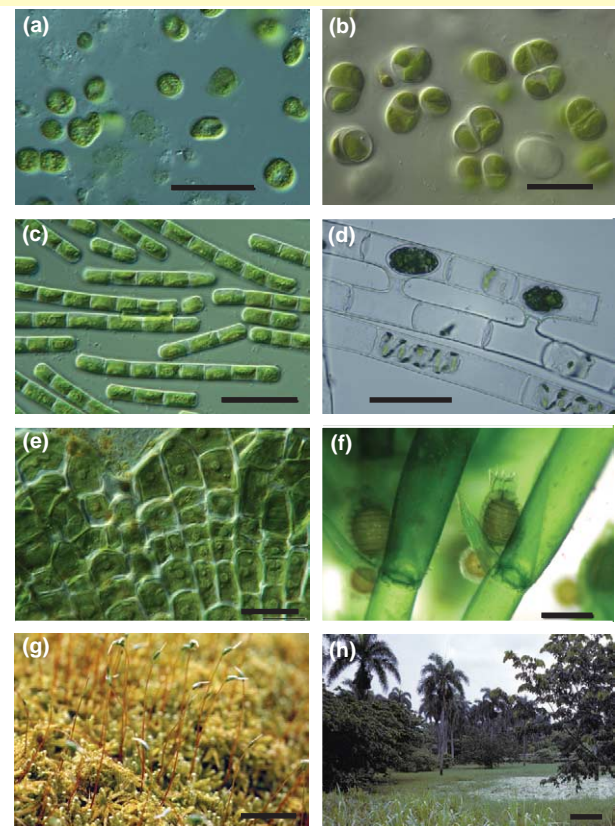
The conjugating green algae exhibit the greatest diversity, if not complexity, in form of the charophyte green algae. More than 4000 species in some 54 genera have been described. Thalli (vegetative bodies) range from unicells to short or long filaments, as well as a few colonial clusters of cells; flagella are unknown in the group. Cells of the more derived conjugating green algae are divided into two symmetrical semicells joined at a middle isthmus, with walls strikingly ornamented by elaborate sculpting of wall layers. Sex occurs through the process of conjugation, which involves fusion of nonswimming gametes that are brought together via tubes or mucilage vesicles that form between vegetative cells. (Figure 1d. Scale bar = 64 µm.)

Coleochaetales (coleochaetophytes) (*Coleochaete pulvinata*)

These small (100–200 µm) discoid or filamentous species grow on stems and leaves of water lilies or other vascular plants and other submerged solid substrates. Only two or three genera exist, but all have cells that bear distinctive sheathed hairs that are extensions of the cell wall. The fertilized zygote is retained on the maternal plant in some species and corticated after fertilization by a layer of sterile cells. The zygote of at least one species receives nourishment via placental transfer cells with wall ingrowths that increase the area in contact with the zygote. The walls of the zygote possess sporopollenin, a highly resistant substance found in the outer wall of pollen [56]. (Figure 1e. Scale bar = 32 µm.)

Charales (stoneworts, charophytes *sensu stricto*) (*Chara braunii*)

Stoneworts are morphologically the most complex charophyte green algae, with central stalks of so-called giant, multinucleate cells with whorled branchlets at a series of nodes. Growth occurs at the apex of the central stalk and branchlet tips. Sexual structures nested in the axils of branchlets produce either eggs (in oogonia) or sperm (in antheridia). A single egg is jacketed by five spiraled sterile cells that come together at the apex in a corona of smaller tip cells. Highly asymmetrical sperm are produced in threadlike cells compacted inside



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Figure 1.

spherical reddish orange gametangia. Only six genera and a few hundred species are extant, although a rich fossil record reveals far greater taxonomic diversity extending back to the Silurian [37]. (Figure 1f. Scale bar = 1 mm.)

Embryophytes (land plants) (moss, *Brachythecium* sp.)

