The branching pattern of major groups of land plants inferred from parsimony analysis of ribosomal RNA sequences

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Abstract. The parsimony and bootstrap branching pattern of major groups of land plants derived from relevant 5S rRNA sequence trees have been discussed in the light of paleobotanical and morphological evidences. Although 5S rRNA sequence information is not useful for dileneating angiosperm relationships, it does capture the earlier phase of land plant evolution. The consensus branching pattern indicates an ancient split of bryophytes and vascular plants from the charophycean algal stem. Among the bryophytes, *Marchantia* and *Lophocolea* appear to be phylogenetically close and together with *Plagiomnium* form a monophyletic group. *Lycopodium* and *Psilotum* arose early in vascular land plant evolution, independent of fem-sphenopsid branch. Gymnosperms are polyphyletic; conifers, Gnetales and cycads emerge in that order with ginkgo joining *Cycas.* Among the conifers, *Metasequoia, Juniperus* and *Taxus* emerge as a branch independent of *Pinus* which joins Gnetales.

The phylogeny derived from the available ss-RNA sequences shows that angiosperms are monophyletic with monocots and dicots diverging from a common stem. The nucleotide replacements during angiosperm descent from the gymnosperm ancestor which presumably arose around 370 my ago indicates that monocots and dicots diverged around 180 my ago, which is compatible with the reported divergence estimate of around 200 my ago deduced from chloroplast DNA sequences.

Keywords. Phylogenetic analysis; rRNA sequences; branching pattern; land plants; age of angiosperms.

1. Introduction

Although there is overwhelming evidence to suggest that land plants evolved from green algal ancestors, the origin and evolution of major groups of plants is not clear. In recent years, the sequences of proteins and nucleic acids are increasingly being used to investigate plant evolution. Hori *et al* (1985) have derived the phylogeny of land plants based on 5S rRNA sequences, by calculating knuc values as described by Kimura (1980) and constructing a phylogenetic tree using the unweighted pair group method. Their analysis suggests that land plants arose from *Nitella-like,* green algae and the pteridophytes and bryophytes are sister groups, separate from seed-bearing plants. Although there have been doubts about the utility of 5S rRNA sequences for phylogenetic analysis (Cavalier-Smith 1989; Steele *et al* 1991), from a detailed study of 5S rRNA sequences Wolters and Erdmann (1989)

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have concluded that 5S rRNA is suited for the study of molecular evolution. Devereux *et al* (1990) have provided new 5S rRNA sequences from three charophycean genera, *Spirogyra, Klebsormidium* and *Coleochaete* and used these sequences along with those of *Nitella,* chlorophycean genera and four land plants to derive a phylogenetic tree based on the unweighted pair group method. Their results also show that *Nitella* and the three charophycean genera are closer to land plants than the chlorophycean genera. Van de Peer *et al* (1990) have determined the nucleotide sequences of 5S ribosomal RNAs from three gymnosperms, *Pinus contorta, Taxus baccata, Juniperus media* and from one fern, *Pteridium aquilinum,* derived a phylogenetic tree of photosynthetic eukaryotes based on the available cytoplasmic 5S rRNA sequences using a distance matrix method (De Soete 1983) and discussed the interrelationships of green plants. Similarly Troitskii *et al* (1991) derived complete or partial nucleotide sequences of five different rRNAs from a number of seed plants and discussed the angiosperm origins and early stages of land plant evolution based on phylogenetic dendrograms using the compatibility (Estabrook 1983) and parsimony methods from the PHYLIP package (Felsenstein 1989).

It has become clear that different macromolecules or even different treeing algorithms may not give the same branching pattern in the phylogenetic trees. Hence it is necessary to construct trees by more than one type of method for different data sets and make a cooperative use of information to arrive at a possible species phylogeny. It has been established that maximum parsimony trees derived from sequence data capture species phylogenies when the ancestral nodes on the tree are separated consecutively by short evolutionary time (Barnabas *et al* 1972) and that a correctly reconstructed parsimony tree can even distinguish between orthologously and paralogously related sequences (Goodman *et al* 1987). Since a large body of data of 5S rRNA sequences from land plants is currently available, parsimony trees from these sequences can be used to capture interrelationships of major groups of land plants.

