

Phylogeny and evolution of charophytic algae and land plants

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Abstract Charophytic algae and land plants together make up a monophyletic group, streptophytes, which represents one of the main lineages of multicellular eukaryotes and has contributed greatly to the change of the environment on earth in the Phanerozoic Eon. Significant progress has been made to understand phylogenetic relationships among members of this group by phylogenetic studies of morphological and molecular data over the last twenty-five years. *Mesostigma viride* is now regarded as among the earliest diverging unicellular organisms in streptophytes. Characeae are the sister group to land plants. Liverworts represent the first diverging lineage of land plants. Hornworts and lycophytes are extant representatives of bryophytes and vascular plants, respectively, when early land plants changed from gametophyte to sporophyte as the dominant generation in the life cycle. *Equisetum*, Psilotaceae, and ferns constitute the monophyletic group of monilophytes, which are sister to seed plants. Gnetales are related to conifers, not to angiosperms as previously thought. *Amborella*, Nymphaeales, Hydatellaceae, Illiciales, Trimeniaceae, and *Austrobaileya* represent the earliest diverging lineages of extant angiosperms. These phylogenetic results, together with recent progress on elucidating genetic and developmental aspects of the plant life cycle, multicellularity, and gravitropism, will facilitate evolutionary developmental studies of these key traits, which will help us to gain mechanistic understanding on how plants adapted to environmental challenges when they colonized the land during one of the major transitions in evolution of life.

Key words charophytes, evolution, gravitropism, land plants, life cycle, multicellularity, origin, phragmoplast, phylogeny, plasmodesmata, the tree of life.

The origin of land plants (embryophytes) was one of the major events in history of life; it irreversibly changed the evolutionary course of life and the environment on earth (Graham, 1993; Gray, 1993; Kenrick & Crane, 1997; Hagemann, 1999; Gensel & Edwards, 2001). To gain a full understanding of how such a major evolutionary transition was unfolded, it is not only necessary to study the event itself, but also essential to investigate other events and processes that led to and happened after the origin of land plants, which undoubtedly contributed to the evolutionary success of this large clade of photosynthetic eukaryotes. A fully resolved phylogeny of major lineages of land plants and their algal relatives represents a foundation for comparative biological research on extant and extinct organisms to elucidate the nature of these events. Until recently, however, critical parts of the phylogeny of land plants and their close algal relatives had remained elusive, despite one and half century's effort by plant systematists on exploring morphological, ultrastructural, phytochemical, and serological characters since Charles Darwin (1859) proposed that all life shared common descent. Over the last

twenty-five years, a rapid progress has been made in molecular systematics, as development of PCR, cloning, automated DNA sequencing technology, and high-speed computing hardware and software has permitted extensive surveys of living organisms. For the first time in the history of biology, an unprecedented amount of historical information encoded in the genomes has become available to rigorous quantitative analysis for resolving many difficult phylogenetic problems. In this paper, I will review the recent progress on phylogenetic reconstruction of charophytic algae and land plants. Because phylogenetic hypotheses play an important role in shaping our understanding of evolution of organisms, I will also discuss implications of the new phylogenetic hypotheses on several key aspects of plant evolution, especially focusing on recent progress in genetic and developmental biological studies of the plant life cycle, multicellularity, and gravitropism. Hopefully, this dual focus approach will bring a more comprehensive understanding on evolution of plants.

1 Phylogeny of charophytic algae and land plants

Reconstructing phylogeny of organisms has been

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one of the main goals of evolutionary biologists ever since the publication of Charles Darwin's theory of evolution (Darwin, 1859). In fact, biologists before the mid-1800's were already pursuing the interconnected relationships among organisms in their classification work (Mayr, 1982). Formulation of cladistic principles by Hennig (1966) and others in the 1960s–'70s established a clear conceptual framework to uncover relationships among organisms through examination of their similarities and differences. The rise of molecular biology and advancement of computer science in the 1980's unlocked an unprecedented amount of information and analytical power for quantitative analyses, making it possible to realize the dream of reconstructing the Tree of Life (Haeckel, 1866). However, the development path of phylogenetics has not been without detour. Early morphological cladistic studies made a great contribution to systematics by establishing the first explicit phylogenetic frameworks for many groups of organisms, but mis-interpretation of character homology and underestimation of homoplasy resulted in some major erroneous hypotheses. During the early phase of molecular systematics, use of single genes, often without extensive taxon sampling, produced a bewildering array of competing phylogenetic hypotheses, creating an impression that molecular phylogenetic analysis was just another one of those methodological innovations that came and went. Fortunately, this dilemma was soon ended with the invention of automated DNA sequencing technology, which enabled most systematists to use several genes, often from one to several cellular compartments, to conduct phylogenetic studies for virtually any group of organisms. With extensive taxon sampling, this multigene approach, dubbed the supermatrix approach, has proven most effective to tackle difficult phylogenetic problems (Delsuc et al., 2005). More recently, genomic scale data have been applied to phylogenetic reconstruction of organisms, but this approach has so far received only mixed results, primarily because of analytical errors amplified by the imbalance of under-sampling of taxa and over-sampling of characters (Leebens-Mack et al., 2005; Brinkmann & Philippe, 2008; Heath et al., 2008). One contribution emerging from phylogenomic studies is the analyses of genomic structural changes, which have been done back in the early days of molecular systematics. This type of analyses, because of character selection based on frequency of changes and a large number of them available in entirely sequenced genomes, can provide independent data sets and is often quite informative in

resolving difficult phylogenetic problems (Kelch et al., 2004; Jansen et al., 2007). Furthermore, as high through-put sequencing technology develops, which allows increased taxon sampling, and our ability to understand and solve problems in phylogenomic studies enhances (Brinkmann & Philippe, 2008), phylogenomic analysis is likely to become more commonly used to resolve difficult phylogenetic issues. Following the tradition in systematics of using all sources of data, modern phylogeneticists have much more information and many more tools to unravel the historical patterns among organisms resulted from evolution. Clearly, a lot of progress has been made on clarifying phylogenetic patterns among organisms by taking an integrated approach.

1.1 Phylogeny of charophytic algae

Characeae, *Coleochaete*, Desmidiaceae, and Zygnemataceae (all of Charophyceae), together with *Fritschiella*, *Oedogonium*, and *Ulothrix* (all of Chlorophyta, see Lewis & McCourt, 2004), were among the green algae that were discussed as potential relatives of land plants from the mid 1800's to the early 1900's (Pringsheim, 1860, 1878; Celakovsky, 1874; Bower, 1890, 1908, 1935; Fritsch, 1916; Svedelius, 1927). Characeae were in fact often mistaken to be higher multicellular plants and placed together with mosses, e.g., in Celakovsky (1874). However, it was not until the discovery of the phragmoplast in *Chara*, *Coleochaete*, and *Spirogyra* around 1970 that the status of these algae as the closest algal relatives of land plants was firmly established (Pickett-Heaps, 1967, 1975; Fowke & Pickett-Heaps, 1969; Pickett-Heaps & Marchant, 1972; Marchant & Pickett-Heaps, 1973). A formal circumscription of Charophyceae by Mattox and Stewart in 1984, based on information from cell division and ultrastructure of the flagellar apparatus, largely defined membership of this important group of green algae, which included: Chlorokybales, Klebsormidiales, Zygnematales, Coleochaetales, and Charales. Recent studies, mostly molecular phylogenetic ones, have accumulated a large body of evidence to support the hypothesis that land plants indeed had a charophytic ancestry (Delwiche et al., 1989; Manhart & Palmer, 1990; Melkonian et al., 1995; Chapman et al., 1998; Karol et al., 2001; Petersen et al., 2006; Lemieux et al., 2007; Turmel et al., 2007). However, two questions have figured conspicuously in studies of these algae over the last twenty-five years. One concerns whether the scaly green flagellate alga *Mesostigma viride* Lauterborn is a member of charophytes or not. The other asks which group of charophytes represents the sister lineage of

land plants.