Land plants dominate the terrestrial ecosystem and are often treated as the plant kingdom *in toto*, although they are a subgroup of green algae phylogenetically. Land plants are the most derived descendants of the charophyte green algae because they produce embryos that are nourished by maternal gametophytic tissue. There are two distinct phases in the life cycle: the gamete-producing, or gametophyte phase (dominant in mosses and so-called lower plants such as shown in Figure 1g); and the spore-producing, or sporophyte, phase that is dominant in the life cycle of seed plants (including the flowering plants surrounding the pond in Figure 1h). The group consists of more than half a million species is primarily terrestrial, but includes many species that have secondarily invaded freshwater, or, more rarely, marine habitats. The image in Figure 1h from the Dominican Republic shows a habitat for all major groups of charophytes, algae and land plants. (Figure 1g,h. Scale bars = 1 cm and = 1 m, respectively.)

or the chloroplast. Several conflicting hypotheses have been proposed [6,17], the differences among which are a result of non-overlapping, small taxon sets; the use of different genes in single-gene analyses; and to different types of analyses (e.g. gene versus protein sequences). For example, sequence analyses of nuclear 18S (small subunit) rDNA and the plastid gene encoding Rubisco large subunit (*rbcL*) produced a variety of phylogenies that proposed

different sister groups to the land plants [17]. The strongest conflict between analyses based on these two genes was the placement of the stoneworts (Order Charales), which are relatively large, complex green algae up to 1 m in height with whorled branches and elaborate sperm- and egg-producing organs (Box 1f). The 18S data, depending on the analysis, suggested that Charales were: (i) sister to land plants [14,16,21], or