This work was undertaken to examine the evolutionary relationships among major groups of land plants by reconstructing the most parsimonious phylogenetic trees as well as bootstrap consensus trees from relevant cytoplasmic 5S rRNA sequences. We believe that these approaches will minimize the effect of homoplasies (multiple occurrences of the same genetic changes). To locate the origin of land plants, we have also included the sequences of green algae with those of a protozoan flagellate as an outgroup species. Our computer program is based on a Zip parsimony approach (Barnabas *et al* 1978) which is adapted from a mathematically proven maximum parsimony method (Moore *et al* 1973); and incorporates an exhaustive swapping procedure to carry out an iterative search to arrive at the parsimony solution (Krishnan *et al* 1990). Zip parsimony involves a 'walk' on a given network and consists of the following operations. For each aligned nucleotide position, the zipping procedure assigns (at the interior points of the network) nucleotides which have the potential of minimizing the network length. Then it computes the total network length. The method also examines alternate networks generated by extensive swapping of branches and identifies the network having minimum total network length. The unzipping procedure is executed on this network to calculate the branch lengths. We have also carried out bootstrap analysis of these sequences using the PHYLIP package. Based on these results, we have made a phylogenetic analysis

of branching pattern of major groups of land plants in the light of paleobotanical and morphological evidences. The 1991 version of aligned 5S rRNA sequences was obtained from the Berlin rRNA data base through the courtesy of Prof. Dr V A Erdmann. We have also carried out parsimony analysis of the available small subunit rRNA sequences (ss-rRNA), (made available through the courtesy of Prof. Dr R de Wachter) for estimating the age of angiosperms.

2. Results and discussion

In our parsimony analysis, we have used 45 relevant cytosolic 5S rRNA sequences including one from *Trypanosoma*. These sequences include 6 from chlorophycean algae, 4 from charophycean algae, 4 from bryophytes, 5 from vascular non-seed bearing plants, 8 from gymnosperms and 17 from flowering plants. Using our algorithm, we obtained 156 equally parsimonious trees having a tree length of 327 nucleotide substitutions (figure la). Most of the parsimonious solutions came from different topological arrangements in angiosperm lineage indicating that the 5S rRNA phylogeny does not capture angiosperm relationships. In order to find out whether the inclusion of large number of angiosperm sequences in 5S rRNA trees blur the interrelationships among major groups of land plants, we have derived phylogenetic trees retaining only 3 out of 17 angiosperm sequences (figure lc). Branching pattern of early radiation of land plants did not significantly change when limited angiosperm sequences were included. For each set of 5S rRNA sequences we have also carried out 500 bootstrap replicates (figure lb, d) and use these trees to arrive at the consensus branching pattern. Thus in our discussions we have used 5S rRNA sequence phylogeny to discern only the earlier phase of land plant evolution. For delineating monocot-dicot divergence and to deduce the time of origin of angiosperms, we have derived a maximum parsimony phylogenetic tree of ss-rRNA sequences (figure 2).