Mesostigma viride was not in Charophyceae as originally circumscribed by Mattox and Stewart (1984), yet the species was shown to have a multilayered structure (MLS) in its flagellar apparatus that is very similar to that of charophytes and land plants (Rogers et al., 1981; Melkonian, 1989). Since these two studies, molecular phylogenetic analyses have obtained conflicting results, with two main different positions for the taxon. Two early molecular phylogenetic studies, analyzing nuclear encoded small subunit (SSU) rRNA gene and actin gene respectively, placed the species at the base of streptophytes (i.e., charophytes + land plants) (Melkonian et al., 1995; Bhattacharya et al., 1998). However, a phylogenomic study analyzing both entire chloroplast genome sequences and genomic structural changes showed that the species represented the first diverging lineage in Viridiplantae (i.e., Prasinophyceae, Chlorophyta, and Streptophyta) (Lemieux et al., 2000). It was quickly pointed out that this result might be an analytical artifact caused by sparse taxon sampling (Qiu & Lee, 2000). Several recent studies, either analyzing sequences of more genes from nuclear, mitochondrial, and chloroplast genomes or surveying distribution of nuclear gene families, have confirmed a streptophytic affinity of the species (Karol et al., 2001; Kim et al., 2006; Nedelcu et al., 2006; Petersen et al., 2006; Rodriguez-Ezpeleta et al., 2007). More importantly, the original authors who published the result of placing *Mesostigma* as the sister to the rest of Viridiplantae have analyzed a data set with significantly increased taxon sampling (including almost all major lineages of land plants as well as many green and other algae) and more varieties of methods. They conclude that the species is indeed a member of streptophytes, specifically being sister to *Chlorokybus atmophyticus*, and that the two taxa constitute the first diverging lineage within streptophytes (Lemieux et al., 2007) (Fig. 1).

Although claimed to be a monophyletic group when formally defined (Mattox & Stewart, 1984), charophytes are now clearly established as a paraphyletic group (Bremer, 1985; Mishler & Churchill, 1985; Sluiman, 1985; Melkonian et al., 1995; Chapman et al., 1998; Karol et al., 2001; Qiu et al., 2006b, 2007; Lemieux et al., 2007; Turmel et al., 2007). A question that has intrigued many botanists who study the green algae-land plants transition is which one of the extant charophyte lineages is sister to land plants. While *Coleochaete* was favored as the closest extant algal relative of land plants in an early cladistic analy-

sis of morphological and biochemical characters (Graham et al., 1991), two recent molecular phylogenetic studies, with sufficient sampling of taxa and genes (from all three cellular compartments), have suggested that Charales are sister to land plants with moderate to strong bootstrap support (Karol et al., 2001; Qiu et al., 2006b) (Fig. 1). This hypothesis is also consistent with the data from group II intron distribution in the chloroplast genome as well as gene content, gene order, and intron composition in the mitochondrial genome of charophytes and land plants (Turmel et al., 2003, 2006, 2007). However, a phylogenomic analysis of 76 chloroplast proteins and genes has challenged this view, indicating that Zygnematales are sister to land plants (Turmel et al., 2006). Two factors might have contributed to this result. One is the highly rearranged chloroplast genomes in the two zygnematalean taxa that have been investigated, *Staurastrum punctulatum* and *Zygnema circumcarinatum*, both of which lack an inverted repeat typically present in the chloroplast genomes of photosynthetic eukaryotes (Turmel et al., 2007). It has been known for some time that loss of the inverted repeat can cause dramatic rearrangement in the chloroplast genome and produce many autapomorphic (unique) structural changes (Palmer & Thompson, 1982). The other is the two large evolutionary gaps involved in the taxa analyzed, one between *Mesostigma/Chlorokybus* and *Staurastrum/Zygnema/Chaetosphaeridium/Chara*, and the other between the latter group and land plants. These large evolutionary gaps can easily lead phylogenetic analysis astray, particularly in phylogenomic analyses of gene-rich, taxon-sparse sequence matrices, where taxon-character imbalance is so severe that systematic biases in the data sets become virtually irremovable (Delsuc et al., 2005; Leebens-Mack et al., 2005; Brinkmann & Philippe, 2008; Heath et al., 2008). While the zygnematalean ancestry of land plants hypothesis certainly should not be abandoned yet, further work in three areas can help to test whether it is an analytical artifact. First, chloroplast genomes of more taxa need to be analyzed, including *Klebsormidium*, *Entransia*, *Coleochaete*, and more members of Charales and Zygnematales. Second, a parallel study of mitochondrial genomes needs to be conducted, as the data from this genome already shows incongruence with those from chloroplast genomes (Turmel et al., 2007). Finally, more analyses using the supermatrix approach to sample many nuclear, mitochondrial, and chloroplast genes can help to distinguish the two hypotheses.

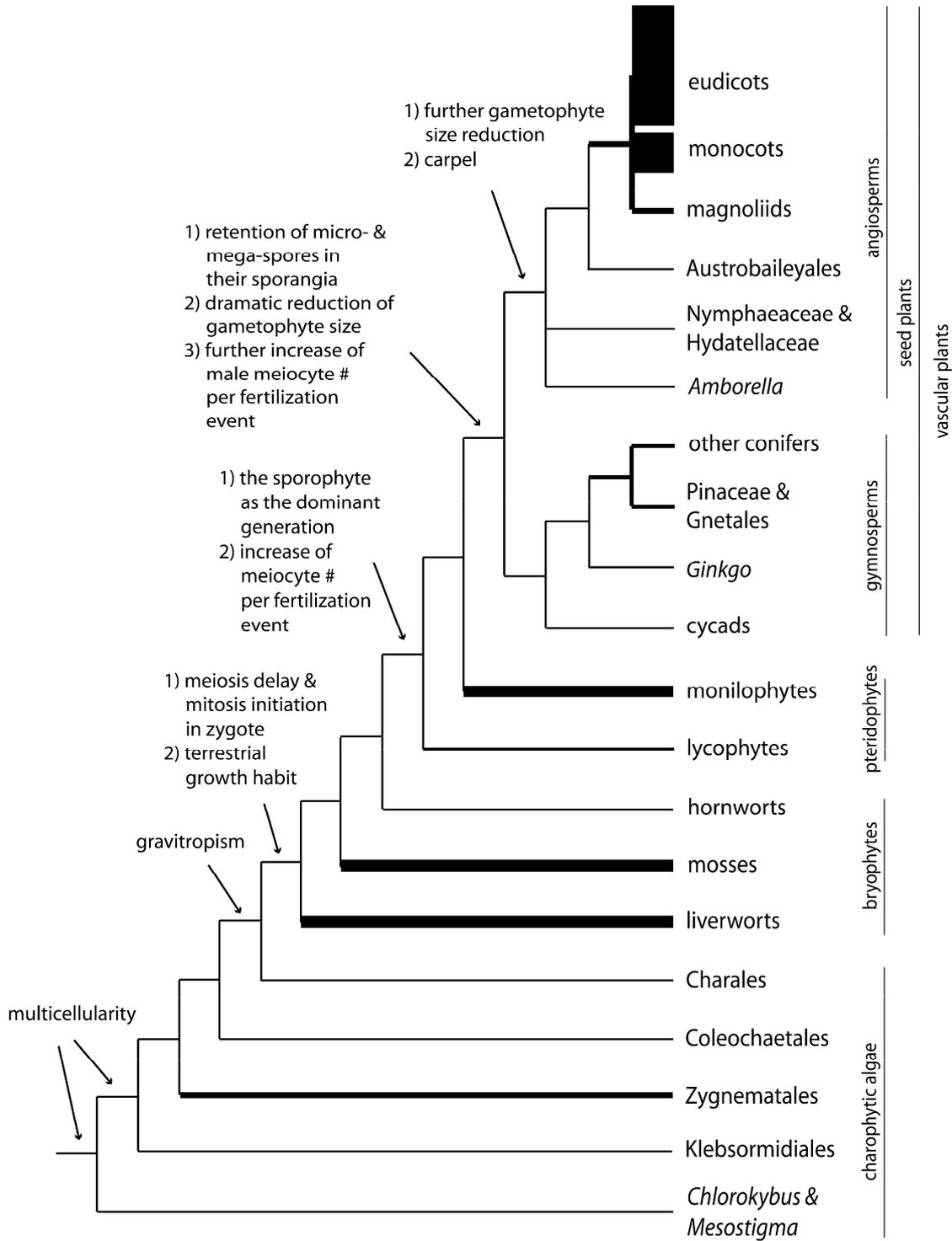


Fig. 1. A representative phylogeny of charophytic algae and land plants. The thicker lines are roughly proportional to the species numbers in the clades (the clades with <500 species are drawn with thin lines). Major evolutionary changes or evolution of major features are labeled at some nodes.