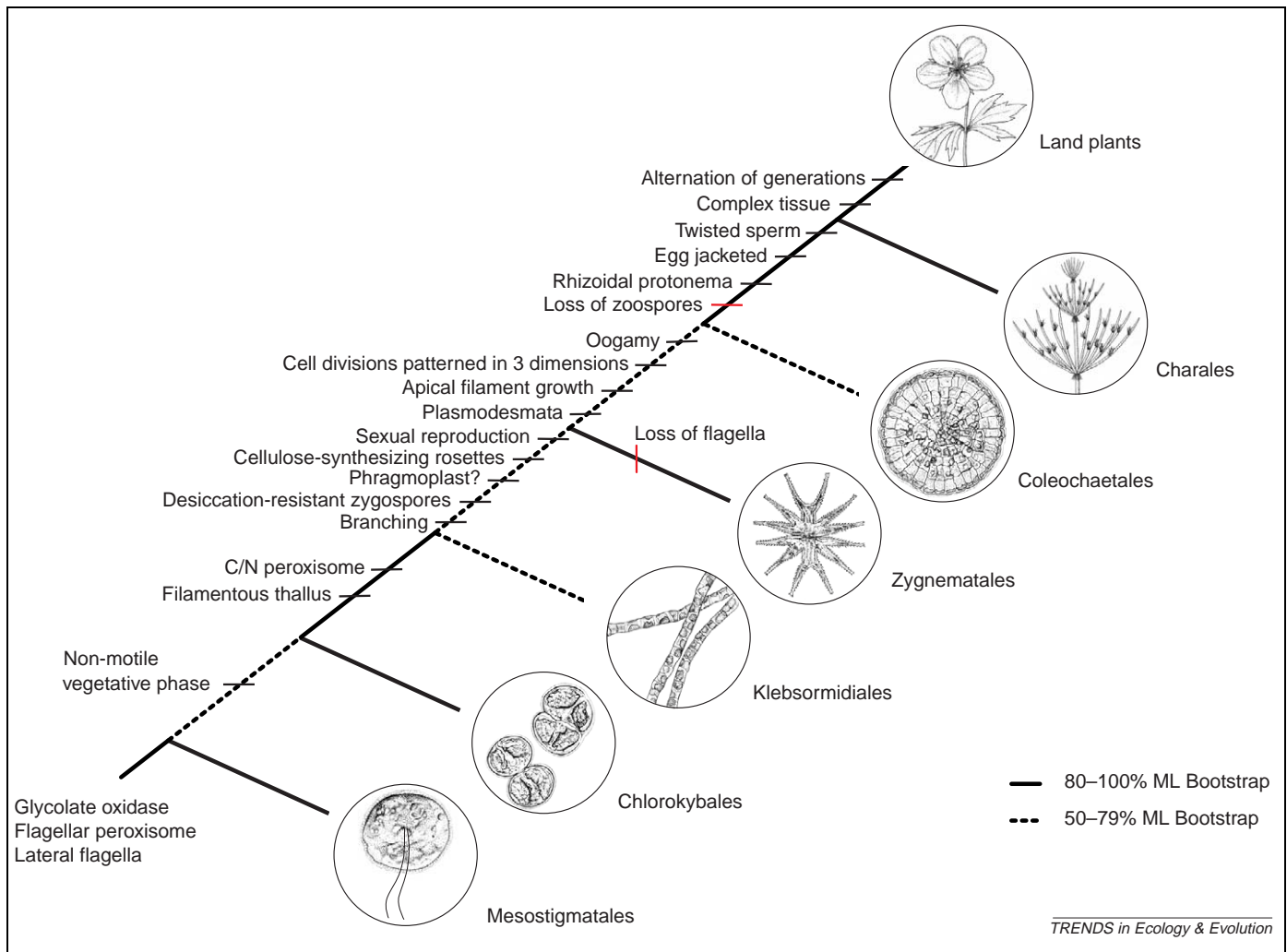


Figure 1. The phylogeny of charophyte algae and land plants based on a molecular analysis by Karol *et al.* [8]. The backbone of the tree is based on data from two plastid genes (*rbcL* and *atpB*), a mitochondrial gene (*nad5*), and a nuclear gene (18S, or small subunit rDNA). The clade shown is sister to other green algae (*Chlorophyta sensu lato*). All branches were strongly supported under Bayesian inference (as measured by posterior probabilities), but maximum likelihood bootstrap support was lower for some branches, as indicated by dotted lines. The ladderlike form of the tree does not imply an inexorable increase in complexity over evolutionary time in this lineage, although that general trend is found on the best-supported tree [8]. *Mesostigma*, the flagellate unicell at the base of the clade shown, is placed as the basal group sister to all other green plants and algae in some genomic analyses [53,54]. The hash marks on branches represent hypotheses of gains (black) or losses (red) of the labeled morphological, biochemical and ultrastructural characters as they would be mapped if the backbone of the tree shown is correct. The order of gain or loss shown between nodes is arbitrary. Thus, hash marks on weakly supported branches (dotted lines) should be viewed as provisional. The '?' following 'phragmoplast' indicates that the phragmoplast type of Zygnematales might be independently derived from that of land plants. For further details of characters, see [2,7].

(ii) one of several groups that were collectively sister to land plants [22], or (iii) on a separate line of evolution that diverged early on from a group that included the other charophyte algae and land plants [15,17,19,20]. These rDNA topologies conflicted with analyses based on *rbcL*, which identified Charales plus Coleochaetales as the sister taxon to the embryophytes [13,17,18,23,24].

The broadest sampling of taxa and genes to date was in a study by Karol *et al.* [8], who sampled several representatives from the six charophyte algal groups (a total of 25 species), plus eight species of land plants, five species of chlorophyte algae, and the glaucocystophyte *Cyanophora paradoxa* as an outgroup. The data from the four genes studied (*rbcL*, *atpB*, *nad5*, and 18S rDNA) were analyzed using Bayesian inference, maximum parsimony, maximum likelihood, and minimum evolution. All methods produced the same tree (Figure 1), although with different levels of support, expressed either as posterior probabilities (PP) in Bayesian [25] and bootstrap

(BS) values in maximum parsimony, maximum likelihood, and minimum evolution methods [26]. All methods strongly supported the stoneworts as the nearest living relative of embryophytes. For other nodes on the tree, support was mixed. Bayesian PP support was uniformly high for all the branches shown in Figure 1, but maximum likelihood BS support was moderate or low for several branches (dotted lines in Figure 1). Monophyly of individual charophyte orders, Zygnematales and Charales, and of land plants was strongly supported (>80%), but support for each of the orders Coleochaetales and Klebsormidiales was lower (<80%). Two other groups, Mesostigmatales and Chlorokybiales, are monotypic and thus monophyletic by default. Their placement at the base of the charophyte algae-land plant clade also had high PP but weak BS support.