2.1 *Bryophytes and vascular plants diverged from charophycean algal stem*
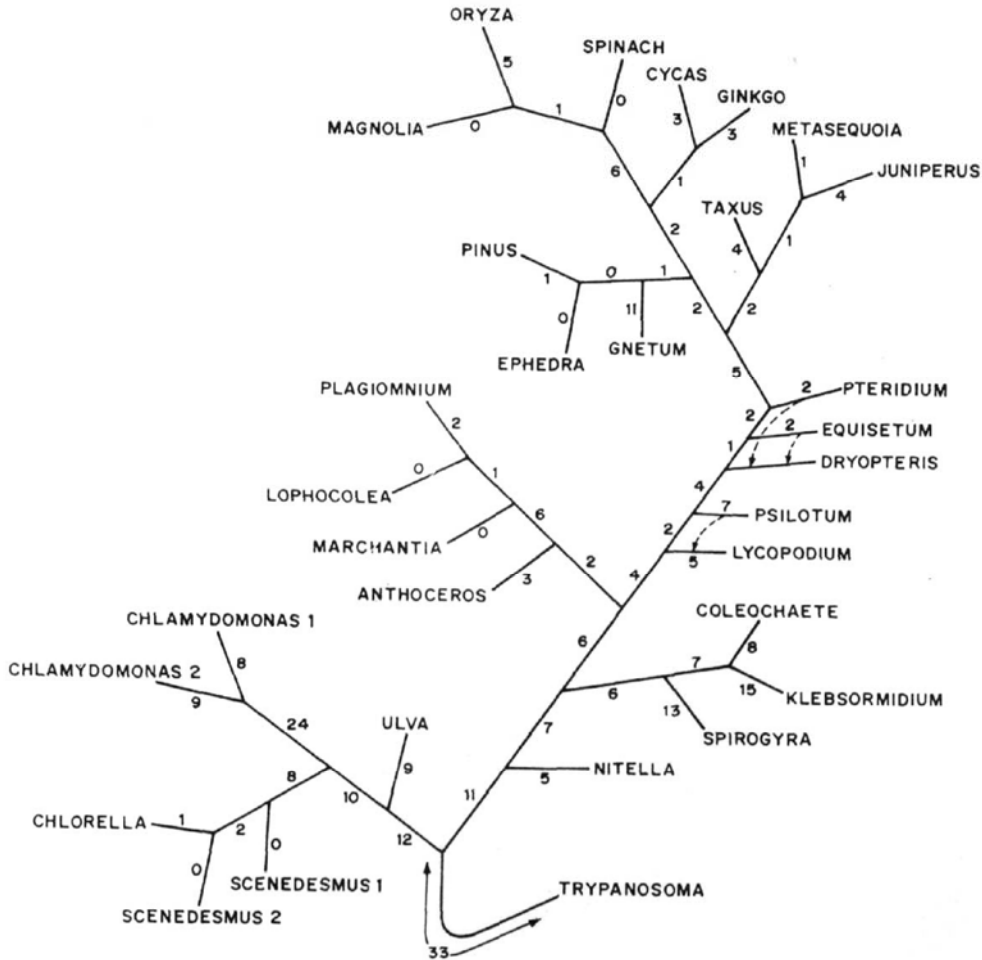
The land dwelling plants have evolved from aquatic algal ancestors and there were perhaps a number of attempts to colonize land (Andrews 1959; Axelrod 1959). Fossils such as *Protosalvinia* (upper Devonian) and *Nematothallus* (lower Devonian) which showed affinities to algae as well as land plants, occupied an environment transitional between water and dry land and are examples of such attempts to adapt to a desiccating terrestrial environment. Based on the studies of mitotic structures of various genera belonging to green algal orders, Pickett-Heaps and Marchant (1972) have suggested that the phragmoplasl type of cell division (with persistent interzonal spindle) present in Charales, *Coleochaete* and related genera, is a primary condition and that higher plants are derived from this line of green algae and the phycoplast type of cell division (with dispersed interzonal spindle and transverse group of microtubules), characteristic of *Chlamydomonas, Ulotrichales* and many other green algal orders arose as a side branch. Stewart and Mattox (1975) have supported this hypothesis and proposed a hypothetical classification of green algae into two lines of evolution based on features of their vegetative and reproductive parts and details of cell division. Charophyceae possess phragmoplast type of cell division and has glycolate oxidase whereas Chlorophyceae possess phycoplast type of cell division and has glycolate dehydrogenase.
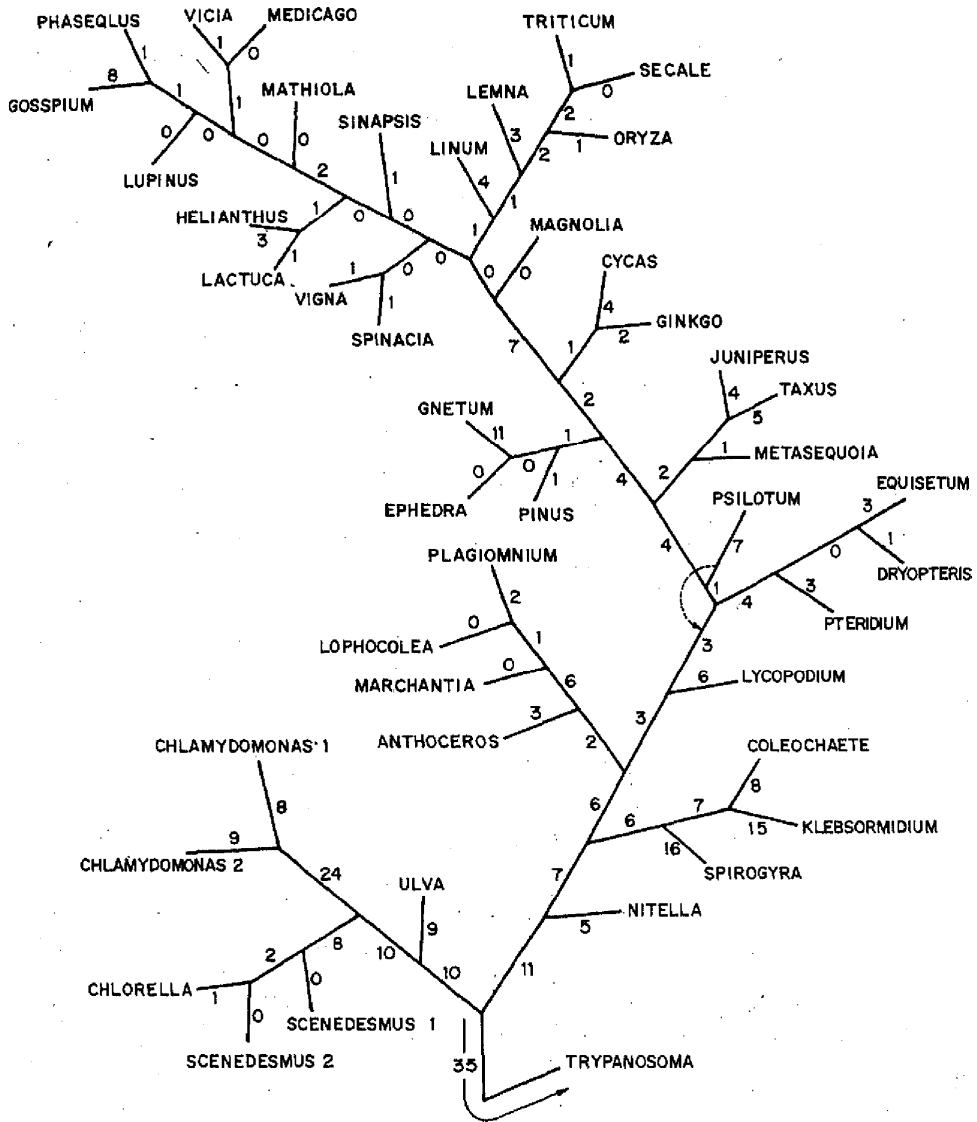
Figure 1a. A phylogenetic tree of 45 relevant cytosolic 5S rRNA sequences. The tree has a length of 327 nucleotide substitutions with 156 equally parsimonious solutions. Numbers represent nucleotide replacements between ancestral and descendant sequences. Curved arrow represents near parsimony solution (+ 1 nucleotide substitution).