In addition to progress on these two major questions, one recent development in phylogenetic investigation of charophytic algae involves the fresh water species *Entransia fimbricata* Hughes. It was only discovered about half century ago (Hughes, 1948) and was placed in Zygnemataceae tentatively (see McCourt et al., 2000). In a major molecular phylogenetic survey of Zygnematales, the species was shown to be placed outside of the order and instead was grouped with *Chaetosphaeridium* with weak bootstrap support (Mccourt et al., 2000). Three later studies with more broad sampling of charophytes showed that *Entransia* was sister to *Klebsormidium* with moderate to strong bootstrap support (Karol et al., 2001; Turmel et al., 2002; Sluiman et al., 2008). The study by Sluiman et al. (2008) also reported that another obscure green algal taxon, *Hormidiella*, might belong to Klebsormidiales, a position originally proposed based on ultrastructural evidence (Lokhorst et al., 2000). These two examples, together with the recent resolution of phylogenetic position for *Mesostigma*, demonstrate that charophytes are vastly under-studied relative to their importance in our quest to understand the origin of land plants. Future research should place some emphasis on diversity exploration in this group of green algae of pivotal importance, as recently stressed elsewhere (Lewis & McCourt, 2004). More missing links may be discovered that will fill large gaps among currently divergent groups and facilitate phylogenomic and other evolutionary studies.

1.2 Phylogeny of land plants—bryophytes

The monophyly of land plants has been robustly established by phylogenetic analyses of morphological and biochemical data (Bremer, 1985; Mishler & Churchill, 1985; Kenrick & Crane, 1997), multigene supermatrices (Qiu et al., 2006b, 2007), and chloroplast genome sequences and gene content (Lemieux et al., 2007), although an early morphological cladistic study suggested that land plants may not be a strictly monophyletic group (Sluiman, 1985). This is one of the few major phylogenetic issues for which there was much controversy in the pre-cladistic days but explicit phylogenetic studies quickly reached a consensus. On the other hand, relationships among basal lineages of land plants have been vigorously debated over the last twenty-five years, from morphological cladistic studies to molecular phylogenetic analyses of genome sequences and multigene supermatrices. Three questions are at the center of the debate. First, do bryophytes constitute a mono- or paraphyletic group? If they form a paraphyletic group, two questions then follow. Which group of bryophytes represents the first

diverging lineage of land plants, and which lineage is sister to vascular plants?

Two early cladistic studies of morphological and biochemical characters concluded that bryophytes were a paraphyletic group (Mishler & Churchill, 1984; Bremer, 1985). This hypothesis was later confirmed by two analyses of somewhat different morphological data sets (Kenrick & Crane, 1997; Renzaglia et al., 2000). However, two studies of spermatogenesis characters reached a conclusion that bryophytes represent a monophyletic group (Garbary et al., 1993; Renzaglia et al., 2000). Two recent studies of entire chloroplast genome sequences, sampling one species each from liverworts, mosses, and hornworts, also recovered a monophyletic group of bryophytes, which is sister to vascular plants (Nishiyama et al., 2004; Goremykin & Hellwig, 2005). On the other hand, an extensive survey of three mitochondrial group II introns across 350 diverse land plants and several red and green algae showed that liverworts exhibit the same condition of lacking the introns as the algae, supporting the paraphyly hypothesis (Qiu et al., 1998). This conclusion was recently reinforced by an expanded study of 28 mitochondrial group II introns in a smaller number of taxa (Qiu et al., 2006b). Most recently, parsimony and likelihood analyses of a multigene supermatrix with extensive taxon sampling of bryophytes and vascular plants have shown that the paraphyly of bryophytes is virtually indisputable (Qiu et al., 2006b). In consideration of all above-cited studies, it is clear that the paraphyly hypothesis of bryophytes has strong support from diverse sources of data, whereas the monophyly hypothesis is only supported by the studies that suffer from weakness in either character or taxon sampling. Hence, the paraphyly of bryophytes can be regarded as one of the most clearly established aspects of the early land plant phylogeny (Fig. 1).

Identifying the earliest diverging lineage of land plants can provide significant insight into the algae-land plants transition. Early cladistic studies of morphological and biochemical data suggested that liverworts occupied such a position (Mishler & Churchill, 1984; Bremer, 1985; Kenrick & Crane, 1997). This hypothesis, however, was challenged by at least four subsequent analyses, one on morphological and developmental characters (Renzaglia et al., 2000) and three on multigene matrices (Nishiyama & Kato, 1999; Nickrent et al., 2000; Renzaglia et al., 2000), which all argued that hornworts represented the sister lineage to the rest of land plants. A large survey of three mitochondrial group II introns, on the other

hand, provided some of the most unequivocal evidence supporting liverworts as the basalmost lineage in land plants (Qiu et al., 1998). This result was later corroborated by an independent study of chloroplast genomic structural changes as well as an expanded survey of 28 mitochondrial group II introns (Kelch et al., 2004; Qiu et al., 2006b). Furthermore, two phylogenomic analyses of entire chloroplast genome sequences, after sufficient taxon sampling of major land plant lineages was achieved, produced the same topology (Wolf et al., 2005; Qiu et al., 2006b). Finally, both parsimony and likelihood analyses of a multigene supermatrix with extensive taxon sampling across all major lineages of land plants resolved the liverworts' basalmost position in land plants with strong support (Qiu et al., 2006b).

The paraphyly of bryophytes and the basalmost position of liverworts in land plants were resolved in three early cladistic studies of morphological and biochemical data (Mishler & Churchill, 1984; Bremer, 1985; Kenrick & Crane, 1997), and they stood tests by numerous molecular phylogenetic analyses over the last twenty-five years. However, the status of mosses as the sister lineage to vascular plants established in those studies was challenged very early on by molecular studies. Hornworts were often recovered as sister to vascular plants in some early single gene analyses (Lewis et al., 1997; Samigullin et al., 2002; Dombrowska & Qiu, 2004). More convincing evidence for this position of hornworts came in several recent studies of chloroplast and mitochondrial genomic structural features (Malek & Knoop, 1998; Kelch et al., 2004; Groth-Malonek et al., 2005) and phylogenomic analyses of entire chloroplast genome sequences (Wolf et al., 2005; Qiu et al., 2006b). In particular, phylogenetic analyses of a supermatrix with dense taxon sampling in charophytes, bryophytes, pteridophytes, and seed plants have provided decisive support to the position of hornworts as the sister to vascular plants (Qiu et al., 2006b). In retrospect, the early morphological cladistic studies mis-interpreted analogy of vascularized conducting tissues in moss sporophytes and vasculature in vascular plants. On the other hand, development of nutritionally largely independent sporophytes in hornworts (Stewart & Rodgers, 1977), a key character syndrome that facilitates completion of alternation of generations during early evolution of land plants, has been greatly under-appreciated (Qiu et al., 2006b, 2007). This is an exemplar case where new molecular phylogenetic results lead to discovery of previously neglected morphological and developmental characters and

consequently greatly enhance our understanding of plant phylogeny and evolution through reciprocal illumination (Hennig, 1966).