A recent study of glucose (GAPDH) sequences placed *Coleochaete* rather than Charales as the sister taxon to land plants [27]. This result agrees with earlier

morphology-based suggestions [1,28], although fewer green-algal taxa were sampled and only for the *GAPDH* gene. Similar to the four-gene study [8], the *GAPDH* tree had high PP support and low BS support values.

Consistently higher Bayesian PP support compared with maximum likelihood BS support on molecular trees has drawn criticism from several simulation studies [29,30], which suggest that Bayesian PP support is too liberal and maximum likelihood BS is too conservative for detecting phylogenetic patterns [29,31]. However, other simulations have reported that Bayesian analysis is more effective in detecting phylogenetic signal than maximum likelihood [32]. The crucial issue is to have enough sequence data, the right model, and broad enough sampling to achieve high support values from various methods. Clearly, more data are needed to resolve fully the phylogeny of charophyte algal groups.

Recent analyses using other genes have been used to test the sister relationship of Charales and land plants shown in Figure 1. A study of large- and small-subunit chloroplast rDNA genes [33] did not find clear support for a sister relationship of Charales and land plants, although one of several topologies with equal likelihood scores positioned Charales as sister to land plants. However, a whole-genome sequencing project for the mitochondrion of the stonewort *Chara vulgaris* [34] included an analysis of the combined sequences of 23 mitochondrial genes, which strongly supported the sister relationship of Charales to land plants. The presence-absence patterns of genes and group I and II introns in the *Chara* mitochondrion [34] exhibited a greater similarity with the respective patterns in embryophytes than they did with those of the other green algae sequenced to date. In addition, A+T content, total gene number, and genome size suggest an intermediate mitochondrial genome structure for *Chara* that is consistent with the sister relationship of Charales and embryophytes [34]. This result from studies of organellar genomes, although based on a large amount of sequence data, remains tentative because of the few taxa sampled (the three embryophytes *Beta vulgaris*, *Arabidopsis*, *Marchantia*; plus the three algae *Chara*, the coleochaetalean genus *Chaetosphaeridium*, and *Mesostigma*).

Likewise, this analysis of the mitochondrial genome did not sample broadly enough to resolve branching order among the charophyte algal groups diverging before Charales and land plants. Nor have other genes or methods of analysis clarified the phylogeny of these groups [8,27,34]. The uncertainties of the phylogeny are indicated by dotted lines in Figure 1. Resolution of the phylogenetic relationships of these remaining groups awaits study of additional taxa and genes.

From simple to complex?

Despite the uncertainty of some of the branches in Figure 1, some inferences can be drawn about the derivation of characters that might have played a role in the morphological elaboration of embryophytes. Groups near the base of the tree are either unicellular (e.g. *Mesostigmatales*) or consist of packets of a few cells (e.g. *Chlorokybus*). Later diverging groups are multicellular and exhibit various types of branching through cell division (Box 1). Similarly,

sexual reproduction is absent or unknown in the early diverging groups, but is present in morphologically more complex charophyte algae, all of which exhibit a haplo-biontic life cycle (the vegetative phase is haploid and produces gametes; only the zygote is diploid [7]). Finally, all these groups are found primarily in shallow freshwater habitats that are subject to periodic drying [2], which makes it probable that the first land plants were derived from a freshwater rather than a marine environment. Dormancy stages are absent from marine algae, but common in freshwater taxa [2,7].

Many of the characters mapped onto the tree in Figure 1 have evolved independently in chlorophyte green algae, which, despite most major groups having at least a few terrestrial members, have largely failed to diversify greatly on land [17,35,36]. The occurrence of characters such as loss of zoospores, evolution of non-motile vegetative phase, sex and oogamy, branching thalli, and resistant zygospores in chlorophyte green algae suggests that these features in the ancestors of land plants were not sufficient to enable the dramatic diversification of the embryophytes on land. Why the charophyte algae gave rise to one broadly successful clade of land plants, whereas other green algae that colonized land maintained a general stasis in diversity and form is an interesting question.