Phaseolus vulgaris, Gossypium hirsutum, Lupinus luteus, Vicia faba, Medicago sativa, Matthiola incana, Helianthus annuus, Lactuca sativa, Sinapsis alba, Spinacia oleracea, Vigna radiata, Linum usitatisimum, Lemna minor, Oryza sativa, Triticuma estivum, Secale cereale, Magnolia cobus, Cycas revoluta, Ginkgo biloba, Pinus contorta, Ephedra kokanica, Gnetum gnemon, Metasequoia glyptostroboides, Juniperus media, Taxus baccata, Psilotum nudum, Ptridium aqilinum, Dryopteris acuminata, Equisetum arvense, Lycopodium clavatum, Plagiomnium trichomanes, Lophocolea heterophylla, Marchantia polymorpha, Anthoceros punctatus, Spirogyra grevilleana, Coleochaete scutata, Klebsormidium flaccidum, Nitella flexilis, Chlamydomonas reinhardii 1 and 2. Chlorella Sp., Scenedesmus obliquus (Scenedesmus 1), Scenedesmus quadricauda (Scenedesmus 2), Ulva pertusa, Trypanosoma brucei.

Figure lb. Bootstrap consensus tree of 45 relevant 5S cytosolic 5S rRNA sequences. 500 replicates were done.

Manhart and Palmer (1990) have provided the first molecular evidence to support the charophycean origin of land plants. They have shown that genes of tRNA[Ala] and tRNA[Ile] of chloroplast DNA present in the two charophycean algae, *Coleochaete orbicularis* and *Nitella axillaris* contain introns in the same position as chloroplast tRNA genes in *Marchantia polymorpha* and other land plants, in contrast to other algal groups and eubacteria which have uninterrupted genes.

Figure 1c. A phylogenelic tree of land plants with limited angiosperm cytosolic 5S rRNA sequences.