1.3 Phylogeny of land plants—pteridophytes

While the knowledge of extinct fossil taxa is essential for our understanding of the origin and evolution of vascular plants, I will limit this review mostly to studies of extant plants in order to keep the paper within a reasonable length. Among several extant basal vascular plant lineages, Psilotaceae were often compared to extinct early vascular plants Rhyniopsida and were suggested to be sister to the rest of extant vascular plants in a morphological cladistic study (Bremer, 1985). However, discovery of a 30 kb inversion in the chloroplast genome shared by all vascular plants except lycophytes, which exhibit the same condition as bryophytes, clinched the status of lycophytes as the earliest diverging lineage among extant vascular plants (Raubeson & Jansen, 1992). This result has recently been confirmed by completely sequenced chloroplast genomes of *Physcomitrella patens* (Sugiura et al., 2003), *Anthoceros formosae* (Kugita et al., 2003), *Huperzia lucidula* (Wolf et al., 2005), and *Psilotum nudum* (Wakasugi et al., unpublished). In *Selaginella uncinata*, however, there is a shorter (20 kb) inversion in the same region of the chloroplast genome that appears to show the same condition as non-lycophyte vascular plants (Tsuji et al., 2007). Nevertheless, adjacent genes immediately outside of this inversion still exhibit the same order as those in *Huperzia lucidula*, thus suggesting that this species acquired a superficially similar inversion via an independent genome rearrangement event. Recently, the position of lycophytes as the sister to other vascular plants has also been corroborated by analyses of a multigene supermatrix with extensive sampling of all major land plant lineages (Qiu et al., 2007) (Fig. 1).

The other major breakthrough in pteridophyte systematics over the last twenty-five years is represented by identification of a major clade that unites *Equisetum*, Psilotaceae and true ferns and placement of this clade as the sister to seed plants (Fig. 1). This clade, named monilophytes, was first recognized in a morphological cladistic analysis on extinct and living taxa, and it possesses one synapomorphy, mesarch protoxylem confined to the lobes of the xylem strand (Kenrick & Crane, 1997). Later, a molecular phylogenetic study identified the clade with strong bootstrap support and also uncovered a highly diagnostic three codon insertion in the chloroplast gene *rps4* (Pryer et al., 2001). A large-scale phylogenetic study

with extensive taxon sampling in bryophytes, pteridophytes, and seed plants have also identified this clade and placed it as the sister to seed plants with strong statistical support (Qiu et al., 2007). Resolution of these relationships represents significant progress toward achieving a complete understanding on the origin of seed plants, as early morphological cladistic studies have had great difficulty to clarify relationships among the so-called fern allies (*Equisetum*, Psilotaceae, and lycophytes), ferns, and seed plants (Bremer, 1985; Garbary et al., 1993).

While placement of *Equisetum* and Psilotaceae with true ferns in the monilophyte clade has clarified relationships among early vascular plants, relationships among these two taxa, two eusporangiate fern families (Marattiaceae and Ophioglossaceae), and the clade of leptosporangiate ferns are still not resolved. The only resolved part here is the sister relationship between Psilotaceae and Ophioglossaceae, which receives strong bootstrap support in molecular phylogenetic analyses (Pryer et al., 2001; Qiu et al., 2007). Future studies that sample more genes from mitochondrial, nuclear, and chloroplast genomes may offer resolution to this problem.

1.4 Phylogeny of land plants—seed plants

Early morphological cladistic analyses of extinct and extant taxa concluded that seed plants were a monophyletic group (Crane, 1985; Doyle & Donoghue, 1986), although there was a possibility of biphyletic origin of seed plants (Doyle & Donoghue, 1986). The seed plant monophyly has now been clearly confirmed by a large-scale molecular phylogenetic study that sampled both non-seed plants and seed plants extensively (Qiu et al., 2007).

The other major finding from several morphological cladistic analyses of seed plants (Crane, 1985; Doyle & Donoghue, 1986; Nixon et al., 1994; Rothwell & Serbet, 1994) was a close relationship between Gnetales and angiosperms, but this result was almost never recovered in any molecular study. Instead, Gnetales were often shown to be sister to Pinaceae (Goremykin et al., 1996; Winter et al., 1999; Bowe et al., 2000; Chaw et al., 2000; Frohlich & Parker, 2000; Gugerli et al., 2001; Magallon & Sanderson, 2002; Soltis et al., 2002; Burleigh & Mathews, 2004; Qiu et al., 2007) (Fig. 1) or sometimes to conifers (Chaw et al., 1997; Burleigh & Mathews, 2004). In analyses of fast-evolving genes or nucleotide positions, typically chloroplast genes or 3rd codon positions of other genes, Gnetales were placed as the sister to all other seed plants (Magallon & Sanderson, 2002; Rydin et al., 2002). Only in a study of nuclear 18S and 26S

rRNA genes, Gnetales were shown to be sister to angiosperms, but with low bootstrap support (Rydin et al., 2002). Given the kinds and number of genes sampled, the diversity of taxon sampling schemes used, and the variety of methods employed in all these analyses, it is difficult to imagine what types of systematic errors were present in all these molecular data sets that would prevent recovery of a close relationship between Gnetales and angiosperms if there was one. Therefore, it seems reasonable to conclude that Gnetales are related to conifers rather than to angiosperms.

One of the most spectacular discoveries in molecular systematics over the last two and half decades is the identification of several basal angiosperm taxa as the earliest diverging lineages among living angiosperms, which include *Amborella*, Nymphaeales, Hydatellaceae, and Illiciales/Trimeniaceae/*Austrobaileya* (ANHITA; Fig. 1) (Mathews & Donoghue, 1999; Parkinson et al., 1999; Qiu et al., 1999; Soltis et al., 1999; Barkman et al., 2000; Graham & Olmstead, 2000; Saarela et al., 2007). Subsequent analyses sampling more genes and employing more varieties of analytical methods have solidified this result (Qiu et al., 2000, 2001, 2005, 2006a; Zanis et al., 2002; Borsch et al., 2003; Hilu et al., 2003; Stefanovic et al., 2004; Leebens-Mack et al., 2005; Jansen et al., 2007; Moore et al., 2007). Until recently, it was thought that the diversification pattern among the earliest angiosperms could never be resolved despite nearly two centuries of research (see Qiu et al., 1993). The divergence gap between ANHITA and euangiosperms (angiosperms exclusive of *Amborella*, Nymphaeales, Hydatellaceae, and Illiciales/Trimeniaceae/*Austrobaileya* (Qiu et al., 1999)) in fact is quite large, and has been identified in most molecular phylogenetic studies that sample several genes (Parkinson et al., 1999; Qiu et al., 1999, 2005, 2006a; Graham & Olmstead, 2000; Soltis et al., 2000; Borsch et al., 2003; Stefanovic et al., 2004; Jansen et al., 2007; Moore et al., 2007). This gap has also been independently corroborated by studies of fossil evidence (Friis et al., 1999) and morphology (Endress & Igersheim, 2000; Williams & Friedman, 2002). In retrospect, early appearance of ANHITA in angiosperm evolution was already detected in the comparative analyses of extant angiosperms (Stebbins, 1974; Endress, 1986) and fossil record (Upchurch, 1984). This is another exemplar case where phylogenetic relationships, once resolved by molecular systematic studies, suddenly reveal a consistent evolutionary pattern in the data that already existed and are being gathered, shedding significant

light on a long-standing evolutionary enigma—the origin of angiosperms in this case.