The fossil record and timing of divergence

The fossil record of most charophyte algae is scarce and provides few clues to phylogeny, and minimum dates for some groups might be greatly underestimated [37]. However, fossils are abundant for Charales and several related extinct groups. Calcified oogonia of these algae (gyrogonites) first occur in deposits of the lower Silurian [425 million years ago (Mya)] and extinct taxa are more diverse than extant forms [24,38]. Fossils of other charophyte algal groups are known only from rocks younger than the Silurian [39–41]. The phylogeny shown in Figure 1 suggests that these groups originated before Charales, and their general lack of calcification might account for the lack of older fossils.

The earliest land plant fossils are small, dispersed spores from the Ordovician (475 Mya) [39,40,42], although other spore-like microfossils (cryptospores) have been reported from the mid-Cambrian (540 Mya) [43,44]. Based on one molecular study, land plants originated an astonishing 700 Mya [45], although another molecular analysis suggested a later, more orthodox age of 450 Mya [46]. In any case, the earliest land plant fossils and proposed age of origin are older than the earliest gyrogonites in the Silurian. The divergence of Charales and related extinct groups from land plants must have occurred before the Ordovician, but current data do not reveal when.

The success of land plants is hypothesized to be linked to symbiotic associations with arbuscular mycorrhizae [45,47]. Fungal material has been found associated with green algae, in particular with Charales fossils [48,49]. Finding additional charophyte algal fossils, and perhaps associated fungal material, will improve our interpretation of phylogenetic patterns and will help calibrate the

timing of major events in the evolution of these algae and their terrestrial relatives.

Fossil data might yet provide the Rosetta Stone for interpreting molecular data and dating divergences of charophyte algae and land plants. But as Chapman and Waters [50] pointed out, charophyte algae have been evolving along several independent lineages for several hundred million years at least. These authors questioned whether the molecular and morphological characters that have been evolving after the lineages diverged might hide the signal of those characters inherited from a common ancestor and make phylogeny impossible to discern. Perhaps, but we believe that too few genes and genomes have been investigated to date to draw such a firmly pessimistic conclusion.

The enigma of *Mesostigma*

The placement of the unicellular *Mesostigma viride*, the sole known member of Mesostigmatales, has been the focus of several studies that have yielded conflicting results. This scaly flagellate unicell was initially classified in a paraphyletic assemblage of unicellular algae (Class Prasinophyceae) [5]. Later study of ultrastructural features led to its placement in the lineage of charophyte algae and land plants [51], which meant it might resemble the unicellular ancestor of the group. Molecular studies of the nuclear genes actin [52] and 18S rDNA, as well as the plastid-encoded *rbcL* gene [18], supported the initial ultrastructural classification of *Mesostigma* as a charophyte alga, although its precise placement remained elusive. These single-gene analyses yielded hypotheses ranging from a position basal to all other charophyte algae and land plants [18] to a position as sister the coleochaetalean genus *Chaetosphaeridium* [15].

Additional sequence data have not solidified the phylogenetic position of *Mesostigma*. The four-gene analysis [8] corroborated the *rbcL* topology [18] (Figure 1), whereas analysis of 53 genes from the plastid genome [53] and 19 genes from the mitochondrial genome of *Mesostigma* [54] placed the genus as a third independent lineage of green algae diverging before the chlorophyte and charophyte algal clades. By contrast, Martin *et al.* [55] performed an analysis of 41 plastid genes that placed *Mesostigma* in a position consistent with the *rbcL* and four-gene analyses (Figure 1).

Clearly, a more thorough sampling of taxa across green algae and embryophytes is needed to resolve the placement of *Mesostigma*. Furthermore, one is left wondering if the discovery of additional enigmatic lineages of green algae might dramatically alter our understanding of the evolution and phylogeny of green algae and land plants.

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