In our dendrograms (figure 1) chlorophycean algae and charophycean algae diverge along separate lines. The multicellular marine alga, *Ulva* diverges early in evolution from the chlorophycean stem which gave rise to two branches, one leading to *Chlamydomonas* and the other leading to *Scenedesmus* and *Chlorella.* In the charophycean algal branch, *Nitella* arises first independently of other charophytes namely *Coleochaete, Klebsormidium* and *Spirogyra,* which share a common ancestry with the land plants. The bryophytes are regarded as being among the simplest of terrestrial plants, most of them being small and lacking complex cellular differentiation. The oldest fossil bryophyte is *Pallavicinites devonicus* (Hueber 1961), a thalloid liverwort from upper Devonian rocks. The earliest known fossil moss, *Musites polytrichaceus,* is from the Carboniferous (Stewart 1983). The earliest vascular plants are around mid-Silurian in age (Chaloner 1967; Edwards and Feehan
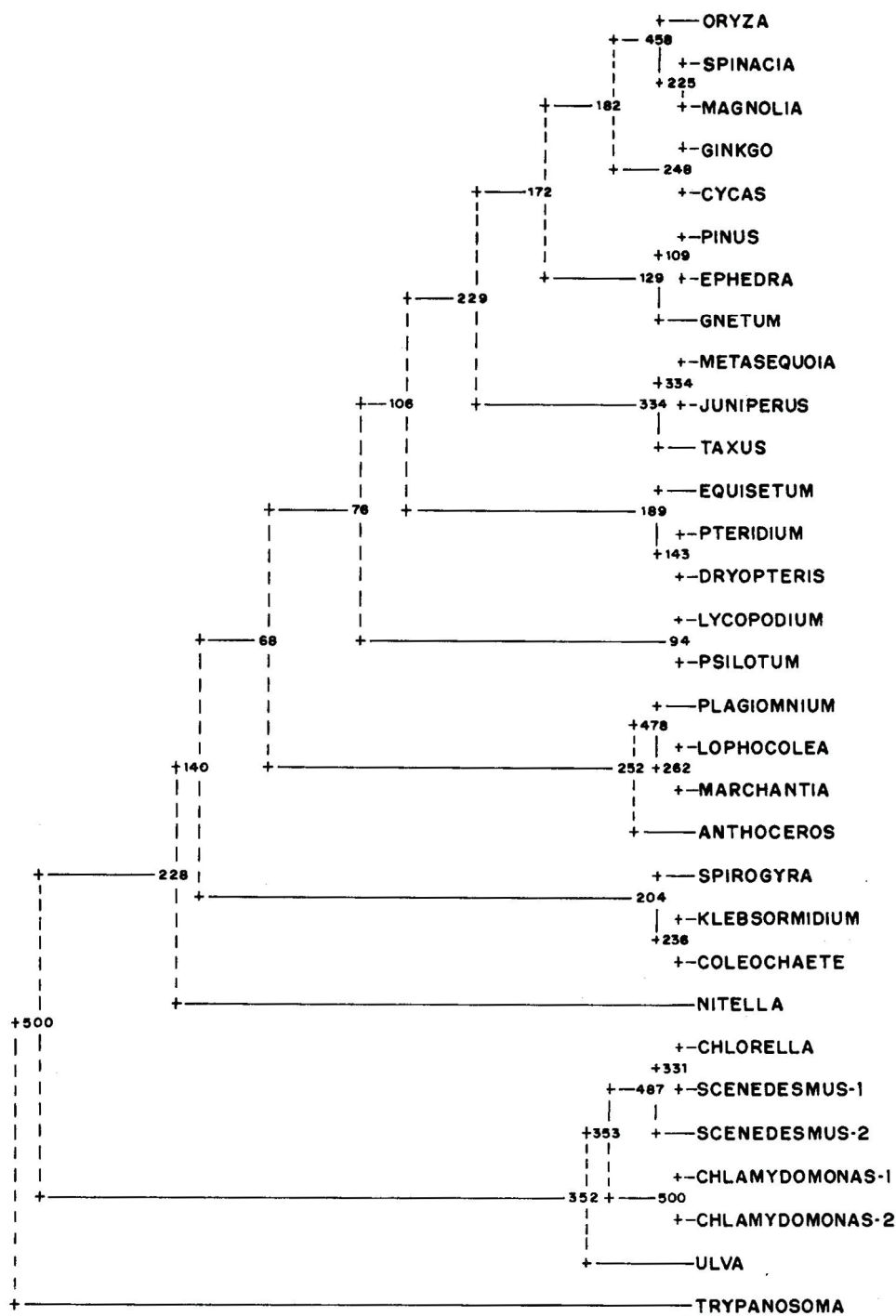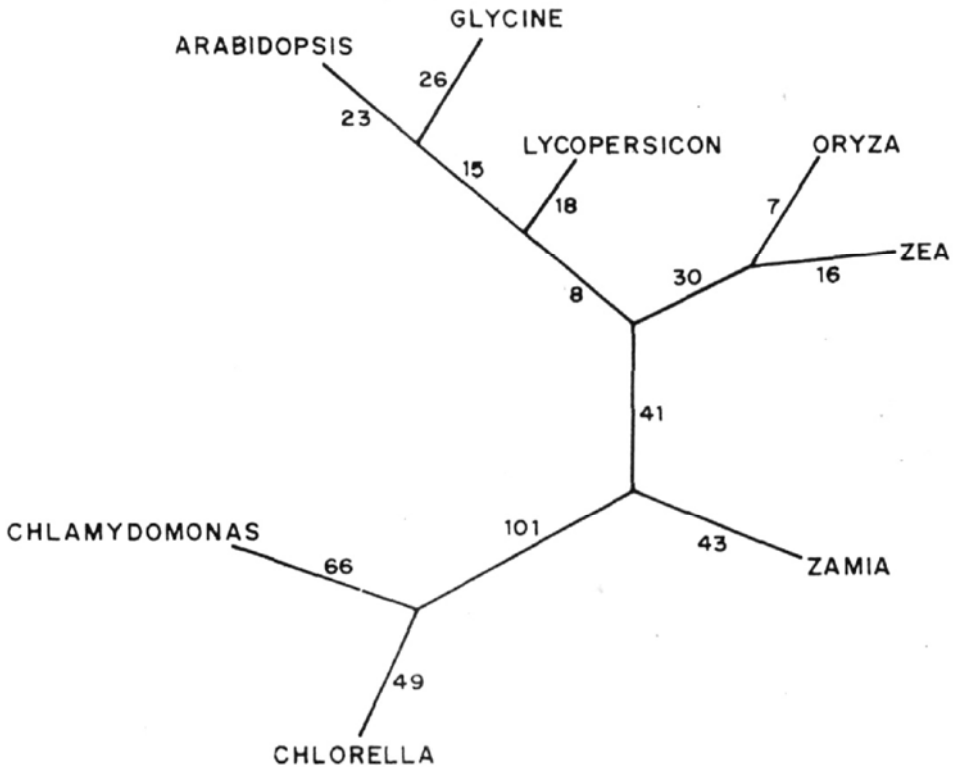
Figure 1d. Bootstrap consensus tree of land plants with limited angiosperm cytosolic 5S rRNA sequences. 500 replicates were done.