Another spectacular discovery in the recent history of plant systematics involves the recognition of a large monophyletic group of angiosperms termed eudicots (or tricolpates) (Doyle & Hotton, 1991), which encompasses 75% of extant angiosperm diversity (Mabberley, 1987) (Fig. 1). It was suggested as early as in the 1930's that angiosperms with tricolpate pollen and derived pollen types may represent a natural group based on extensive surveys of extant and fossil angiosperm pollen (Wodehouse, 1935, 1936). Several other authors later supported this hypothesis from their comparative studies of plant morphology and pollen (Bailey & Nast, 1943; Hu, 1950; Walker & Doyle, 1975). In an explicit cladistic analysis of basal angiosperms using morphological data, the monophyly of eudicots was established for the first time (Donoghue & Doyle, 1989). However, limited taxon sampling in that study prevented this finding from being widely recognized. The first large-scale molecular phylogenetic analysis of angiosperms using sequences of the chloroplast gene *rbcL* established monophyly of this large group beyond any doubt (Chase et al., 1993). All major molecular phylogenetic studies of angiosperms since then have shown that monophyly of eudicots is one of the best established aspects of the angiosperm phylogeny (Qiu et al., 1999, 2006a; Savolainen et al., 2000; Soltis et al., 2000; Hilu et al., 2003; Jansen et al., 2007; Moore et al., 2007). Evolution of tricolpate pollen turns out to be such an infrequent event that it happened only twice outside eudicots, once in Illiciaceae and once in Arecaceae (the palm family), and in both cases the pollen developmental pattern is actually different from that in eudicots (see Cronquist, 1981; Qiu et al., 1993). The history of discovery of this large clade of land plants, spanning more than half century, demonstrates that our ability to explore the nature is highly dependent on advancement of technology. In this particular case, invention of light microscope, electron microscope, DNA sequencing techniques, and computer all had an instrumental role in the eventual recognition of this major clade of angiosperms.

Besides these two major findings, recent molecular phylogenetic analyses of large data sets with extensive sampling of genes and taxa have greatly clarified relationships among angiosperms (Hilu et al., 2003; Qiu et al., 2005, 2006a). Overall, euangiosperms can be divided into five monophyletic groups: *Ceratophyllum*, Chloranthaceae, eudicots, magnoliids (which include two pairs of sister taxa, Canel-

ales/Piperales and Magnoliales/Laurales), and monocots. Currently, relationships among three large clades with significant diversity, magnoliids, monocots, and eudicots, have been resolved differently in studies using multigene and phylogenomic data sets. In a study of analyzing 8 mitochondrial, chloroplast, and nuclear genes from 144 taxa with a compatibility method, magnoliids and eudicots were shown to be sister to each other, and they were sister to monocots (Qiu & Estabrook, 2008). However, in two analyses of entire chloroplast genome sequences, monocots were sister to eudicots, and together they were sister to magnoliids (Jansen et al., 2007; Moore et al., 2007). Because the power of the compatibility method in resolving deep phylogenetic patterns remains relatively untested, and phylogenomic studies have often suffered from systematic errors in data sets (Delsuc et al., 2005; Leebens-Mack et al., 2005; Brinkmann & Philippe, 2008; Heath et al., 2008), it is best to view relationships among the major angiosperm lineages as unresolved at present.

2 Evolutionary implications of a newly reconstructed phylogeny of charophytic algae and land plants

From the above review, it is clear that our understanding on the phylogeny of charophytic algae and land plants has been significantly improved over the last two and half decades (Fig. 1). Several cladistic analyses of mostly morphological characters for the first time formulated explicit phylogenetic hypotheses on relationships among major lineages of these photosynthetic eukaryotes (Mishler & Churchill, 1984, 1985; Bremer, 1985; Crane, 1985; Doyle & Donoghue, 1986; Donoghue & Doyle, 1989; Graham et al., 1991; Kenrick & Crane, 1997). These hypotheses served as paradigms for guiding evolutionary studies of various aspects of these organisms during this period, for example, origin of sporopollenin in charophytes (Delwiche et al., 1989), fertilization in Gnetales and angiosperms (Friedman, 1990) and auxin metabolism in early land plants (Sztein et al., 1995; Cooke et al., 2002, 2004). Undoubtedly, these morphological cladistic studies represented a major step forward from traditional taxonomy, enforcing a rigorous criterion on identifying homologous characters and defining strictly monophyletic groups. Nevertheless, these studies also had their limitations, especially in making mis-interpretation of some of the morphological characters and under-estimating the

extent of homoplasy in plant evolution. Molecular phylogenetic studies, in particular those based on supermatrices and infrequent genomic structural changes, have a greater resolution power because of access to a much larger amount of historical information and higher quality characters (Manhart & Palmer, 1990; Raubeson & Jansen, 1992; Chase et al., 1993; Qiu et al., 1998, 1999, 2006a, b, 2007; Bowe et al., 2000; Chaw et al., 2000; Graham & Olmstead, 2000; Savolainen et al., 2000; Soltis et al., 2000; Karol et al., 2001; Pryer et al., 2001; Hilu et al., 2003; Burleigh & Mathews, 2004; Kelch et al., 2004). These molecular studies have remedied to a good extent the weakness of morphological cladistic studies, by circumventing the problem of relying on a few morphological characters that might have experienced convergent evolution due to similar selection pressure. As a result, the combined use of morphology and molecules in rigorous quantitative analyses over the last twenty-five years has led to one of the most rapid growth periods in our knowledge on evolutionary relationships among organisms.

The significantly improved organismal phylogeny is providing a momentum for the pendulum of evolutionary research to swing back to the study of mechanisms and processes. It allows tracing macro-evolutionary patterns among major clades of organisms on large scales and helps formulating hypotheses on mechanisms and processes of some major evolutionary transitions. The emergence of evolutionary developmental biology will further catalyze this transformation by providing experimental approaches to test the hypotheses. This interplay among studies of phylogenetic patterns, developmental mechanisms, and evolutionary processes in a large diversity of organisms is likely to lead to a new level of understanding on functioning and evolution of life in general. Charophytes and land plants together represent one of the major lineages in eukaryotic evolution, which spans the diversity from unicellular aquatic algae to highly evolved multicellular angiosperms. Studies of their evolutionary patterns and developmental mechanisms under a new phylogenetic framework will help us not only to understand how this major clade has evolved, but also to learn how eukaryotes in general adapt to environment challenges during several major evolutionary transitions that were not unique to plants, e.g., from unicellularity to multicellularity, from a gravity-water buoyancy environment to a gravity-air buoyancy environment, and from a haploid gametophyte to a diploid sporophyte as the dominant generation in the life cycle. Below, I will

review and discuss recent progress on genetic, developmental, and cell biological studies of several plant traits, which, when investigated with an evolutionary developmental approach under the new phylogenetic hypotheses, are likely to further our understanding of plant evolution.

2.1 Evolution of life cycle in land plants

One of the most interesting and important, but somehow recently neglected, aspects of plant evolution is the change of life cycle in various lineages of charophytic algae and land plants. The phylogeny of these organisms as currently understood (Fig. 1) and their order of appearance in the fossil record (Gray, 1993; Taylor & Taylor, 1993; Kenrick & Crane, 1997; Wellman et al., 2003) clearly demonstrate a trend of expansion of the diploid sporophyte generation with concomitant reduction of the haploid gametophyte generation. However, had the phylogenetic pattern not been clear, it would have been much more difficult, if not impossible, to detect this trend based purely on fossil evidence.