Figure 2.   Phylogenetic tree of small subunit rRNA sequences. Tree length = 443.
Oryza sativa, Zea Mays, Lycopersicon esrulentum. Glycine max, Arabidopsis thalina, Zamia pumila.

1980). Examples of some early vascular plants which belonged to the class Rhyniopsida are: *Cooksonia* (mid-Silurian, Wenlockian), *Steganotheca* (lower Devonian) and *Rhynia* and *Horneophyton* from the lower Devonian (Siegenian/Emsian) Rhynie Chert beds. Rhyniopsida were small and simply organized plants with three-dimensional dichotomising naked axis terminating in sporangia, containing isospores. There was a central vascular cylinder which was a circular protostele with annular tracheids (Lang 1937).

In figure 1 as well as in the phylogenetic tree of Van der Peer *et al* (1990), bryophytes and vascular plants diverge from each other from the charophycean stem. Remy (1982) has described gametophytic plants from the lower Devonian of Scotland and Germany having sporophyte-like characteristics. He suggests that these plants, along with several of Rhynie Chert sporophytes having a few characteristics of bryophytes might be remnants of a plant group which preceded both bryophytes and pteridophytes. Perhaps these anatomically preserved plants are represented in figure 1 of Peer *et al* (1990) as the most common ancestor of bryophytes and vascular plants or its immediate descendants. Unlike the suggestion by some investigators (Steere 1958; Lacey 1969) regarding the bryophytes being polyphyletic, in our analysis they are a monophyletic group. *Marchantia* and *Lophocolea* together with *Plagiomnium* appear to be phylogenetically close and together with *Anthoceros* form the bryophyte lineage.

2.2 *Lycopodium and Psilotum arose early in vascular land plant evolution independent of fern-sphenopsid branch*

Lycopsida are regarded as having originated from the mid-Silurian-lower Devonian Rhyniopsida and the group of Devonian lycopsid plants represented by Drapanophycales and Protolepidodendrales is regarded as being ancestral to the three extant orders of the lycopsids: Lycopodiales, Sellaginellales and Isoetales (Stewart 1983).

Psilotum (class Psilopsida) exhibits a number of primitive features like the absence of roots and vascularized leaves, with eusporangiate sporangia producing isospores, borne on short lateral axes. The underground rhizome possesses a protostele with exarch arrangement of primary xylem and there is similarity in structure between the sporophyte and the gametophyte; some of the gametophytes possess vascular tissue (Bierhorst 1953). Because of similar primitive characters, Psilopsida were regarded as being extant relatives of the Silurian-Devonian Rhyniopsida. Although psilopsids show primitive features they have derived characters such as relative complex vasculature and fused sporangia.

In our maximum parsimony trees (figure la, c) , *Lycopodium* is the most ancestral branch in the vascular plant lineage. In figure lc *Lycopodium* is followed by *Psilotum* and a separate branch consisting of *Dryopteris, Pteridium* and *Equisetum;* shifting *Psilotum* to the *Lycopodium* branch is a near-parsimony solution (+1 nucleotide substitution). In figure la, *Psilotum* emerges immediately following the separation of fern-sphenopsid and seed plant lineages. However, shifting it to the base of the fern-sphenopsid stem or to the position just prior to the base of the vascular plant following the separation of *Lycopodium* is a near-parsimony solution. In the bootstrap consensus tree, *Lycopodium* and *Psilotum* cluster together as a branch prior to the separation of fern-sphenopsid branch. In a recent paper, Raubeson and Jansen (1992) have shown that lycopsids share the orientation of a 30 kb region in chloroplast DNA with *Marchantia,* whereas all other vascular plants including *Psilotum* share the inverted gene order. Based on this result, they suggest that bryophyte-lycopsid orientation is ancestral whereas the non-lycopsid vascular plants have the derived condition. The consensus branching arrangement in our analysis suggest that *Lycopodium* and *Psilotum* arose early in land plant evolution independent of fern-sphenopsid branch.