Three issues in the phylogeny of charophytic algae and early land plants, all resolved over the last several decades, can directly affect interpretation of evolution of life cycle in land plants. First, charophytes, rather than Ulvophyceae in Chlorophyta, are identified as the closest algal relatives of land plants. Although this relationship was recognized based on surveys of cell division and ultrastructure of the flagellar apparatus among green algae and land plants (Pickett-Heaps, 1975; Mattox & Stewart, 1984), phylogenetic analyses of morphological and molecular data provided robust assurance and independent corroboration to this result (Manhart & Palmer, 1990; Melkonian et al., 1995; Chapman et al., 1998; Karol et al., 2001; Lemieux et al., 2007; Turmel et al., 2007). Second, the monophyly of land plants was much less certain in the pre-cladistic time, and an early morphological cladistic study even expressed doubt on the issue (Sluiman, 1985). However, cladistic studies of morphological and biochemical data firmly established monophyly of land plants (Bremer, 1985; Mishler & Churchill, 1985; Kenrick & Crane, 1997). Recent phylogenetic analyses of molecular data have provided further and more convincing evidence to support this conclusion (Qiu et al., 2006b, 2007; Lemieux et al., 2007; Turmel et al., 2007). Finally, the paraphyly of bryophytes, though established by some early morphological cladistic studies (Mishler & Churchill, 1984; Bremer, 1985), has been challenged by both morphological and molecular phylogenetic analyses from time to time (Garbary et al., 1993;

Renzaglia et al., 2000; Nishiyama et al., 2004; Goremykin & Hellwig, 2005). The recent large scale analyses of multigene supermatrices and a broad survey of mitochondrial group II introns, all with extensive taxon sampling across land plants, have decisively resolved this issue (Qiu et al., 1998, 2006b, 2007). Failure to resolve any of these issues would have resulted in a much less clear phylogenetic pattern, impeding investigation of the origin of land plants and evolution of alternation of generations. Alternatively, if some of these three issues were resolved with different outcomes, e.g., Ulvophyceae were identified as the closest algal relatives of land plants, or bryophytes were shown to form a monophyletic group sister to vascular plants, an entirely different hypothesis, the homologous hypothesis (Pringsheim, 1878), than the one discussed below would have to be considered to explain the origin of land plants and evolution of life cycle in land plants.

The phylogenetic pattern among charophytic algae and early land plants inferred from morphological and molecular data sheds significant light on two major events in the history of plant life: colonization of land and change from a haploid gametophyte to a diploid sporophyte as the dominant generation in the life cycle. One school of thoughts, often known as the antithetic hypothesis, first developed Celakovsky in 1874 and later greatly expanded by Bower (1890, 1908, 1935) and others (Campbell, 1924; Svedelius, 1927; Smith, 1955), actually used a phylogenetic scheme that is largely congruent to what is reconstructed now to explain evolutionary changes at these two major events. These authors examined and compared developmental patterns of life cycle in various algal and plant lineages by following this phylogenetic scheme. They hypothesized that land plants originated as a consequence of interpolation of a new phase (sporophyte generation) into the life cycle of some green algae that were more likely related to today's charophytes. They further suggested that as early land plants (mosses as recognized by those early botanists) evolved, the sporophyte generation expanded through structural elaboration and progressive sterilization of potentially sporogenesis tissues and ultimately became a free-living dominant generation as seen in the life cycle of ferns and seed plants. It is noteworthy that the different ploidy levels of gametophyte and sporophyte generations (Strasburger, 1894) and meiosis (Van Beneden, 1883) (see Hamoir, 1992) were both discovered after Celakovsky (1874) had proposed this hypothesis. Hence, one has to be amazed by the power of comparative developmental biology, which can

only be realized when there is a correct phylogenetic framework to guide interpretation of the observed pattern.

The third major event in plant evolution is the origin of seed plants (and the origin of angiosperms can be regarded as an extension of this process). This event has not been so much targeted in the study of evolution of life cycle in land plants, particularly in the debate between the antithetic (Celakovsky, 1874; Bower, 1890; Campbell, 1924; Svedelius, 1927; Smith, 1955) and the homologous hypotheses (Pringsheim, 1878). Equally puzzling is that despite intense interest in the origins of seed plants and angiosperms throughout the entire last century, few have looked at the problems from a life cycle evolutionary developmental perspective, with perhaps one exception (Takhtajan, 1976), who alluded to neoteny as one of the possible mechanisms contributing to the origin of angiosperms. What has received most attention is emergence of new structures such as seeds and flowers (Crane, 1985; Doyle & Donoghue, 1986; Frohlich & Parker, 2000; Theissen et al., 2000), but equally important aspects during this phase of land plant evolution are reduction of gametophytes and further increase of male meiocyte number per fertilization event (this number, in a non-heterospory situation, was already greatly increased when the sporophyte became a dominant generation during the origin of vascular plants). These evolutionary changes are obvious when reproductive cycles of seed plants, monilophytes, and lycophytes (Gifford & Foster, 1989) are compared under a phylogenetic framework, which again has only been available from recent phylogenetic analyses of morphological and molecular data (Kenrick & Crane, 1997; Pryer et al., 2001; Qiu et al., 2007). More importantly, these changes fit the trend of sporophyte expansion and gametophyte reduction since plants colonized the land (Fig. 1). Adaptive significance of this trend in life cycle change lies in the fact that it allows generation of a larger number of genetically different gametes through increase of the meiocyte number, which then leads to occupation of more variable environmental niches on the land than in the water by more genetically variable offspring after fertilization (Svedelius, 1927).

Once this macro-evolutionary trend is revealed, it becomes relatively straightforward to design an integrated experimental strategy to explore developmental mechanisms that have shaped the pattern of life cycles in extant land plants, though it will take many years to elucidate these mechanisms. The study of developmental events in the life cycle, especially

the control of timing of meiosis initiation, has been pursued for many years in the fungal system, *Schizosaccharomyces pombe*. One particular gene that has been identified to play an important role in meiosis initiation is *mei2*, which encodes an RNA-binding protein that is essential for premeiotic DNA synthesis and the commitment to meiosis (Watanabe & Yamamoto, 1994). This gene is likely to be conserved among protists, fungi, and plants (Jeffares et al., 2004). In green algae and land plants, it appears that the gene has undergone a major duplication event, with one gene family (*TEL*) involved in cell differentiation in shoot and root meristems (Jeffares et al., 2004; Veit et al., 1998) and the other (*AML*) playing a role in vegetative meristem activity as well as meiosis in *Arabidopsis thaliana* (Kaur et al., 2006). It is no coincidence that both meiosis and mitosis are targets of action by this gene family, as cell divisions in vegetative and reproductive growth are key processes to regulate in order to mold the life cycle of a certain lineage. Hence, genes controlling the timing of mitosis and meiosis should be high priority targets to investigate in the effort to understand evolution of the life cycle in land plants. Since the primary focus of this review is on the phylogeny, no other genes will be discussed here though many have been identified (Ma, 2005; Hamant et al., 2006). The above example is provided merely to show that it is feasible to take an evolutionary developmental approach to study mechanistic aspects of evolution of life cycle in land plants.

2.2 Transition from unicellularity to multicellularity in streptophytes

Land plants represent one of the most successful groups and the most sophisticated kinds of multicellular organisms (Hagemann, 1999). The transition from uni- to multicellularity actually took place before they came onto land, and the current phylogeny suggests that it happened either in the common ancestor of all streptophytes or shortly after the origin of this clade (Fig. 1). Placement of *Mesostigma viride*, a unicellular organism, is critical to pinpoint the origin of multicellularity in this part of eukaryotic evolution. Since there is no longer any dispute on its inclusion within charophytes (Karol et al., 2001; Kim et al., 2006; Nedelcu et al., 2006; Petersen et al., 2006; Lemieux et al., 2007; Rodriguez-Ezpeleta et al., 2007), it is reasonable to suggest that multicellularity evolved in the common ancestor of streptophytes or later, because the green algae below this node on the phylogeny, Prasinophyceae, are all unicellular (Baldauf, 2003; Lewis & McCourt, 2004). On the other hand, whether *Mesostigma* alone (Karol et al., 2001;

Petersen et al., 2006) or together with *Chlorokybus* (Qiu et al., 2006b; Lemieux et al., 2007) is sister to all other streptophytes has not been clearly resolved. Hence, it is also likely that multicellularity evolved shortly after the origin of streptophytes, particularly in consideration of the sarcinoid organization in *Chlorokybus atmophyticus*, which is a packet of a few cells held together by a gelatinous matrix without plasmodesma connection (van den Hoek et al., 1995)—a quasi-state of multicellularity. Regardless of these topological variations, the currently resolved phylogenetic relationships among early diverging charophytic algae provide a sound evolutionary framework under which the transition from uni- to multicellularity can be meaningfully investigated.

Multicellularity evolved more than two dozens of times in bacteria, archaea, and eukaryotes (Bonner, 1999; Grosberg & Strathmann, 2007). It confers several advantages to organisms by enhancing their metabolic and reproductive capabilities (Niklas, 1997; Grosberg & Strathmann, 2007). First, the sheer increase of physical size allows the organisms to use resource from the environment better than their unicellular competitors. Because the cell size increase has an upper bound constrained by physico-chemical properties of phospholipids, proteins, and other compounds that make up the plasma membrane and cell wall, multicellularity provides the only solution to the problems of out-competing other organisms when resource stays the same or decreases in the environment, or increasing metabolic activity in a resource-richer environment. Multicellularity also ensures better protection of genetic material than unicellularity. Second, once multicellularity emerges, functional differentiation and specialization (division of labor) among cells in an organism will confer a greater fitness to its metabolism and reproduction. Complexity will be achieved through structural (morphological), metabolic (chemical, physiological, and behavioral), and reproductive differentiation, with formation of tissues, organs, and member groups within a social group. Indeed, empirical analyses have detected a positive correlation between size and complexity (Bell & Mooers, 1997; Bonner, 2004). Finally, because the life span of a cell is constrained by physico-chemical properties of carbohydrates, phospholipids, fatty acids, amino acids, proteins, nucleic acids, and other compounds that make up the cell, multicellular organisms have an advantage of out-living unicellular competitors.

Development of multicellularity depends on two basic processes at the cellular level, cell cohesion and

cell-cell exchange of information and materials (Alberts et al., 1989). At present, little is known about cell cohesion during the transition from uni- to multicellularity in early streptophytes. In comparison, more information is available on plasmodesmata-cytoplasmic bridges that connect adjacent cells and allow exchange of hormones, RNAs, carbohydrates, proteins, and other compounds between cells (Lucas & Lee, 2004). In eukaryotes, plasmodesmata have evolved several times independently, in Fungi, Phaeophyta, Chlorophyta, and Streptophyta (Lucas et al., 1993; Raven, 1997). Evolution of this cell-cell communication/transportation device in early streptophytes has undoubtedly contributed to the success of building large complex multicellular organisms in this lineage of eukaryotes. Among all extant charophytes, *Mesostigma viride* is probably the only ancestrally unicellular organisms. *Chlorokybus atmophyticus* is sarcinoid (no plasmodesma), exhibiting a primitive type of multicellularity. The three genera of Klebsormidiales, *Klebsormidium*, *Entransia*, and *Hormidiella* (Lewis & McCourt, 2004; Sluiman et al., 2008), all contain unbranched filamentous species (Hughes, 1948; van den Hoek et al., 1995; Lokhorst et al., 2000; Cook, 2004). There are no plasmodesmata connecting cells in species of *Klebsormidium* (van den Hoek et al., 1995) and *Entransia* (M. E. Cook, personal communication); no information about plasmodesmata is currently available in *Hormidiella*. Zygnematales contain a large number of unicellular, colonial, or unbranched filamentous species. The phylogenetic distribution of plasmodesmata in charophytes (Mccourt et al., 2000; Karol et al., 2001) suggests that unicellularity in this group might have been secondarily derived. So far, no plasmodesmata have been reported in any species of Zygnematales (van den Hoek et al., 1995). Coleochaetales and Charales are the only multicellular charophytic algae that have plasmodesmata connecting their cells (Franceschi et al., 1994; van den Hoek et al., 1995; Cook et al., 1997). From their distribution on the currently resolved phylogeny of charophytes and land plants (Fig. 1), it seems reasonable to suggest that plasmodesmata evolved in the common ancestor of Coleochaetales, Charales, and land plants.

Another structure that has likely contributed to evolution of multicellularity in streptophytes, and particularly formation of the three-dimensional plant body, is the phragmoplast, which is a unique arrangement of vesicles and microtubules during cytokinesis whereby microtubules are oriented perpendicular to the plane of cytokinesis (Fowke &

Pickett-Heaps, 1969; Pickett-Heaps, 1975). It is found primarily in Zygnematales, Coleochaetales, Charales, and land plants (Pickett-Heaps, 1967; Fowke & Pickett-Heaps, 1969; Marchant & Pickett-Heaps, 1973); elsewhere it is only found in Trentepohliales of Chlorophyta (Chapman & Henk, 1986). *Klebsormidium*, though lacking a clear phragmoplast, exhibits two characteristics required for evolution of the structure: an ingrowing cleavage furrow and a persistent system of interzonal microtubules separating daughter nuclei and derived from a spindle apparatus (Floyd et al., 1972; Pickett-Heaps & Marchant, 1972). Hence, it seems that the phragmoplast evolved shortly after streptophytes originated, as can be inferred from its distribution on the phylogeny of charophytes and land plants (Fig. 1). It has been suggested that the phragmoplast is perhaps essential for organisms to achieve a two- to three-dimensional pattern of cell division, instead of a one-dimensional, filamentous type of simple division, to develop complex plant bodies (Hagemann, 1999; Pickett-Heaps et al., 1999). Hagemann (1999) in particular has argued that the type of cell division involving formation of a phragmoplast is related to the unique way of cell wall construction in land plants, which are multicellular organisms that grow against the direction of gravity in a less buoyant medium (air, in comparison to water for most algae) and have few, if any, parallels in eukaryotes. These ideas are certainly consistent with the pattern of morphological complexity of plant bodies exhibited by various charophyte and land plant lineages on the phylogeny (Fig. 1).

The plasmodesmata and phragmoplasts, the evolution of which seems not dependent on each other, may have been largely responsible for evolution of multicellularity in streptophytes (Lucas et al., 1993; Franceschi et al., 1994; Hagemann, 1999). Identification of genes encoding various components of both structures will significantly increase our understanding on how multicellularity was achieved step by step during the transition of photosynthetic eukaryotes from the aquatic to the terrestrial environment. The knowledge accumulated from cell biology research over the last several decades has laid down a solid foundation (Pickett-Heaps et al., 1999; Lucas & Lee, 2004), but fine-scale evolutionary genetic and developmental studies are needed to elucidate the process of uni- to multicellularity transition in streptophytes. Finally, it should be added that uni- to multicellularity transition actually happened twice during streptophyte evolution: once at the gametophyte whole organism level in early evolution of charophytes and another at

the sporophyte level during the origin of land plants (only part of the organism was involved in Characeae and liverworts). For the latter, a life cycle with the diploid sporophyte generation being dominant (Svedelius, 1927; Coelho et al., 2007; McManus & Qiu, 2008) and origin of lignin (for cell cohesion) probably have also contributed to the building of large and complex multicellular plant bodies.