Sphenopsids are represented by only one living form; *Equisetum.* The Sphenopsida are regarded as being derived from the upper Silurian, lower and middle Devonian Rhyniopsida (Stewart 1983) through the Trimerophytopsida (a class of Tracheophyta), a group closely related to the Rhyniopsida (Banks *et al* 1975). From the Hyeniales, a transitional group derived from trimerophyte ancestors, Equisetales originated and the only living genus (family - Equisetaceae) is derived from this line, in the Carboniferous (Good 1975).

The middle Devonian Hyeniales also showed affinities with the Cladoxylales (lower Devonian to lower Carboniferous), putative ferns belonging to the class Filicopsida (Leclercq and Schweitzer 1965). Schweitzer and Giesen (1980) have shown that the sporangiophores of *Calamophyton primaevum,* one of the two genera belonging to Hyeniales are identical to the sporangium-bearing organs of *Cladoxylon scoparium.* A relationship between the fern lineage and the sphenopsids is supported by our tree; *Equisetum* arises from the same stock as the fern lineage

(figure 1). The coenopterid ferns existed in the upper Devonian, Carboniferous and lower Permian and probably had their ancestry in Trimerophytiopsida with whom they share many features like, similar branching patterns, presence of protosteles with centrarch or mesarch primary xylem and sporangial characters (Stewart 1983). In our dendrograms the common stock of ferns and sphenopsids could represent the trimerophytopsid/rhyniopsid stock.

2.3   *Gymnosperms are polyphyletic*

The evolution of the seed habit was a very significant development in the evolution of vascular plants and has made them the most successful of land plants. The gymnosperms, the earliest seed plants, are regarded as having originated from the progymnosperms (Beck 1976), a group of Devonian fossil plants which combined the features of gymnosperm-like secondary wood and pteridophyte-like reproduction. The progymnosperms may have affinities with lower and middle Devonian Trimerophytopsida since there are similarities in branching, vascular elements and reproduction parts (Stewart 1983). *Oocampsa,* an early middle Devonian (Eifelian) plant is regarded as an intermediate between the trimerophytes and progymnosperms (Andrews *et al* 1975). The Aneurophytales are regarded as an ancestral group which gave rise to two lineages in the upper Devonian-lower Carboniferous, one leading to the conifers through the Archaeopteridales and the other leading to the cycads through the gymnosperm group Petridospermales (Scheckler and Banks 1971; Bonamo 1975; Beck 1976). The traditional view is that progymnosperms gave rise to Coniferophyta, Ginkgophyta and Gynetophyta on the one hand, and Cycadophyta and angiosperms on the other. In our phylogenetic dendrograms, conifers, Gnetales and cycads emerge from the gymnosperm stem in that order with ginkgo clustering with cycads. Also, *Metasequoia, Juniperus* and *Taxus* emerge as a branch independent of *Pinus.* An equally parsimonous solution is obtained when *Pinus* joins Gnetales or separates just prior to the separation of Gnetale branch. The Gnetopsida are represented in figure 1 by *Ephedra* and *Gnetum.* Their fossil record is poor and does not throw much light on their origin and evolution. The gnetopsid megafossils have been found in Tertiary (Wodehouse 1934) and pollen has been found in late Triassic (Scott 1960), Cretaceous and Tertiary (Srivastava 1967). In the most parsimonious alternative tree as well as in the bootstrap consensus tree (figure 1), Gnetales-*Pinus* branch separates prior to the emergence of cycad-ginkgo branch. The double fertilization present in *Ephedra* (Friedman 1990) as well as in angiosperms, is considered to be an ancestral condition which appeared before the divergence of Gnetopsida and angiosperms. This is not supported by our trees, although shifting Gnetales-*Pinus* branch in figure la to the angiosperm stem is a near-parsimony solution.

   Ginkgo appears to combine the characteristics of the cycads and the conifers and perhaps arose from Paleozoic pro-gymnosperm ancestors which also gave rise to conifers and pterydosperms (Stewart 1983). In our trees, the ginkgo-cycad branch emerges just prior to the separation of angiosperms. However, shifting ginkgo to the base of the conifer branch is a near-parsimony solution. Although cycads are a sister group to the angiosperms in our dendrograms, Gnetales also show a near-parsimony affinity to the flowering plants. Thus the 5S rRNA phylogenies do not clearly indicate the position of Gnetales in relation to angiosperms. Based on

a numerical cladistic study, Doyle and Donoghue (1987) have concluded that though Gnetales and angiosperms are closely related, angiosperms are not derived from Gnetales, but angiosperms, Bennettitales and Gnetales were derived from a common ancestor. The angiosperms have also been related to the two Mesozoic seed fern families, Caytoniaceae and Corystospermaceae (Stebbins 1974; Doyle 1978) and the Permian glassopterids of Gondwana (Stebbins 1974; Retallack and Dilcher 1981).

2.4 *Angiosperms arose in the Triassic*

Angiosperms are a highly successful plant group whose adaptive radiation resulted in significant diversification of species by the Neogene (Niklas *et al* 1983). An important distinguishing feature of the angiosperms is double fertilization, during which one sperm nucleus fuses with the egg nucleus giving rise to the embryo while a second fuses with the polar nuclei of the embryo sac, resulting in the formation of a polyploid nourishing tissue, the endosperm. This reproductive adaptation of the angiosperms is regarded to have contributed to their evolutionary success (Stebbins 1974). From recent fossil evidence (Taylor and Hickey 1990) the ancestral angiosperms appeared to have been small herbaceous plants with apetalate flowers consisting of ovaries and overlapping bracts and bracteoles, diminutive alternatively arranged leaves with a multistrand vascular element entering the leaf blade. These fossils shared characters with the extant Magnoliidae and basal monocots.

In the 5S rRNA tree (figure la), there are many parsimonious solutions resulting from different topological arrangements of angiosperm lineage. In one of the most parsimonious trees (figure la) the monocot-dicot divergence is discernable although *Linum* emerges from the base of the monocot branch. However, shifting *Linum* to a number of positions in the dicot lineage gives near parsimony solutions. The position of *Magnolia* is not clear in our dendrograms. There are 11 maximum parsimony solutions for *Magnolia.* It can either emerge as the most ancient angiosperm branch (figure la) or can occupy different positions among the dicots. In our bootstrap consensus tree *Magnolia* as well as monocot branch emerge from among the dicots (figure lb). In the bootstrap consensus tree containing limited number of angiosperms, the separation of *Magnolia-Spinacia* branch occurs after the emergence of *Oryza* (monocot) (figure 1d). Clearly 5S rRNA sequence phytogeny is not suitable for discerning the relationships of flowering plants.

It is generally accepted that ss-rRNA sequences are well suited for deriving species phylogeny. Hence we have derived a parsimony phylogenetic tree based on the available ss-rRNA sequences to discern angiosperm relationships (figure 2). Although the tree contains only two monocots (*Oryza* and *Zea*) and three dicots (*Lycopersicon, Glycine* and *Arabidopsis*)*,* the monocots and dicots separate as distinct lines from the angiosperm stem. The gymnosperm *Zamia,* is seen as a sister group to angiosperms, when the Chlorophycean algae (*Chlamydomonas* and *Chlorella*) are used as outgroups. Based on 1722 aligned sites which were used in the construction of the parsimony tree, we have calculated nucleotide replacements during angiosperm descent. Assuming that the progymnosperm ancestor arose around 370 my ago (Wolfe *et al* 1989), we have calculated both the average nucleotide replacements from the gymnosperm ancestor to angiosperm species (81·8) and the time span per mutation (4·52) as shown in table 1. Based on this estimate, the divergence date

Table 1. Nucleotide replacements during angiosperm descent based on 1722 aligned sites of small subunit rRNA sequences.

Evolutionary period	No. of species	Average nucleotide replacements	Time span per mutation (my)	Divergence date of monocots and dicots (my)
From gymnosperm angiosperm ancestor to angiosperm species (370 to 0 my)	5	81·8	4·52	–
From gymnosperm ancestor to angiosperm ancestor	5	40·8	–	185·3
From angiosperm ancestor to angiosperm species	5	40·8	–	184·4

of monocots and dicots works out to be around 180 my ago, which is compatible with divergence date of about 200 my ago (with an uncertainty of 40 my) established by Wolfe *et al* (1989) based on the phylogenetic trees from chloroplast DNA sequences. However, based on the divergence estimate of glyceraldehyde—3 phosphate dehydrogenase gene sequences in plants, animals and fungi, it has been suggested (Martin *et al* 1989) that angiosperm ancestors underwent diversification more than 300 my ago, which is much earlier than the appearance of flowering plants in the fossil record. Although angiosperm had achieved widespread floristic dominance by about 90 my ago, they were present in the fossil record around 120 my ago or even earlier (Lidgard and Crane 1988) and the most parsimonious cladistic trees derived by Doyle and Donoghue (1986) based on morphological character states have implied a late Triassic origin for angiosperms.

Acknowledgements

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