2.3 Origin and evolution of gravitropism in streptophytes

Land plants, as the major primary producers in the terrestrial ecosystem, have developed a body plan of a vertical axis with photosynthetic organs (leaves) in the air and absorption-anchorage organs (roots or rhizoids) in the soil. Although this body plan is best manifested in seed plants (Cooke et al., 2004), prototypic forms are found in pteridophytes, bryophytes, and Characeae. Gravitropism has played an instrumental role in the origin and evolution of this body plan, as streptophytes undergo the transition from free-swimming/planktonic green algae such as *Mesostigma viride* and Zygnematales to aquatic rhizophytic Characeae (Raven & Edwards, 2001), and to land-grown bryophytes, pteridophytes and seed plants. Essentially, two types of cells, "root" and "shoot" meristematic cells that respond to gravity positively and negatively, are responsible for building this body plan. Though gravity has always accompanied life on earth (Volkman & Baluska, 2006), in no time has it figured so conspicuously in influencing evolution of organisms as during the water-land transition of plants, since no other organisms have built a body with the size and mass of giant sequoia or eucalyptus trees. Hence, elucidating the origin and evolution of gravitropism in streptophytes will not only help us to understand the origin and evolution of land plants, but also provide insight into the role of gravity in shaping evolution of life.

The phylogeny of charophytic algae and land plants as currently reconstructed (Fig. 1) shows that gravitropism in streptophytes likely evolved in the common ancestor of Characeae and land plants, because both groups are rhizophytes (Raven & Edwards, 2001) and other charophytes are either free-swimming/planktonic or epiphytic in aquatic or terrestrial habitats (van den Hoek et al., 1995). In some early phylogenetic studies of streptophytes and green algae using nuclear 18S rDNA data, Characeae were shown to be the first diverging lineage among charophytes (Kranz et al., 1995; Friedl, 1997). The cladistic analysis of morphological and biochemical characters placed *Coleochaete* as the closest extant

algal relative of land plants (Graham et al., 1991). Both topologies suggest either two independent origins of gravitropism in Characeae and land plants separately or loss of the trait in some charophytes. These scenarios are not entirely without any merit since rhizophytes are also found in Chlorophyta (Raven & Edwards, 2001), and gravitropism has clearly evolved more than once in eukaryotes. However, the current strong support from two multigene analyses clearly favors the position of Characeae as the sister to land plants (Karol et al., 2001; Qiu et al., 2007). Thus, the best hypothesis at present is that gravitropism evolved only once in streptophytes.

Gravitropism in Characeae has been studied at the cellular level in great details (Braun & Limbach, 2006). In general, actin has been shown to be intimately involved in gravity sensing and polarized cell growth in this system. Actomyosin plays a key role in gravity sensing by first coordinating the position of statoliths, which are BaSO₄-crystall-filled vesicles (different from starch-filled amyloplasts in angiosperms). Upon a change in the cell's orientation relative to the direction of gravity, it directs sedimenting statoliths to specific areas of the plasma membrane, where contact with membrane-bound gravisensor molecules elicits short gravitropic pathways. In controlling polarized cell growth, actin and a steep gradient of cytoplasmic free calcium make up crucial components of a feedback mechanism. So far, only limited knowledge on the role of auxin in regulating rhizoid growth and gravitropism has been obtained in *Chara* (Klambt et al., 1992; Cooke et al., 2002).

In *Arabidopsis thaliana*, a lot more information has been learned about gravitropism from fine-scale genetic analyses conducted over the last ten years. A family of genes (named PIN after pin-formed mutants) encoding auxin efflux carrier proteins have been isolated (Galweiler et al., 1998; Paponov et al., 2005). It is demonstrated that upon gravity stimulation, the PIN3 protein, positioned symmetrically at the plasma membrane of the root columella cells, rapidly relocalizes at the lateral plasma membrane surface and to vesicles that cycle in an actin-dependent manner, which provides a mechanism for redirecting auxin flux to trigger asymmetric growth (Friml et al., 2002; Palme et al., 2006). Recently, it has been shown that five PIN genes (PIN1, 2, 3, 4, and 7) collectively control auxin distribution to regulate cell division and expansion in the primary root, and that they work with another family of genes (PLT for PLETHORA) to specify the meristematic identity of cells in the root (Blilou et al., 2005). In another recent paper, sterol

composition has been implicated in polar localization of the PIN2 protein, which also encodes an auxin transporter and directs root gravitropism (Men et al., 2008). This body of work basically confirms the classical view that auxin, via its basipetal transport, is the main secondary messenger regulating gravitropic growth (Boonsirichai et al., 2002), but has revealed much more genetic insight on mechanisms how plants respond to gravity.

The detailed genetic and cell biological studies of gravitropism in Characeae and *Arabidopsis* set a stage for broad-scale evolutionary investigation of the phenomenon. A third line of work that may aid this research is the isolation of various gravitropic mutants in two mosses, *Physcomitrella patens* (Knight et al., 1991) and *Ceratodon purpureus* (Wagner et al., 1997; Cove & Quatrano, 2006). Given the momentum of research on *Physcomitrella*, it is conceivable that the genes will be isolated from these mutants soon. Thus far, the PIN genes have been found in both eudicots and monocots (Paponov et al., 2005). It will be desirable to extend isolation of this family of genes and the PLT genes, which mediate patterning of the root stem cell niche in *Arabidopsis* (Aida et al., 2004), to gymnosperms and pteridophytes, since the root apical meristem first appeared at the beginning of vascular plant evolution. Further, the search of the PIN genes should be pursued in bryophytes and Characeae. Although there is lack of evidence at present to support a hypothesis that gravitropism in Characeae and all land plants is controlled by the same genetic machinery, it is logical to expect so. A generally consistent evolutionary pattern of auxin metabolism and transport in Characeae, bryophytes, pteridophytes, and seed plants offers some optimism in this hypothesis (Cooke et al., 2002). When the algae colonized the land, among various challenges they face (desiccation, nutrient shortage, less buoyancy, UV, and sperm-locomotion hindrance), absorption of water and nutrients was the first they had to deal with in order to survive, and gravitropism had to have evolved before the algae moved onto the land so that underground organs could develop to overcome this challenge. Moreover, a brief examination of morphology of the organisms along the phylogeny also supports such a hypothesis. Characeae, liverworts, mosses, hornworts, and vascular plants all have rhizoids or roots, which are all gravity-sensing and underground organs. The shoot negative gravitropism has not been studied as much as the root positive gravitropism, but the evolutionary history of the shoot portrays a similar and virtually universal role of negative gravitropism in

shoot development. At the gametophytic level, Characeae, *Haplomitrium*, some leafy liverworts, *Takakia* and acrocarpous mosses all have a vertical upward growing axis. At the sporophytic level, *Haplomitrium*, simple thalloid and leafy liverworts, all mosses, hornworts, and vascular plants also have such an organ. In development of multicellular organisms, cell polarity is the fundamental problem that organisms have to solve during evolution (Cove, 2000). During evolution of streptophytes, gravity had clearly provided the most decisive environmental cue for polarity establishment once these plants became anchored on the soil. Therefore, the current exquisite genetic information obtained from *Arabidopsis* and the emerging results from studies of Characeae and *Physcomitrella* provide a solid foundation to investigate the origin and evolution of gravitropism in charophytic algae and land plants.

In summary, the last twenty-five years witnessed one of the most rapid growth periods in the history of systematics. For the phylogeny of charophytic algae and land plants, not only the backbone, which was proposed even before morphological cladistic studies, has been confirmed with rigorous quantitative analyses of morphological and molecular data, but also many important details crucial for our understanding of some major evolutionary transitions have been clarified by mostly molecular phylogenetic studies. This progress on one hand brings us closer toward our goal of reconstructing the Tree of Life (Haeckel, 1866), and on the other hand sets the stage for new endeavors to explore how major evolutionary transitions in evolution of plant life happened. Advances in genetics and developmental biology in particular offer a great opportunity to integrate the knowledge from model organisms such as *Physcomitrella* and *Arabidopsis* and the new phylogenetic information on lineages that preceded and followed some major transitions, e.g., Coleochaetaceae/Characeae and the origin of gravitropism, and hornworts/lycophytes and alternation of generations in the life cycle of early land plants. This integrated multidisciplinary approach is likely to help us to gain mechanistic understanding on how some of the major evolutionary events in the plant history were unfolded.

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